Chapter-5

Discussion

DISCUSSION

5.1. Pilot Study

Adaptogens modulate physiological responses to physical, environmental and emotional stress, and thus help to regulate and support the interconnected functions of neuroendocrine and immune system. Transient hyperthermia triggered by diverse stressful stimuli is a physiological response, the intensity of which is reduced by benzodiazepines therapeutically used for treatments exaggerated anticipatory anxiety. Although many *Rasayana* herbs also possess such efficacy in animal models, unlike conventionally known anxiolytics they do not induce hypothermia, muscle relaxation and other neurological deficits even after their higher doses. Results of this reported pilot experiments reveal that even the highest daily oral dose of AP tested (800 mg/kg) was well tolerated by experimental animals and did not induce muscle relaxation and other adverse effects quantified in unstressed animals. However, clear dose and duration of treatments dependent suppressive effects of AP on stress-induced hyperthermia was apparent after its repeated daily doses. Quantitatively though, efficacy of the extract observed on the days 5 and 7 of the treatments after its highest two doses (400 and 800 mg/kg/day) were lower than those of its lower tested ones. However, such was not the case on day 10 of the treatments when efficacies of all doses of AP higher than 200 mg/kg/day were almost equal.

Another interesting observation made during the stress-induced hyperthermia test was that the daily handling-induced increase in basal rectal temperature observed in the control group was completely absent in all AP treated groups. Since analogous observations were often made in our laboratories with a few other adaptogenic plant extracts this seems to be a common characteristic of adaptogens. It must be noted that maximal possible effects of AP against daily handling-induced increase in basal rectal temperatures were observed after its lowest dose tested, which was not the cases for foot shock stress triggered transient hyperthermia. These observations could indicate that anti-stress efficacy of AP in regulating or maintaining body temperature depends on the stress intensity felt by the animals.

Body temperature regulation is a fundamental homeostatic function that is governed by CNS in animals and human, and abnormalities in the central dopaminergic system causes malignant hyperthermia (Nakamura, 2011; Ahuja and Cole, 2009). Dose dependent antagonistic effects of AP observed after its ten daily oral doses in the apomorphine-induced cage-climbing test could indicate the involvement of central dopaminergic system in the observed anti-hyperthermic effects. It must be noted though that unlike in the stress-induced hyperthermia test no significant effects of the extract were observed after in the cage-climbing test on the 5^{th} and 7^{th} treatment days. The possibility that this discrepancy is due to the use of different rodent species cannot be ruled out. It remains certain though, that repeated daily treatments with AP is necessary for observing its brain function modulating activities, and that AP treatments can ultimately depress the sensitivity of the CNS against stress and dopaminergic stimuli.

Stress-induced hyperthermia tests are sensitive and specific one for identifying benzodiazepines-like anxiolytics, with sedative and anti-epileptic activities. Observations that 10 daily oral doses of AP also significantly reduce spontaneous motility and potentiate pentobarbital-induced sleep indicate its benzodiazepines-like sedative effects after its 100 or 200 mg/kg daily oral doses. However, in both the seizure models used significant protective effects of such treatments were observed only after its highest dose tested, and its efficacy was much lower than those of the standard antiepileptic drugs tested simultaneously. These observations could indicate that prolonged treatments with AP could be useful for helping patients with exaggerated anxiety, and other stress triggered mental health problems commonly associated with numerous chronic diseases. That such could indeed be the case is also indicated by the observations made in the behavioural tests for therapeutically used anxiolytics and antidepressants. It was interesting to note that statistically significant anxiolytics and antidepressant-like effects of AP were observed even after its lowest dose tested (50 mg/kg), whereas significant effects of the extract in all other tests used in this study were observed only after is 100 mg/kg, or higher doses. Only other statistically significant effects of AP observed after 50 mg/kg daily doses during

our efforts was its ability to completely block daily handling triggered elevation in the basal core temperature. These observations could indicate that alterations in the sensitivity of thermoregulatory mechanisms and processes could be involved in the therapeutically interesting neuro-psychopharmacological activity profile of the tested extract. In any case, the observations reported in this thesis add further experimental evidence to our conviction that clinically observed therapeutic benefits of *Andrographis paniculata* extract could as well be due to their modulating effects on central sensitivity to external stimuli, and that the stress-induced hyperthermia test can be a useful starting point for identifying adaptogenic potentials of test agents.

It is well known that apart from its anxiolytic activity, diazepam also possess diverse other CNS function modulating effects and that depending on its dose administered for protracted treatments tolerance liability of diazepam vary considerably (Divljakovic *et al.*, 2012). Our observations clearly revealed that all physiological responses quantified in the bioassay used are antagonized by diazepam after its 5 mg/kg repeated daily oral doses only, and that its activity profile observed after this dose of the anxiolytic administered for more than 5 days is almost identical to that observed after 10 mg/kg/day oral treatment regimen of pure andrographolide. Therefore, it could seem reasonable to assume that the biological processes and mechanisms involved in their observed effects of the two agents must have some similarities.

In any case, it remains certain that qualitatively the activity profile of AP observed earlier in our laboratories in the bioassay used is identical to that of pure andrographolide observed in the experiments (Thakur *et al.*, 2014a). However, the ED_{50} values of pure andrographolide estimated in this study were always significantly lower than those calculated from the dose response curves of andrographolide administered with its tested extract and using the same bioassay procedure (Thakur *et al.*, 2014a). Therefore, it appears that some other bioactive constituents present in the tested extract modulate the stress response desensitizing effects of andrographolide observed in the bioassay.

The possibility that bioactive agents could have adaptogenic properties evolved from pharmacological observations made initially in Russia with a structurally well defined molecules during 1940s (Brekhman and Dardymov, 1969). Since then the concept that adaptogenic properties of herbal remedies are involved in their medicinal benefits has been well accepted also by almost all scholars, researchers and practitioners of integrative medicine well trained in modern medical sciences (Winston and Maimes, 2007). By definition, adaptogens are nontoxic, or well tolerated, bioactive substances capable of counteracting or preventing homeostatic disturbances triggered by metabolic, environmental and mental stress. The facts that andrographolide is a nontoxic substance are evident from a recently reported toxicological study (Bothiraja *et al.*, 2013), and are reconfirmed by the observations made in the pilot experiment described in this thesis. This experiment was conducted to estimate the pharmacologically interesting dose and treatment regimen of andrographolide using its daily oral doses up to 300 mg/kg/day. Even after its highest dose tested, no apparent behavioural and other apparent toxicities of andrographolide were observed in this experiment during the 11 observational days. On the other hand, even the lowest andrographolide dose tested (3 mg/kg/day) completely prevented the daily handling and intermittent foot-shock triggered body weight losses and slight elevation of core body temperatures within physiological ranges observed in control animals. Moreover, dose and treatment regimen dependent partial protection against transient foot-shock triggered hyperthermia with ED_{50} values around 10 mg/kg/day were observed in the pilot as well as in both the confirmatory experiments. Since the dose and duration of treatment dependence of these observed effects of andrographolide were not identical, it seems reasonable to assume that different physiological thermoregulatory mechanisms and biological processes are involved in its diazepam-like beneficial effects observed only after their fairly low daily oral doses.

Numerous behavioural alterations induced by daily handling of experimental animals have since long been known to experimental pharmacologists (Claassen, 1994), and it is well recognized also that almost all handling procedures necessary for *in vivo* bioassays drastically alter their metabolic status, basal core

temperature, and numerous other physiological parameters (Balcombe *et al.*, 2004). However, till now daily handling stress-induced homeostatic and behavioural alterations have seldom been used for identifying adaptogenic or anti-stress potentials of test agents. However, stress-induced hyperthermia paradigm is now regularly used by numerous drug discoverers for identifying acute dose effects of structurally and functionally novel psychoactive drugs potentially useful for treatment of exaggerated anticipatory anxiety (Adriaan Bouwknecht *et al.*, 2007; van der Heyden *et al.*, 1997), or for overall estimation of stress responses in experimental and other animals (Careau *et al.*, 2012). Since clear dose dependent diazepam-like effects of andrographolide against foot-shock stress-triggered hyperthermia were apparent after its repeated daily doses, it seems reasonable to assume that neurotransmitter mechanisms modulated by such treatments are analogous to those of conventionally known benzodiazepine-like anxiolytics. The observations that daily treatments with andrographolide also potentiate pentobarbital-induced sedation and hypnosis, and that its dose response curves for such effects are almost identical to those observed on the 7^{th} and 10^{th} observational days in the stress-induced hyperthermia test are in agreement with this possibility. Since both benzodiazepines and barbiturates are well known modulators of central $GABA_A$ receptors, it could as well be that modulation of the functions of GABAergic system is also involved in the observed effects of andrographolide after its repeated daily doses.

Maximal suppressive effects of andrographolide against hyperthermia induced by daily handling was observed after its five daily doses of 3 mg/kg, and its 30 daily such doses have been reported to be its maximally effective doses in three different gastric ulcer model (Saranya *et al.*, 2011). However, antihyperglycemic and anti-diabetic activities of pure andrographolide have also been observed after its oral doses as low as 1.5 mg/kg (Nugroho *et al.*, 2012; Yu *et al.*, 2003). Our observations taken together with others reported to date on pharmacological activity profile of andrographolide after its repeated oral doses strongly suggest that it is an orally active antihyperglycemic agent with gastric ulcer protective and anti-stress activity, and that its structural modulations are not essential

prerequisites for obtaining such therapeutic benefits from this naturally abundant phytochemical. Therefore, efforts to experimentally verify the possibility that andrographolide itself could as well be useful for prevention and cure of metabolic disorders triggered by or associated with environmental and metabolic stress seems to be an urgent therapeutic necessity. During such efforts due attention has to be paid to the adaptogenic effects of andrographolide induced after its repeated daily doses.

5.2. General Neuropharmacological Screening

CNS active properties of drugs may be assessed by checking the effect of drug on pentobarbital-induced loss of righting reflex. The drugs having sedative properties may potentiate pentobarbital sedation by reducing the onset of sleep (loss of righting reflex) and prolonging the duration of sleep (Carvalho-Freitas, 2002). In our study, pre-treatment with AP also caused loss of righting reflex in a dose dependent manner, which indicates that it has sedative action. Locomotor activity is considered as an index of wakefulness or alertness of mental activity and a decrease may lead to calming and sedation as a result of reduced excitability of the CNS. Drugs acting on CNS have been identified to influence the locomotor activity in men and animals. In the case of sedative drugs, they attenuate the motor activity and many authors have used interruption of the light beams as lateral movements of rats or mice in a cage as a tool to investigate CNS active drugs (Dew, 1953; Saelens *et al.*, 1968). As AP attenuated motor activity possibly by its central sedative action, it is quite possible that observed antianxiety effect of AP is responsible (Thakur *et al.*, 2014a).

The maximal electroshock (MES) test in animals is used to screen compounds, which are effective in grandmal epilepsy where as pentylenetetrazole (PTZ)-induced convulsions are used primarily to evaluate antiepileptic drugs likely to be used in petitmal epilepsy. A typical MES-induced convulsion consists of a brief tonic flexor convulsion (flexion) and a prolonged tonic extensor convulsion (extension), particularly of the hind limbs. These tonic convulsions may be terminal or may be followed by clonic convulsions. Blockade of the hind-limb tonic extensor component of the convulsion indicates an anticonvulsant effect

(Giardina *et al.*, 2009). In MES test, tonic hind extensions are evoked by electric stimuli, which are suppressed by anti-epileptics and other central active drugs. The MES and low-dose PTZ assays are primary screening tests that identify compounds that prevent seizure spread and raise seizure threshold, respectively (Peterson, 1996; Rudzik *et al.*, 1973). Compounds that block seizure spread also block hind-limb extension in the MES tests, whereas compounds that block clonic convulsion in the PTZ test raise the seizure threshold. AP at higher dose used in the study altered the flexor, extensor and clonic phases of MES seizures and offered complete protection against PTZ-induced convulsions. However, it has been shown that most anxiolytic agents are also able to prevent or antagonise PTZ-induced convulsions and here also AP (200 mg/kg, p.o.) has shown anticonvulsant activity in MES and PTZ-induced convulsions probably by its sedative/anxiolytic action similar to benzodiazepine (Thakur *et al.*, 2014a; Thakur *et al.*, 2012b).

5.3. Antidiabesity Activity

Obesity is a risk factor not only for diabetes and other metabolic disorders, but also for numerous other life threatening non-communicable diseases including cardiovascular diseases, cancer and dementia. The term diabesity was initially coined during 1970s to describe strong pathogenic links between type-2 diabetes and obesity (Sims *et al.*, 1973), and it is now well recognized that diabesity is the spreading epidemic of the 21st century (Farag and Gaballa, 2011). However, despite extensive efforts, as yet no very effective, or universally acceptable, therapeutic strategy for prevention of this co-morbid condition has evolved (Colagiuri, 2010).

Although during recent years a few reports revealing anti-hyperglycemic activity of diverse types of *Andrographis paniculata* extracts in animal models have appeared (Agarwal *et al.*, 2005; Nugroho *et al.*, 2012; Subramanian *et al.*, 2008; Xu *et al.*, 2012), as yet little attention has been paid to their therapeutic potentials for combating diverse other pathologies commonly encountered in obese patients. In view of the situation, it was of interest to test whether AP by virtue of its anti-stress and anti-dopaminergic activities could as well be a

starting point for obtaining a therapeutic lead, or a phyto-pharmaceutical, potentially useful for combating diverse spectrums of obesity associated pathologies.

Induction of diabetes is associated with the characteristic loss of body weight, which is due to increased muscle wasting (Swanston-Flat *et al.*, 1990; Mastrocola *et al.*, 2008) and loss of muscle proteins due to persistent hyperglycemia (Russell *et al.*, 2009). Rats treated with AP or glibenclamide showed an increase in body weight as compared to the diabetic control rats, which may be due to protective effect on muscle wasting or due to better glycemic control and carbohydrate homeostasis. Our results revealed that even ten oral daily 50 mg/kg doses of AP significantly reduced blood glucose clearance in glucose tolerance test in normal laboratory rats, and this effect of the extract increased after higher doses. However, the effects of AP treatments observed after its highest dose tested (200 mg/kg) were qualitatively much lower than that observed for 10 mg/kg/day dose of the standard anti-diabetic drug glibenclamide. Moreover, unlike in the glibenclamide treated group, blood glucose decay-curves observed between 30 and 120 min after glucose load in all AP treated groups were always parallel to that observed in normal control group. This observation indicates that the mode and site of action of AP in regulating glucose metabolism is most probably unlike those of glibenclamide.

Observations made in the type-2 diabetes model revealed again that 50 mg/kg/day treatment regimen of AP is effective in reducing all metabolic alterations observed in vehicle treated hyperglycemic and hypo-insulinemic animals and that these observed effects of the extract always increased with its increasing doses. All observed effects of AP on every metabolic parameter assayed were dose-dependent, and were qualitatively analogous to those observed in the glibenclamide treated diabetic animals. However, the histopathological observations made in drug treated diabetic animals were not similar. Although the pancreatic pathologies were less severe in both AP or glibenclamide treated groups, such were not the cases in liver, spleen, and kidney. Unlike in the AP treated groups, no protective effects of glibenclamide treatments against the pathologies of these three later mentioned organs were

observed. These observations reaffirm that the mode(s) and site(s) of actions of AP are unlike those of pancreatic insulin stimulating agents like glibenclamide. In any case as previous study (Zhang and Tan, 2000a; Zhang and Tan, 2000b; Yu *et al.*, 2003), the observations made in this model clearly reveal that daily oral administration of AP is not only effective in compensating metabolic abnormalities and bodyweight losses in diabetic animals, but also afford protection against free radicals mediated organ damages.

Diabesity associated hyperglycemia and hyperlipidemia are well recognized risk factors of cardiovascular diseases (Braun *et al.*, 2013). Reported observations made in the type-2 diabetes model clearly revealed dose-dependent anti-hyperglycemic and anti-hyperlipidemic effects of AP, and during the course of our studies two reports revealing such effects of very high doses of another type of *Andrographis paniculata* extract, or of fairly low doses of pure andrographolide or of neoandrographolide in other rodent models have appeared (Nugroho *et al.*, 2013; Yang *et al.*, 2013a). Observed effects of AP on hyperglycemia and hyperlipidemia in both the obesity models used in our studies were quite analogous to those observed in the type-2 diabetes model. Animal models are useful tools for obesity research as they readily gain weight, when fed high-fat diets (Buettner *et al.*, 2007). The rats fed with high fat develop obesity, hyperphagia, hyperleptinemia, hyperinsulinemia, hyperglycemia, and hypertriglyceridemia (Chang *et al.*, 1990). The physiological aspects of this model replicate many of the features observed with the human obesity syndrome (Levin *et al.*, 1997). The hyperinsulinemia in the HFD fed obese rats results due to development of insulin resistance, a common feature of human obesity and critical factor in the progression of diabetes and cardiovascular disease (Woods *et al.*, 2003). Hyperinsulinemia and insulin resistance are persistent features of obesity (Ginsberg and Huang, 2000). Hypercholesterolemia may be attributed to increased dietary cholesterol absorption from the small intestine following the intake of HFD (Colca *et al.*, 1991). The increase in adipocytes mass and accompanying decreased insulin sensitivity associated with obesity has multiple effects on lipid metabolism. More free fatty acids are delivered from the expanded adipose tissues to the liver, where they are re-

esterified in hepatocytes to form triglycerides, which are packaged into VLDLs for secretion into the circulation. The increased insulin level promotes fatty acid synthesis in the liver. All these factors exert negative impact on the glucose utilisation by peripheral tissues like skeletal muscle and adipose tissue and further worsen the insulin resistance (Husain, 2011a). Fructose feeding induces moderate obesity and several adverse metabolic effects, including hypertriglyceridemia, hyperinsulinemia and weight gain in rodents (Zavaroni *et al.*, 1980; Lee *et al.*, 1994; Elliott *et al.*, 2002). The abnormalities and the disease progression in fructose fed rats resemble the human condition of metabolic syndrome. Fructose-fed rats have been used extensively to study influences of various treatment interventions on the metabolic syndrome (Husain, 2011a). However, body weight gain and circulating insulin level in the AP treated type-2 diabetes rats were higher than those of the vehicle treated ones, whereas both weight gain and insulin level in AP treated obese rats were lower than those observed in vehicle treated obese rats. These observations strongly suggest that the effects of AP treatments on insulin levels and body weights depend largely on metabolic status of animals. Further, it indicates that modulating effects of the extract on biological mechanisms and processes regulating insulin secretion and metabolism could as well be involved in its modes of actions. Since AP and several of its components modulates the productions of diverse mediators of inflammation (Chandrasekaran *et al.*, 2010; Chandrasekaran *et al.*, 2011), we speculate that the observed therapeutically interesting anti-diabetic and anti-obesity effects of AP are due to its anti-inflammatory activities.

Our results revealed that the effects of repeated daily intake of *Andrographis paniculata* extract on plasma insulin levels and body weight gain depend on the metabolic status of the animals, and that AP could as well be a therapeutic possibility for combating life threatening metabolic disorders commonly associated with, or caused by, disturbances of glucose and lipid metabolism. In this respect, it was interesting to note that plasma insulin level of type-2 diabetic rats increased after AP treatments, whereas the elevated plasma insulin level observed in both the obese rat models were suppressed in the extract treated obese groups. Thus, AP seems to be a pharmacologically novel type of regulator

of glucose homeostasis with some functional commonalities with the therapeutically used anti-hyperglycemic or anti-hyperlipidemic drugs.

5.4. Co-morbid CNS Disorders

Although depressive symptoms, anxiety and cognitive dysfunctions are now well-established comorbidities in diabetic patients (Pouwer, 2009) but until now more attention has been paid to depression only. This might be due to the fact that available evidences from extensive population studies suggest that the relationship between diabetes and depression is bi-directional (Golden *et al.*, 2008) and that such is not necessarily the case for diabetes and anxiety (Bouwman *et al.*, 2010) or diabetes and memory impairment. However, despite numerous efforts, no definitive statement can yet be made on the complexity of the cause effect relationship between diabetes (and other metabolic disorders) and diverse types of psychosomatic and/or mood related disorders. Our observations are concomitant with earlier studies that diabetic animal showed increased anxiety, depression and memory-impairment compared to nondiabetic rats.

5.4.1. Antidepressant Activity

Behaviour despair/forced swim, and learned helplessness tests are two most commonly used validated models of rodent to assess anti-depressant drugs. Porsolt *et al.*, 1978, developed behavioural despair model and he suggested that rats forced to swim in a restricted space from which they cannot escape, exhibit a characteristic immobility. This immobility reflects a state of despair that can be alleviated by several agents, which are clinically effective in human depression. In this study, nondiabetic rats treated with all doses of AP were showed significant reduction in immobility period, which indicates potential antidepressant activity. In learned helplessness test, exposure of rodents to inescapable and unavoidable electric shocks later results in situation where they fail to escape shock in a different situation when escape is possible (Kumar *et al.*, 1999; Overmier and Seligman, 1967). This phenomenon was evaluated as a potential animal model of depression. A drug is considered effective antidepressant when learned helplessness is reduced and the number of failures

to escape is decreased (Sherman *et al.*, 1979). Nondiabetic rats treated with AP at all three dose levels were show significant decrease in escape failures as shown by imipramine.

Diabetes mellitus in rats, as well as in humans is associated with alterations in brain monoaminergic system. Streptozotocin-induced diabetic rats have shown a significant inhibition of serotonergic functions in different brain regions and these neurochemical alterations were reversed when diabetic rats received insulin replacement therapy (Chu *et al.*, 1986). Similar changes in serotonergic system are also linked to depression (Price *et al.*, 1991). Therefore, a functional link between diabetes and depression (Rodin, 1983) may exist at the level of the brain monoamines system. Unfortunately, currently available antidepressant and other psychoactive drugs do not meet the therapeutic demands of diabetic patients and many of them are even contraindicated for patients with diabetes (Andersohn *et al.*, 2009; Brown *et al.*, 2008; Deuschle, 2013; Goodnick, 2001). Therefore, efforts were made in our laboratories to identify an adaptogenic herb that could be used as a starting material for identifying novel therapeutic strategies for treatments of diabetes-associated depression. In view of the observed antihyperglycemic activity in type-2 diabetic animals (Thakur *et al.*, 2013c; Thakur *et al.*, 2014b) and antidepressants-like efficacies of AP in nondiabetic animals (Thakur *et al.*, 2014a), it was of interest to experimentally verify the possibility whether it could also be useful for suppressing the exaggerated depressive state in diabetic animals.

In a very first report indicating such possibilities, sedative and some other unspecific brain function altering activities of fairly high acute intra-peritoneal doses of an extract of the plant in rodent models were described (Mandal *et al.*, 2001), and in another recent report appearing after more than a decade the effects of daily doses of another such extracts as immunostimulant, cerebroprotective and nootropic agents in normal and type-2 diabetic rats was described (Radhika *et al.*, 2012a). However, no analytical data or standardisation protocols for the extracts used for the studies were mentioned in both these reports, and no attempts were made to detect their potential antidepressants or anxiolytics-like therapeutic potentials. Similar, or analogous, have also been the

cases for numerous other reports describing diverse therapeutic potentials of different types of extracts obtained from this traditionally known medicinal plant. Recent efforts made in our laboratory to define the psychopharmacological activity profile of an analytically well standardises and therapeutically used *Andrographis paniculata* extract, rich in andrographolide (>30% w/w), were the very first ones revealing its therapeutic potentials for treatments of depression, anxiety, and other psychopathologies characterized by exaggerated symptoms of both anxiety and depression in nondiabetic animals (Thakur *et al.*, 2014a).

One medical condition commonly associated with such comorbidities is diabetes, i.e. an endocrine disorder resulting from inadequate release and/or reduced insulin sensitivity. Diabetes and depression are two major chronic diseases with bidirectional relationship (Lin *et al.*, 2010), and both of them are spreading like epidemics in almost all countries around the global. Co-occurrence of these two pathologies in same patients has strong negative impacts on their quality of life, and shortens their life span (Dirmaier *et al.*, 2010; Roy *et al.*, 2012). Depression has been found also to be associated with alterations in diverse other diabetes related psychological and physiological processes (de Groot *et al.*, 2001; Lustman *et al.*, 2000), and it has been reported that prevalence of depression in diabetics is higher than prevalence of depression in normal population (Anderson *et al.*, 2001). Numerous structural, behavioural and biochemical alterations of the CNS are observed in diabetic patients, and diverse such alterations are observed also in rodent models of diabetes where exaggerated symptoms of depression, anxiety, and cognitive deficits are observed also (Hilakivi-Clarke *et al.*, 1990; Husain *et al.*, 2011b; Rowland and Bellush, 1989; Thakur *et al.*, 2013a; Thakur *et al.*, 2013b; Thakur *et al.*, 2014d).

Although complex interactions of physical, psychological and genetic factors that contribute to such associations still remain to be properly defined, available evidence strongly suggest that depression could as well a consequence of persistent metabolic abnormalities (MacKenzie and Trulson, 1978; Trulson and Himmel, 1985). However, it has been reported also that depression actually doubles the risk of type-2 diabetes, and that depression could as well be an

independent risk factor for type-2 diabetes (Eaton *et al.*, 1996; Kawakami *et al.*, 1999; Nouwen *et al.*, 2010).

In the present study, the depressive state of vehicle treated type-2 diabetic rats in both the tests were more pronounced than nondiabetic ones, and imipramine-like antidepressant activity of AP in nondiabetic and diabetic rats were also observed in both the tests. Even 50 mg/kg/day dose of AP completely antagonized the exaggerated depressive behaviour of nondiabetic and diabetic rats in the behavioural despair test and its antidepressants-like efficacies observed in this test after its 100 or 200 mg/kg/day doses were quantitatively almost equal to that of the tested imipramine dose (15 mg/kg/day). Analogous were also the behavioural observations made on the first test day in the learned helplessness test. Efficacy of AP in this test increased somewhat on the subsequent two testing days, and efficacies of 200 mg/kg/day dose of AP were always higher than that of imipramine on all the three test days in nondiabetic and diabetic animals. These differences could as well be due the difference in the modes of actions of imipramine and AP.

The behavioural despair test is more specific for imipramine and other inhibitors of synaptic monoamine reuptake, whereas the learned helplessness test is sensitive also to diverse other psychoactive agents with antidepressant, anxiolytics, cognitive function modulating, or stress response-modulating agents (Takamori *et al.*, 2001; Yan *et al.*, 2010). Initial neuropsychopharmacological observations made in our laboratories have revealed that even 25 mg/kg daily oral doses of AP completely blocks the handling and mild footshock stress-triggered physiological responses and that it also possess anxiolytics-like efficacies in other behavioural tests using in nondiabetic rodents (Thakur *et al.*, 2014a). Thus, it seems reasonable to assume that AP is a stress response modulating or adaptogenic agent with a broader spectrum of therapeutically interesting psychopharmacological activity profile than synaptic monoamine reuptake inhibitors.

That such is indeed the case is evident also from several other observations made during this study. Unlike nondiabetic rats, the observed beneficial effects

of AP against body weight losses, hyperglycemia, hypo-insulinemia in diabetic rats. AP is also beneficial in management of worsened oxidative status and effectively reduced MAO activities studied in brain regions of nondiabetic as well as diabetic animals. Clear dose-dependent effects of AP on all these parameters were observed, whereas no significant effects of the tested antidepressant dose of imipramine were observed in diabetic animals. However, both AP and imipramine antagonized the lower hippocampal levels of all three monoamines quantified in the diabetic control rats. Although qualitatively these observed effects of AP were quite analogous to those of the antidepressant, here again the efficacy of the highest AP dose tested was higher than that observed for imipramine. Thus, it seems reasonable to assume that although AP is not an imipramine-like psychoactive agent, its observed antidepressant-like efficacy in animal models are due to its modulating effects on central monoaminergic neurotransmitter systems. Such effects of the extract seem to be due its suppressive effects on brain mitochondrial monoamine oxidase activities.

Taken together with other reports on therapeutically interesting bioactivities of *Andrographis paniculata* extracts, our observations strongly suggest that the behavioural effects of AP in diabetic animals is due to its beneficial effects against oxidative damages caused by hyperglycemia and insulin deficiency. Both anti-hyperglycemic and anti-oxidative effects of *Andrographis paniculata* extracts and andrographolide in diabetic animals have often been reported (Chao *et al.*, 2010), and recently a report revealing cerebroprotective and nootropics-like efficacy of an *Andrographis paniculata* extract in type-2 diabetic rats has appeared also (Radhika *et al.*, 2012a). However, in this later mentioned and diverse other preclinical reports the tested extract were administered intraperitoneally, and as yet no reports on the effects of *Andrographis paniculata* extracts and their constituents on brain monoamine levels, oxidative status and mitochondrial monoamine oxidase activities, or on the depressive state of diabetic and other animals, have appeared. The observed bio- and neuro-chemical alterations reported in this thesis revealed that even the lowest oral AP dose tested (50 mg/kg/day for ten days) was effective in reversing the altered enzymatic activities of both oxidative (MAO-A and MAO-B) as well as anti-

oxidative (SOD and catalase) enzymes and lipid peroxide levels in the brain samples of diabetic animals to those observed in nondiabetic ones. This dose of AP was also effective in partially reversing the lower hippocampal levels of the three quantified monoamines (NE, DA and 5-HT) in nondiabetic and diabetic rats.

Thus, the observations reported in this thesis reveal not only that antidepressants-like efficacy of AP is maintained in nondiabetic and diabetic rats, but also that its minimal effective psychoactive oral doses are not higher than 50 mg/kg/day. Moreover, they add further experimental to the convictions that modulating effects of the extract on brain functions are also involved in its clinically observed symptomatic relief after treatments with this and other *Andrographis paniculata* extracts (Panossian *et al.*, 2013), and that AP could as well be a therapeutic herbal alternative for treatments of depression and diverse other psychopathologies commonly associated with or caused by type-2 diabetes and other metabolic disorders. Since currently available antidepressants and all other psychoactive drugs do not properly meet the therapeutic demands of such patients, and are often contraindicated for such purposes, further efforts to more precisely define its sites and modes of actions and its bioactive principles, are now being made in our laboratories.

Observation made to date to identify the antidepressant and adaptogenic components of AP have revealed that andrographolide is indeed is the quantitatively major such component of the extract. Although the efficacy of pure andrographolide have not yet been tested in a type-2 diabetic animals, it has been reported that even very low daily oral doses (1.5 mg/kg/day) of pure andrographolide possess anti-hyperglycemic activity in diabetic but not in normal rats (Yu *et al.*, 2003). Therefore, it seems reasonable to assume that the observed effects of AP on brain monoamines and oxidative status of diabetic animals is primarily due to its effects on some biological processes and mechanisms involved in deregulation of glucose homeostasis. Information on available on oral bioavailability of pure andrographolide (Ye *et al.*, 2011) and other bioactivity constituents of *Andrographis paniculata* extracts (Panossian *et al.*, 2000; Wang *et al.*, 2014) strongly suggest that the primary sites of actions

andrographolide and other bioactive constituents of AP most probably is not the brain tissue itself. It is now well recognized that gut microbial ecology and the so called microbiota-gut-brain axis are involved in physiological regulation of brain functions and metabolic processes (Montiel-Castro, 2013; Kelsen and Wu, 2012; Nicholson *et al.*, 2012; McFall-Ngai *et al.*, 2013; Thakur *et al.*, 2014e). Therefore, it seems reasonable to assume that high efficacies of AP and andrographolide observed in our studies could as well be due to its regulatory effects on gut microbial ecology and enteric nervous system.

That such could indeed be the case is suggested also by the fact that andrographolide and other constituents of possess antibacterial and antiviral activities and that andrographolide and its structural analogues present in AP are extremely bitter substance with high affinity to specific bitter receptors present also in the entire gastrointestinal tract and other organs (Behrens *et al.*, 2009; Clark *et al.*, 2012. It has been reported also that andrographolide forms strong covalent bonds with endogenous thiols and macromolecules involved in regulation of oxidative and other processes leading to metabolic disturbances (Xia *et al.*, 2004). Therefore, it can be expected that the pharmacologically pleiotropic or polyvalent and therapeutically interesting bioactivities of AP and andrographolide observed in experimental animals after their daily oral doses are due to their irreversible interactions with biologically important macromolecules within the gastrointestinal tract. Efforts to identify such macromolecules and their biological functions could eventually lead to identification of novel pharmacological targets involved in the therapeutically interesting activity profile of AP reported in this thesis and earlier reports on this extract.

Thus, the observations reported in this thesis have revealed not only a novel therapeutic potential of AP for prevention or cure of diabesity associated depression and other psychopathologies, but also have supplied us with a tool potentially useful for identifying novel pharmacological targets potentially useful for discovering novel therapeutic leads against co-morbid psychopathologies commonly associated with metabolic disorders and other chronic diseases. It must be mentioned though, that like all other plant extracts, AP also contains

other phytochemicals with known brain function modulating and hyperglycaemic activities worth following further not only for drug discovery purposes, but also for better understanding of the biological processes involved in the modes of actions of the traditionally known and still widely used medicinal plant *Andrographis paniculata.*

5.4.2. Anxiolytic Activity

The results of the present study indicate that STZ-induced experimental diabetes in rats led to increased anxiety, as assessed by the various paradigms used *viz*. social interaction test, light-dark box test, and elevated plus maze test. All these behavioural paradigms have been validated for exogenous and endogenous anxiogenic agents (Bhattacharya and Mitra, 1991, Ramanathan, 1998; Kumar *et al.*, 2000; Mishra *et al.*, 2013).

Total time spent by the diabetic rat pair was less in social interaction test, including sniffing, following, grooming, kicking/boxing, biting and crawling under or over the partner compared to nondiabetic control rats. The latencies to entry, number of entries, and time spent in light box are the parameters generally evaluate for anti-anxiety activity tested drugs during light-dark box test. In our observations, diabetic rats demonstrated exaggerated anxiety in this test, and had fewer activities in light box. However, AP treatment in nondiabetic and diabetic animals demonstrated preference to entry in light box as latency time, number of entry and time spent increased compared to respective control animals. The elevated plus maze is a widely used behavioural assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents, and to define brain regions and mechanisms underlying anxiety-related behaviour (Walf and Frye, 2007). During elevated plus maze test, total time spent in open arms was less in diabetic control rats showing exaggerated anxiety might be due to novelty and risk/fear. Some earlier investigations have also reported increased anxiety in STZ diabetic rats in the plus maze (Ramanathan *et al.* 1989).

Anti-oxidant therapy has proved to be remarkably beneficial to remedy reactive oxygen species (ROS)-induced injury in the CNS (Tsakanova *et al.*, 2011). A study

suggests that hyperglycemia leads to increased oxidative stress, which in turn diminishes antioxidant defense system and short-term supplementation of vitamin C is safe and beneficial for reducing anxiety levels in diabetic patients through alleviating oxidative damage (Mazloom *et al.*, 2013). In our finding, AP treatment successfully reversed hyperglycaemic condition in diabetic animals and the activities of anti-oxidant enzymes in brain toward normal level in nondiabetic and diabetic rats compared to respective control animals.

The AP treatment produced dose-dependent anxiolytic activity in nondiabetic and diabetic animals. However, lorazepam partially attenuated the anxiogenic behavioural patterns in diabetic rats, indicating a differential effect of lorazepam in nondiabetic and diabetic rats. These observations indicate that prolonged treatments with AP could be useful for helping patients with exaggerated anxiety, and other stress-triggered mental health problems commonly associated with numerous chronic diseases.

5.4.3. Nootropic Activity

Cognitive disturbances are not only the hallmarks of dementia, but also are often encountered in patients suffering from many comorbid pathological conditions (Sareen *et al.*, 2006; Kroenke *et al.*, 2007). Most antidepressant, anxiolytic and other psychotherapeutics currently widely prescribed for combating cognitive function associated psychopathologies does not properly meet the therapeutic demands and adverse effects of such psychotherapeutics on cognitive functions are well known. Moreover, during recent decades modern drug discovery strategies have consistently failed to identify novel psychotherapeutic leads or pharmacological targets potentially useful for helping patients with cognitive function associated comorbidities and other neurological disorders (Schoepp, 2011). In view of the situation, the observed antioxidative and cholinesterase enzyme modulating actions of AP and andrographolide could be of considerable interest for drug discovery purposes.

In light of evidence indicating that many diabetics experience loss of short term memory, impaired memory, as well as difficulties in concentration (Perlmuter, 1984), and that cognitive deficits and memory dysfunctions are often associated

with cholinergic hypoactivity (Bartus *et al.*, 1982), it is reasonable to postulate that some of the behavioural consequences of diabetes are due to deficits in cholinergic neurotransmission. Streptozotocin is reported to cause memory deficit in rodents (Flood *et al.*, 1990), and reduced cholinergic transmission due to a decrease in acetylcholine level is reported in cognition deficits of STZ-induced diabetic animals (Welsh and Wecker, 1991). Our present result showed a significant improvement in memory function of diabetic rats by AP and andrographolide treatments in water moris-maze tasks. As cholinergic pathways play an important role in learning and memory processes, AP and andrographolide cause increase in central cholinergic transmission and might be involved in the nootropic and anti-amnesic effects in diabetic rats. In the observations described in this thesis clearly showed that AP (50 mg/kg/day) and andrographolide (15 mg/kg/day) dose regimen of the test drugs were high enough for beneficial effects of drugs on learning and memory as days dependent significant decrease in escape latencies to find out the platform in morris water-maze test paradigm by nondiabetic and diabetic animals and decreased AChE enzyme activity in brain tissues.

Taken together with the observed normoglycemic and enhanced antioxidative status in the brains of AP and andrographolide treated diabetic rats, these behavioural findings indicate that cellular oxidative mechanisms involved in regulation of cognitive functions could as well be the primary targets. Moreover, acetylcholine esterase enzyme activity in different brain regions (prefrontal cortex and hippocampus) of the AP and andrographolide treated nondiabetic and diabetic animals were lower than those of their respective control animals.

5.5. Other neuropsychopharmacological tests

Locomotor activity is considered as an index of wakefulness or alertness of mental activity and a decrease may lead to calming and sedation as a result of reduced excitability of the CNS, and CNS acting drugs have been identified to influence the locomotor activity in animals (Singh *et al.*, 2011; Thakur *et al.*, 2014a). In the case of sedative drugs, they attenuate the motor activity and many authors have used interruption of the light beams as lateral movements of rats or

mice in a cage as a tool to investigate CNS active drugs (Dew, 1953; Nakatsu and Owen, 1980). As AP and andrographolide attenuated motor activity in nondiabetic and diabetic rats possibly by its central sedative action, it is quite possible that observed antianxiety effect is responsible. The 5-HTP-induced head twitch response in mice is indicative of central serotonergic activity (Ernst, 1972). Serotonin is known to be involved in the sleep stages of the sleep wake cycle. The nondiabetic and diabetic mice treated with AP or andrographolide were showed significant increase in number of head twitches, similar to standard antidepressant drug imipramine. There was also significant effect of AP and andrographolide on L-dopa-induced hyperactivity in mice, indicating putative effect of AP and andrographolide on dopaminergic system as well. A number of clinical studies have reported that amphetamine (dopamine/noradrenaline uptake inhibitors) can induce predominantly stereotypic behaviour (Snyder, 1973), a psychosis that resembles schizophrenia (Bell, 1965). In AP and andrographolide treated nondiabetic and diabetic rats decreased amphetamine-induced stereotype movement were observed. These observations were concomitant to the observed monoaminergic activity of AP on nondiabetic and diabetic brain by increased monoamine levels and decreased MAO-A and MAO-B activity. These observations indicate that AP and andrographolide are involved in modulation of central serotonergic and dopaminergic transmission and responsible for observed anti-depressant activity in nondiabetic and diabetic animals.

5.6. Anti-inflammatory and Analgesic Activity

Animal models used for the purpose were the ones commonly used for identifying anti-inflammatory agents with analgesic activities (Kumar *et al.*, 2001a; Campos *et al.*, 2002; Trongsakul *et al.*, 2003). Qualitatively, the observed effects of repeated daily AP and andrographolide doses in the animal models used were quite analogous to those of the reference drugs used in our studies. In any case, it remains certain that inflammatory mechanisms involved in pellet granuloma, swelling, edema, and pain behaviours are suppressed by repeated minimum dose of AP (100 mg/kg) and andrographolide (30 mg/kg). After this dose, significant effects of AP and andrographolide were observed in both the

rodent models of inflammation, and such were also the cases in the tail flick and hot plate tests. Although the observed effects of the tested drugs in these tests increased after its lower dose, its dose effect relationships in the dose range tested were never very steep. These tests are widely used for identifying peripherally as well as central acting analgesics and anti-inflammatory agents, sedatives and other psychoactive agents are known to be effective in inhibiting the inflammation and pain. Since sedative and other behavioural effects of AP becomes apparent after its repeated daily doses (Thakur *et al.*, 2014a), its observed effects in this and other model of analgesia could as well be due to its modulating effects on CNS functions.

Pain commonly accompanies a variety of conditions, including peripheral nerve injury, central nervous system injury, viral infections, tumors and metabolic disorders such as diabetes mellitus (Clark *et al.*, 2013). Hyperglycemia is an important factor in pain hypersensitivity characterized by allodynia and hyperalgesia in diabetes (Courteix *et al.*, 1993; Calcutt and Chaplan, 1997; Barrier *et al.*, 2012). The observations described in thesis clearly revealed out that the diabetic rodents showing hyperalgesic conditions and the inflammatory response is exaggerated compared to nondiabetic rats. Interestingly though, response times of single dose of AP or andrographolide treated nondiabetic and diabetic animals in the hotplate and tail flick tests observed were always similar to those observed in their respective control animals. However, its dose dependant analgesics-like effects were observed in both the tests and this effect of the AP or andrographolide were persistently observed in the tail flick test and hot plate test as well as decreases formalin-induced flinching behaviour in even three hours after its last dose similar to standard central analgesic drug used. All these models are well known for their predictive validity for identifying centrally acting analgesics. Therefore, it can be said that AP or andrographolide possess centrally acting analgesic-like activities. Efforts to clarify the major active constituents i.e. andrographolide and modes of actions involved in it analgesics-like efficacy in these models using nondiabetic and diabetic animals could as well lead to novel pharmacological targets for pain therapy. Other studies reported in this thesis have revealed that after repeated daily doses of AP or

andrographolide, oxidative statuses as well as cytokine expression are altered both in the circulating blood as well as in the brain. Therefore, peripheral as well central mechanisms might be involved in its mode(s) of action(s).

Pain is a cardinal symptom of inflammatory swelling of almost all peripheral organs. Dose dependant anti-inflammatory effects of AP or andrographolide were apparent in both the models used. Moreover, the efficacies of andrographolide of corresponding doses were higher than the AP doses. It is now well recognized that oxidative processes and cytokines are intrinsically involved in the pathogenesis of inflammatory swelling. Repeated daily doses of AP or andrographolide have successfully augment anti-oxidative capacity and cytokine expression both in circulating blood and brain of stress rats. Since these effects of the AP or andrographolide was accompanied with the increased cellular antioxidative capacity, it could as well be that its observed anti-inflammatory and analgesic activities might be the consequences of its effects on metabolic processes controlling cellular cytokine expression. The fact that AP and andrographolide having beneficial effect as analgesic and anti-inflammatory activity in nondiabetic as well as diabetic rats together with restore elevated glucose level in this study suggest that the action(s) against pain or inflammation is not only due to a normoglycemic effect. Bioactive components of AP are known to possess analogous propertied are andrographolide, isoandrographolide (Chandrasekaran *et al.*, 2011) which now well recognized for their therapeutic potentials against diverse inflammatory pathologies (Chandrasekaran *et al.*, 2010; Parichatikanond *et al.*, 2010). However, it cannot be ignored that AP contains numerous other bioactive constituents with pharmacological activity profiles (Chao and Lin, 2010). Therefore, it could as well be that the observed efficacy of AP might be due to modulating actions of diverse active components of the extract.

Observations reported in this thesis add further experimental evidences to the conviction that the broad spectrum of psychopharmacological activity profile of AP observed after its repeated daily doses is mainly due to its modulating, or inducing effects on peripheral and central mechanisms of inflammatory processes. Repeated daily doses of AP is necessary for observing its anxiolytic as

well as antidepressant-like activities and that unlike other anxiolytics and antidepressant drugs this extract actually improves cognitive functions in nondiabetic as well as diabetic experimental models. Moreover, its antidepressants-like activities were also apparent in mentally stressed nondiabetic animals. Thus, it seems reasonable to assume that modulation of biological processes, or mechanisms, involved in anti-inflammatory effects of AP and andrographolide, leads to adaptive responses in the central control mechanisms involved in exaggerated depression and central stress responses. That such could indeed be the case is well supported by the postmodern concepts of psycho-neuro-immunology on comorbid mental health conditions (Leonard and Myint 2009; Juster *et al.*, 2011; Anisman, 2011). Therefore, AP and andrographolide could be a starting point for discovering novel therapeutic leads and pharmacological targets urgently needed for combating inflammation-associated psychopathologies.

5.7. Anti-stress Activity

Stress can be defined as a state of impaired homeostasis. This state is elicited by various stimuli that are usually referred to as stressor signals (Selye, 1973). A subchronic pretreatment with adaptogens causes normalization of stress hormone levels and generally decreased stress predisposition in behavioural tests. The general aims of adaptogen treatment are reduction of stress reactions during the alarm phase of the stress response, prevention or at least delay of the state of exhaustion and hence a certain level of protection against long term stress as described by Selye (1998). A variety of stress situations has been employed in animals to evaluate anti-stress activity and these stress-induced neurochemical and behavioural effects mainly depend on duration and type of stressors (Tannenbaum *et al.*, 2002). A short duration stress can be modulated by the physiological, biochemical and endocrine responses as defensive mechanism to counter stress effects. However, if the stress is applied for a prolonged period of time, the body fails to acclimatize and results in stress-related illnesses like gastric ulcerations, behavioural perturbations together with biochemical and endocrine imbalance (Bhattacharya and Muruganandam, 2003; Chrousos and Gold, 1992).

Many herbs reported in the ancient literature have potent anti-stress activity and their utilities in the current scenario need to be unveiled. The drugs of plant origin are gaining increasing popularity and are being investigated for the remedies of a number of disorders including adaptogenic (anti-stress) activity (Wagner *et al.*, 1994). Since last two decades, supplementation with various macro and micronutrients and herbal preparations has been evaluated for their adaptogenic activity during exposure to a stressful environment (Bhattacharya and Muruganandam, 2003; Bhattacharya *et al.*, 2000b; Kumar *et al.*, 2001b; Bhattacharya *et al.*, 2002; Muruganandam *et al.*, 2002; Sumanth and Mustafa, 2009; Nade *et al.*, 2009; Joshi *et al.*, 2012; Singh, 2012; Singh *et al.*, 2012). Pharmacologically, adaptogens are characterized by their broad spectrum of bio-activities in animal models commonly used for identifying anti-stress, nootropic, immune-functions modulating and antioxidant properties of test agents. Modern herbalists for prevention and cure of mental health problems (Winston and Maimes, 2007) now recommend numerous better-scrutinized adaptogenic herbs. Due to increased physical and psychological demands in the present day life style and advent of various stress related disorders, there is an urgent need to develop agents to overcome these abnormalities.

Daily treatments with AP or andrographolide afford protection against all chronic foot-shock stress-triggered pathologies studied, and that their efficacies are qualitatively quite analogous to that of another Ayurvedic herb *Withania somnifera* (WS). Moreover, they also suggest that their regulating effects on cytokine homeostasis are most probably involved in their observed unspecific protective effects against chronic stress-triggered pathologies. However, observed activity profile of WS on cytokine expressions in blood was not identical to those of AP or pure andrographolide. Although qualitatively the observed activity profile of AP was quite analogous to that of pure andrographolide, not all observed efficacies of AP could be explained by its analytically quantified andrographolide content only. These observations strongly suggest that that bioactive constituents other than andrographolide present in the extract modulate the efficacy of this quantitatively major constituent of the extract.

It remains certain though, that both AP and andrographolide are effective desensitizers of numerous pathologies resulting from chronic unpredictable stress exposures, and that both of them afford protections against over expression of inflammatory as well anti-inflammatory cytokine producing RNAs associated with such exposures. Our earlier observations (Thakur *et al.*, 2014a) had revealed that the dose response relationship of AP for its anti-stress activities against different types of stress vary considerably. In that study, the lowest oral dose (10 mg/kg/day) of AP tested completely prevented bodyweight losses and slightly elevated core temperatures of mice induced by daily handling and intermittent short periods (1 min) of foot-shock stress, whereas its minimal effective daily oral doses for inhibiting foot-shock triggered transient hyperthermia was 50 mg/kg. The minimally effective daily oral doses of AP observed in another study revealing its anti-diabetic, antihyperlipidemic and anti-obesity activity was 50 mg/kg (Thakur *et al.*, 2014b). However, this daily dose of the extract was not always its minimally effective ones for all observed protective effects observed in the present study.

Although statistically significant protective effects of 50 mg/kg/day doses of AP against all quantified chronic stress-induced pathologies were observed, often its efficacy did not increase much with its increasing daily doses. Such was specially the cases for its observed effects against stress-induced elevated IL-10 and IL-1β expressions in blood and TNF-α and IL-10 expressions in brain. All these three elevated cytokine expressions quantified in stressed animals were almost completely absent even after its lowest oral dose tested (50 mg/kg/day), whereas effects of AP treatments on blood TNF-α and brain IL-1β expressions increased with its increasing daily doses. These observations taken together with the similar one made with pure andrographolide strongly suggest that their lowest doses used in this study were much higher than their minimally effective ones necessary for defining their minimally effective ones against stress-induced cytokine over expressions. Therefore, a dose finding experiments has been conducted with pure andrographolide in the bioassay used earlier for estimate pharmacologically interesting doses and dosing regimen of AP (Thakur *et al.*, 2014a). The results of these experiments revealed that like for AP minimal

effective dose of andrographolide vary considerably with the duration of stimuli and that the minimal effective daily oral doses could as well be 3 mg/kg/day and lower (Thakur *et al.*, 2014f). These observations strongly suggested that therapeutically interesting dose ranges of andrographolide and AP are depend on the severity of environmental stress-triggered pathologies encountered in a given patients and that biological processes leading to stress-triggered alterations in cytokine homeostasis are involved in their modes of actions (Thakur et al., 2014g).

It is well recognized that depending on the nature, intensity and duration of stress diverse spectrums of homeostatic alterations are induced (Everds *et al.*, 2013; Koolhaas *et al.*, 2011; Korte *et al.*, 2005; Lucini and Pagani, 2012), and that such alterations eventually leads to diverse spectrums of co-morbid psychopathologies accompanying almost all inflammatory diseases commonly associate with altered cytokine expressions and functions (Boonstra, 2013; Hennessy *et al.*, 2014; Hueston and Deak, 2014; Reber, 2012). Amongst the three cytokine expressions quantified in this study, IL-10 is now well recognized to be a key regulator of chronic stress-triggered immunological functions (Hu *et al.*, 2014; Saraiva and O'Garra, 2010). Its observed elevated expressions of IL-10 in both blood and brain of stressed animals were completely suppressed by the lowest tested daily doses of both AP and andrographolide. Although WS (100 mg/kg/day) also completely suppressed stress-triggered IL-10 expression in the brain, it had only marginal such effects in blood. These observations strongly suggest that the primary modes and sites of actions of AP and andrographolide are not identical to those of WS.

Despite such mechanistic differences, functionally the effects of WS were quite similar or analogous to those observed for AP or pure andrographolide. Therefore, it remains certain that both AP and andrographolide are functionally novel and orally active anti-stress agents with potent immune function regulating activities. Since diabesity and associated neurological disorders are the spreading epidemics of the 21st century, till now we have concentrated our efforts to estimate the doses and treatment regimen of AP necessary for prevention and cure of such disorders only. Observations reported in this thesis

strongly suggest though, that both AP and pure andrographolide could also be effective herbal therapies for diverse other medical conditions commonly caused by prolonged exposures to chronic unavoidable stress.

5.8. Cytokines and TLRs expression in HL-60 Cell line

The concentration of AP (IC_{50}= 48.50 µg/ml) and andrographolide (IC_{50}= 38.62 µM) at which the cell viability of HL-60 was about 50%. In further cytokines and TLRs expressional study, andrographolide 10 µM concentration was used at which cell viability of HL-60 was normal and safe. As earlier study with AP and andrographolide, the non-cytotoxic concentrations were in the same range for cell line study published elsewhere (Chandrasekaran *et al.*, 2010; Chandrasekaran *et al.*, 2011). This andrographolide concentration (10 µM) was sufficient to inhibit TNF-α and IL-1β where the expression of IL-10 is not inhibited rather some higher expression than control. These observations strongly suggest that modulation of cellular processes involved not only in expressions of inflammatory and anti-inflammatory cytokines but also in those of TLR-7 and TLR-8 are involved in the modes of actions of this extremely bitter secondary metabolite of *Andrographis paniculata* and might be a potential candidate for management of neuro-inflammatory disorders associated with neuro-immune functions.