CHAPTER – 5

DISCUSSION

DISCUSSION

The experiments and observations reported in this thesis constitute a part of phyto-pharmacological studies with Ayurvedic medicinal plants ongoing in our laboratories since the 1990s. The very first preclinical reports from our research group revealing the antioxidant activity of withanolides isolated from *Withania somnifera* and beneficial effects of a *Withania somnifera* containing Ayurvedic formulation in hyperglycemic rats appeared in 1997 [S.K. Bhattacharya et al., 1997a; 1997b]. Triggered by the preclinical observations reported in them, the very first exploratory clinical study with *Withania somnifera* root powder in diabetic patients was conducted and reported in 2003 [B. Andallu and B. Radhika, 2000]. The possibility that anti-diabetic and other metabolic benefits of *Withania somnifera* extracts could be due to their protective effects against chronic stress triggered alterations in insulin sensitivity and glucose homeostasis was then suggested by other preclinical observations from our laboratories as well [S.K. Bhattacharya and A.V. Muruganandam, 2003]. That study had revealed also that repeated daily oral doses (25 and 50 mg/kg/day) of a *Withania somnifera* root extract in rats effectively suppresses diverse stress responses triggered by repeated exposures to unpredictable foot shock stress for several days and of fairly short duration.

More recently reported observations in our laboratories made in diabetic mice occasionally exposed to fairly short durations (< 1min) of foot shock stress (only four times during 10 days) had ascertained that although daily oral 25 mg/kg doses of a currently commercially available *Withania somnifera* suppresses hyperglycemia, such treatments do not have any significant effects on hypoinsulinemia or circulating cortisol levels in diabetic animals [A.K. Thakur et al., 2015a]. Since fairly low daily oral doses of *Withania somnifera* root extracts are used in Ayurvedic formulations for diverse medicinal purposes, the aim of the dose

finding experiments reported in this thesis was to identify bioassay systems and biomarkers that could be used for better understanding low dose pharmacology of the root extracts of the plant. Results of these experiments reported in this thesis have revealed that 10 mg/kg daily oral doses of such extracts are high enough for suppressing diverse stress responses quantified (see section 4.1 of the results chapter of this thesis), and that even 3.3 mg/kg/day doses of the extract devoid of withanolides is highly effective in protecting the animals against stress triggered body weight losses and elevations in body temperature (see subsection 4.3 of the result section of this thesis).

Therefore, further experiments were conducted to identify a convenient bioassays system universally acceptable for detecting and quantifying stress response suppressing potentials of diverse types of extracts obtainable from *Withania somnifera* like adaptogenic plants and their bioactive constituents. These efforts eventually led to the identification of the marble burying tests in stressed mice as a convenient screening test for such purposes and also for obtaining blood and other organ samples of experimental animals necessary for *ex vivo* bioassays and mechanistic studies. Potential uses of this test in foot shock stressed mice for comparing stress response desensitizing effects of low daily oral doses of some quantitatively minor food phytochemicals reported to be present in diverse types of *Withania somnifera* extracts (Fumaric acid, quercetin and triethylene glycol) were also verified during these efforts [N. Shrivastava et al., 2015]. However, the question whether these observations more often made in our laboratories in normal mice and analogous ones often reported by others could also be valid in hyperglycemic and other laboratory rodents, or in clinical trials, still remained open. Since larger samples of body fluids and organs (necessary for more detailed mechanistic studies with phytochemicals and plant extracts acting directly and indirectly

acting on many organs, receptors and other biological targets) can easily be obtained from rats, several such experiments were also conducted in diabetic rats.

In this chapter, the results of the mice experiments reported in this thesis are discussed and interpreted first, and thereafter those conducted in diabetic rats are dealt with.

5.1. Mice studies

5.1.1. Pilot methodological and dose finding experiments: Dose finding experiments conducted earlier with a commercially available *Withania somnifera* root extract in our laboratories had revealed that 25 mg/kg daily oral doses of the extract high enough for in increasing stress resistance in normal and diabetic mice, as well as counteracting hyperglycemia and hypoinsulinemia in diabetic animals [A.K. Thakur et al., 2015a]. Diverse spectrums of so called "central sensitivity syndromes" are encountered in patients suffering from, or at risk to, numerous chronic and slowly and silently progressing metabolic disorders, and that hypersensitivity to pain is more often encountered in such patients [M.B. Yunus, 2015]. Therefore, the very first pilot cum dose finding experiment (results of which have already been published [A. Dey et al., 2016a] reported in this thesis was conducted for verifying whether or not daily oral doses of WSR lower than 25 mg/kg could as well be used as an herbal alternative for prevention and cure of such syndromes. The design of the experiment was based on the observation made in a study comparing analgesic, anti-inflammatory, and anxiolytic and antidepressants like activities of daily low oral doses (20 mg/kg/day) of aspirin and mono-hydroxybenzoic acids encountered also in *Withania somnifera* extracts [S.A. Khan et al., 2015; 2016]. Those experiments, had revealed that although after such low daily doses aspirin like suppressive effects of stress trigger central hypersensitivity to pain and other responses are quantitatively and qualitatively similar to those of all three mono-hydroxy benzoic acids, only aspirin possess both anxiolytics and

antidepressants like activities in conventionally known rodent models (elevated plus maze and forced swimming tests) often used for obtaining drug leads.

From the results described in the subsection 4.1.1 of the previous chapter of this thesis it is apparent that daily oral 10 mg/kg WSR doses afford maximally possible protection against unpredictable foot shock stress-triggered elevation in basal core temperature, and that effectiveness of such treatments against all other stress responses quantified increases steadily with increasing numbers of treatment days. Eleven daily treatments with this WSR dose also suppressed depressive behaviour in the tail suspension test. Although this test is still often used for identifying therapeutic leads potentially useful for treatments of depressive disorders, it is now well recognized that this test system used in the experiment is not very specific for quantification of depressive state of rodents [V. Krishnan and E.J. Nestler, 2011]. Moreover since this daily dose also facilitated sleep induction by a hypnotic dose of pentobarbital, it could as well be that the observed low dose effects of WSR in the tail suspension test is due to its sedative or calming effects. *Withania somnifera* has since long been well recognized to be a sleep inducing plant without sedative or hypnotic effects [Archana et al., 2015; U. Pingali et al., 2013; B. Choudhary et al., 2015], It has recently been pointed out indeed, that while most herbal adaptogens possess stimulating effects, *Withania somnifera* is a calming adaptogenic herb [D. Winston and S. Maimes, 2007]. Since several preclinical and a few clinical studies have consistently revealed and re-confirmed beneficial effects of diverse types of *Withania somnifera* extracts against stress triggered psychopathologies leading to sleep disturbance [K. Chandrasekhar et al., 2012; G.A. Ashok and M.B. Shende, 2015; Archana et al., 2015; M.A. Pratte et al., 2014; N.J. Dar et al., 2015; R. Wadhwa et al., 2016], the bioassay procedure used in this experiment seems to be well

suited for comparing bioactivity profiles of diverse types of plant extracts obtainable from *Withania somnifera* and other adaptogenic plants.

One novel and therapeutically interesting result of the experiment was that the highest tested WSR doses (40 mg/kg/day) observed after one single oral treatment possesses centrally acting analgesics like effects in hot plate test conducted in mice preselected for uniformity of their responses in hot plate test and also foot shock stressed once (i.e. in multiply stressed mice). It was interesting to note also that such dose dependant effectiveness of the extract was also observed after its repeated daily lower oral doses (10 and 20 mg/kg/day), and that the antinociceptive effectiveness of it 40 mg/kg daily doses continued to increase after daily treatments. These observations are unlike those often reported for morphine and many other centrally acting analgesics, the pain killing effectiveness of which actually decreases after their prolong uses, and can even induce hyperalgesia [M. Lee et al., 2011]. Therefore, it seems reasonable to assume that WSR like extracts could be promising herbal alternative not only for pain killing but also for prevention of pain hypersensitivity, and that they could be a more realistic therapeutic options for combating co-morbidities of pain, depression, and other mental health problems.

Recent reports from other laboratories not only suggest such possibilities [A. Orrù et al., 2014; 2016], but also the possibility that *Withania somnifera* extracts could also be a therapeutic alternative for obsessive compulsive disorders [B.P. Kaurav et al., 2012]. In this later mentioned report, marble burying tests and single intra-peritoneal doses (25 mg/kg) of the extracts were used for quantifying their brain function modulating effects. Usefulness of this test for quantifying delayed anxiogenic effects of stress has also been recently reported [G. Dagytė et al., 2011; S. Kedia and S. Chattarji, 2014]. Observations made during standardization of two versions of the test under our laboratory conditions had suggested that

its standard version (more often used for pharmacological screening purposes) is sensitive enough for quantifying stress triggered alterations in anxiety or depressive sates of the mice, whereas its two-zone version is more sensitive for quantifying conventionally known antidepressants with anxiolytics activities [L.B. Nicolas et al., 2006]. Therefore, both versions of the test were included in the second pilot methodological cum dose finding experiment (the results of which has already been published [A. Dey et al., 2016b], and are described in more details in the subsection 4.1.2 of the results chapter of this thesis) conducted to reaffirm low dose protective effects of WSR against stress triggered alterations in body weight, thermoregulatory processes, and behavioural changes. Results of this test in the experiment suggest that the standard version of the test is sensitive enough for quantifying protective effects of 10 mg/kg daily oral WSR dose against stress triggered exaggerated state of anxiety and depression, whereas antidepressants or anxiolytics like effectiveness of this and lower daily WSR dose can also be estimated in the two-zone version of the test.

Although the predictive validity of the tail suspension test for antidepressants is well established, face and construct validity of the test has often been questioned [H.M, Abelaira et al., 2013]. Even though the predictive validity of marble burying test is not very specific for antidepressants, its face and construct validities as well as reliability and reproducibility has been well recognized for identifying potential therapeutics against obsessive compulsive disorders [D. Joel, 2006]. Observations made during efforts to verify reproducibility of the two versions of the test, and the results of the second dose finding experiment made with WSR suggest that the standard version of the test is well suited for quantifying alterations in obsessive compulsive behaviour of chronically stressed mice, and that its two zone version is more sensitive for detecting modulating effects of fairly low oral doses of substances with brain function modulating activities.

Amongst all animal models now available for assessing and quantifying the depressive state of laboratory rodents and neurobiological basis of depression, the ones most often used in many laboratories for identifying antidepressants like activities of test agents are those using mice and rats subjected to chronic unpredictable or mild stress [P. Willner, 2017a; 2017b]. For such purposes after exposing the animals for more than two or three weeks to diverse stressful conditions and often water and food deprived (or restricted) they are tested in diverse behavioural and other models and diverse physiological parameters and biomarkers are quantified during the course of the experiments [S. Monteiro et al., 2015]. Such experimental procedures are not only time consuming, but also necessitates elaborate laboratory facilities not readily available in most herbal research laboratories like ours interested in better understanding of psychopharmacology of traditionally known medicinal herbs and their bioactive constituents. Observations made during the pilot methodological cum dose finding experiments with WSR, taken together with numerous others reported recently from our research groups [A.K. Thakur, et al., 2013a; 2013b; 2014a; S.A. Khan et al., 2016; S. Verma et al., 2017; 2015; V. Yadav et al., 2015; A.J. Langstieh et al., 2014; A. Shakya et al., 2014], strongly suggest that the foot shock stress paradigm and the experimental procedures used in these experiments are less time consuming, reliable, and reproducible alternative for quantifying the protective effects of numerous herbal extracts and their bioactive constituents against chronically stressed laboratory rodents.

One unexpected observation made during the second pilot experiment was that unlike in the first one, even a single higher oral WSR dose (40 mg/kg) significantly suppressed foot shock stress triggered transient hyperthermic responses. Such were also not the observations in an earlier dose finding experiment conducted with even higher dose (100 mg/kg) of another batch of commercially available *Withania somnifera* extract also standardized on is total

withanolides contents [A.K. Thakur et al., 2015a]. Since the bioactivity profiles of single or repeated daily doses of structurally diverse such individual molecules can vary in extracts obtained from different batches of the same plant part, this unexpected observation could as well be due to the presence of different quantities of structurally different withanolides in the two batches of the extract tested. Such will certainly be true for extracts obtained from different parts of the plant. Therefore, the next sets of experiments were conducted to compare the activity profiles of *Withania somnifera* extracts obtained by the same extraction and processing procedures from roots (WSR), stems only (WSS), and other aerial parts (WSA) of the plant. Results of these experiments are discussed in the following.

5.1.2. Adaptogenic potentials of three *Withania somnifera* extracts: Many aspects of brain functioning exhibit important sex difference that affect behaviour, mental health and mental disorders [P. Palanza and S. Parmigiani, 2017]. A vast majority of preclinical reports on adaptogenic or stress response modulating activity of *Withania somnifera* extracts and structurally and functionally diverse phytochemicals encountered in them have been conducted mainly in male laboratory animals. Therefore, in the first set of experiments comparing adaptogenic potentials of fairly high (but well tolerated) daily oral doses of WSR, WSS, and WSA using the same experimental procedures and methodologies were conducted for verifying whether their activity profiles in male and female mice are similar or not. Except for the observed hyperthermic effect of a single WSR dose males (see figure 4.3A in the result chapter), all other observed effects of the extracts, or of the reference drugs tested, there were negligible quantitative or qualitative differences in the observed activity profiles of any of the test agents. All stress triggered responses assessed in the experiments were also both quantitatively and quantitatively almost identical in males and females. These observations reaffirm that neither the stress responses nor the anti-stress effects of the tested

doses of the extracts and reference drugs depend on the sexes of mouse colony used in our laboratories.

These results revealed in addition, that such effects of the tested doses of aspirin, metformin, and nicotinic acid (niacin) in antagonizing body weight losses, basal core temperature, transient hyperthermic responses, and reaction times in hot plate test were both qualitatively and quantitatively quite similar to those of all the three extracts tested. However, such was not the observed effects of these three drugs in the tail suspension and pentobarbital hypnosis tests. These observations strongly suggest that not all biological targets and physiological mechanisms involved in the brain function modulation effects the extracts are the same as those involved in such effects of these three drugs often used for prevention and cure of diabesity (i.e. obesity associated diabetes) or metabolic syndromes associated physical and mental health problems.

It must be noted also, that unlike the extracts and these three plants derived drugs, other two reference drugs, i.e. the psychoactive drugs imipramine and diazepam, had no protective effects against stress triggered body weight losses. Otherwise, all other observed effects of imipramine, diazepam, and the extracts tested, except in the pentobarbital tests, were qualitatively similar. These observations suggest that the body weight regulating effects of the extracts and the threes reference drugs metformin, aspirin, and nicotinic acid are due to their protective effects against either stress triggered alterations in the digestive process, or hyperphagaia induced by fairly short durations of unpredictable foot shock stress, or both. Since body weights of stressed control groups continue to decrease steadily after further stress exposures, it seems reasonable to assume also that laboratory rodents are unable to adapt themselves or get habituated to the alterations in eating behaviour or digestive functions after repeated exposures to unpredictable foot shock stress for less than one minute.

Since protective effects of even very low daily oral doses of both WSR and its fraction devoid of withanolides (results discussed later) against stress triggered body weight losses were observed, it is evident that *Withania somnifera* extract constituents other than withanolides are also very potent stimulators of digestive processes supplying essential nutrients necessary for body weight gains. The observations that effectiveness of higher daily oral doses of all three extracts (with their total contents in withanolides varying between 1.5 and 3%) in shortening sleep induction and prolonging duration of sleep induced by pentobarbital were of the same order of magnitude indicate in addition, that all of them are almost equally active as modulators of biological processes and mechanisms involved in sleep inducing activities of the hypnotic. Results of two further comparative experiments discussed in the following are not only in agreement with these inferences, but also reaffirm that analytical standardization of *Withania somnifera* extracts on their contents of total withanolides cannot reasonably guarantee the consistency of their stress resistance increasing, or adaptogenic, potentials, or of many of their other therapeutically interesting bioactivities observed after their lower or higher daily oral doses.

The other two experiments conducted to compare the effectiveness of the three extracts after their five times lower daily oral doses (10 mg/kg/day) in the two bioassay systems used in the pilot dose finding experiments indicate that although qualitatively the bioactivity profiles of WSS and WSA are very similar to that of WSR, there might be some quantitative differences in their potencies. More definitive inferences from these observations are possible only when the dose response relationships of all three extracts between their 1 and 50 mg/kg daily oral doses are defined. It was interesting to note though, that effectiveness of 10 mg/kg daily WSR dose in both versions of the marble burying test was somewhat higher than WSA or WSS, and that WSS was the least effective one amongst the three tested extracts. Since the

total withanolides content in WSS (1.5%) was also the lower than in WSA (3.0%) or in WSR (2.7 %), it could as well be that the withanolides modulate the bioactivities of other *Withania somnifera* extract constituents (which constitute more than 95% of all three tested extracts).

The possibility that total contents of these chemotaxonomic markers biosynthesized and stores in different quantities in different parts and cultivars of the plant are not the major ones involved in traditionally known medicinal uses of *Withania somnifera* as tonics or for healthy life extension is apparent from the results of the other experiments. Results of one such experiment conducted in stressed mice are discussed next.

5.1.3. Adaptogenic activity of a *Withania somnifera* extract devoid of withanolides

Results of this experiments revealed that only 3.3 mg/kg/day is a maximally effective daily oral doses of withanolides free *Withania somnifera* roots extract (WFWS) in suppressing basal core temperature elevations, and that its 3.3 as well as 10 mg/kg/day doses partially protected the animals from stress triggered body weight losses and transient hyperthermic responses. However, both these lower daily doses of WFWS had no significant effects in both versions of marble burying test. Except for the observations made in the standard version of the test, such was also the observed effects of 10mg/kg daily oral doses of tri-ethylene glycol (TEG) used in the experiment as a reference antiviral agent. Since these doses of WFWS and TEG did not have any significant effects on relative weights of adrenal glands and mean values of circulating cortisol levels, it seems reasonable to assume that their low dose protective effects against stress triggered alterations in body weight and thermoregulatory processes are most probably due to their modulating effects on enteric nervous system regulated by gut microbiota and not entirely due stress triggered hyper-secretation of the hormone. The observation that unlike the same daily doses of TEG, 10 mg/kg daily WFWS treatments had no significant effects in the standard version of marble burying test could

indicate that this extract is a more potent stress response modulating agent than TEG. Observations made in a recently published study [N. Shrivastava et al., 2015], had revealed that although even 5mg/kg daily TEG doses possess anxiolytics or antidepressants like activities in tail suspension test, unlike quercetin (one of the many plant phenolics encountered also in *Withania somnifera* extracts), this dose of the structurally simple virucidal and bactericidal agent does not have any significant effects in both versions of marble burying test in stressed mice. Therefore, it seems reasonable to assume that even if TEG would be a bioactive constituent of WFWS, most probably it is not one of the more potent brain functions modulating bioactive constituent of the extract involved in its antidepressants and anxiolytic activities.

In any case, the observed adaptogens like activity profile of WFWS reaffirm that presence of withanolides in *Withania somnifera* extracts is not essential for increasing stress tolerance, and that its activity profile is qualitatively as well as quantitatively very similar to those of withanolides containing extracts as stress resistance increasing agents. Results of the more detailed experiments, to be discussed hereafter, added further experimental evidence supporting this inference. Although numerous vitamins and food phytochemicals other than withanolides encountered in *Withania somnifera* have already been identified as stress resistance increasing substances, as yet little concentrated efforts have been made to identify rodent bioassays necessary for quantifying possible synergetic or antagonistic interactions between them after their daily oral intakes. Therefore a further set of analogous experiments were conducted to verify whether or not marble burying tests in stressed mice could also be used for quantifying such possible interactions between WSR and TEG co-administered for several days. Results of these experiments are discussed in the following.

5.1.4. The co-administration experiments

To date, only one report revealing carcinostatic potentials of orally administered TEG in experimental animals has appeared [R. Wadhwa et al., 2013]. However, due to its bactericidal and virucidal activities, this toxicologically safe and water soluble dehydrating agent is often used in sprays for air disinfection purposes [O.H. Robertson, 1949]. A more recent report reaffirming that TEG is highly effective in inactivating influenza viruses [S.N. Rudnick et al., 2009], suggests that it could also be used for prevention of influenza pandemics. Microbiologists and virologists have now well recognized that differential host response plays a crucial role in the pathogenicity of influenza viruses [P.S. Askovich et al., 2013], and many molecular determinants of influenza pathogenesis have also been identified inside their digestive tract of mice as well [R.P. Kamal et al., 2014]. Many of the easily quantifiable clinical features of influenza and other viral or bacterial infections, i.e. exaggerated pain sensitivity, myalgia, malaise (sickness feelings and related behaviours) anorexia (characterized by body weight losses), altered thermoregulation etc., are easily quantifiable in diverse bioassay procedures using foot shock stressed mice.

Persistence of such symptoms often leads to metabolic disease and other chronic diseases and their syndromes. Both *Withania somnifera* extracts [V.B. Trivedi et al., 1981; M Owais et al., 2005; L. Kambizi et al., 2007; M. Pant et al., 2012] as well as TEG [W. Lester et al., 1952; 1949; S.N. Rudnick et al., 2009] have since long been well recognized to possess bactericidal and virucidal activities. Very recently, it has been reported also that unlike two withanolides (Withanone and Withaferin F) triethylene glycol and water soluble *Withania somnifera* leaf extracts increases non-rapid eye movement sleep in mice in a dose-dependent (10-30 mg/mouse) manner [M.K. Kaushik et al., 2017]. This repot suggests also that triethylene glycol is an active sleep-inducing component of Ashwagandha leaves that could potentially

be useful for insomnia therapy. Observations made an earlier dose finding experiment in our laboratories [N. Shrivastava et al., 2015] that daily oral doses of TEG between 5 and 400 mg/kg in stressed mice for eleven days dose dependently shortens sleep induction time induced by pentobarbital, and also significantly prolongs the duration of sleep induced by the hypnotic [N. Shrivastava et al., 2015]. Therefore, these threes experiments were conducted to verify whether combinations of TEG with *Withania somnifera* extracts could also be an alternative for prevention of physical and mental health problems often accompanying or caused by sleep disturbances accompanying adverse health conditions [C.M. Baldwin et al., 2010].

Results of these experiments revealed that even 1 or 3 mg/kg daily oral WSR doses are highly effective in antagonizing unpredictable foot shock stress triggered elevation of basal core temperature and bodyweight losses in mice. Both these and the two other quantified stress response suppressing effects of very low WSR doses (i.e. against transient hyperthermic responses and marble burying behaviour in the standard version of the test) were also potentiated dose dependently by increasing co-administered TEG doses. However, although the observed effects of 10 mg/kg daily doses of WSR in the two zone version of marble burring test were also more pronounced in all TEG co-administered groups, such co-administration effects of TEG did not increase with its increasing co-administered daily doses. These and the observed effects of treatments on adrenal gland weights and circulating cortisol and glucose levels indicate that co-administration of TEG can slightly potentiate stress resistance increasing effects of WSR, but not the anxiolytic or antidepressants like effectiveness of the extract.

An alternative theoretical possibility for explaining this observation could as well be that the effectiveness of WSR, or of higher TEG daily doses, in suppressing marble burying

behaviour decreases by their co-administrations. Such negative interactions between bioactive constituents of herbal extracts have often been observed in our laboratories [A.K. Thakur et al., 2015b; 2015c; S. Verma et al. 2016] and elsewhere. In any case, high effectiveness of extremely low daily oral doses of WSR in antagonizing stress triggered bodyweight loses and alterations in physiological thermoregulatory processes observed in these experiments reaffirm that all biological processes and mechanisms involved in these effects of WSR are most probably not identical to those involved in antidepressants and anxiolytics like activities of its higher daily doses. Since the observation made with very low and high daily oral WFWS doses were also quite analogous to those of WSR, it seems reasonable to assume that withanolides are not the major *Withania somnifera* metabolites involved in the broad spectrums of therapeutically interesting bioactivity and safety profiles its extracts. This inference is further supported by the results of the rat experiments discussed in the following.

5.2. Experiments in diabetic rats

These final sets of experiments were conducted for identifying pharmacological targets and mechanisms potentially involved in stress resistance increasing effects of WSR and WFWS, and to verify whether *Withania somnifera* extracts could therapeutic alternatives for prevention and cure of diabetes associated comorbidities. Choices of the parameters quantified and behavioural models for these experiments were based on current knowledge on the modes of actions of *Withania somnifera* and other adaptogenic or stress response suppressing medicinal plants and their bioactive constituents. Hereupon, due attention was paid to the fact that almost all plant extracts, adaptogenic or not, possess anti-oxidative and bactericidal activities, and to the possibility that their brain function modulating activities [D.O. Kennedy, 2014a; 2014b; V. Murugaiyah et al., 2015] could as well be due to their

modulating effects on physiological functions of the so called "gut-microbiota-brain axis" or "the second brain" [K. Winek et al., 2016; E.A. Mayer, 2011; P.C. Konturek et al., 2011; V. Ridaura and Y. Belkaid, 2015; B. Herpertz-Dahlmann et al., 2017]. Currently available preclinical and clinical information on metformin, strongly suggest that some of the pharmacological targets of this antidiabetic drug of first choice also reside inside the digestive tract [L.J. McCreight et al., 2016; H. Wu et al., 2017]. Observations made during efforts in our laboratories to identify metformin like bioactive constituents of Ayurvedic medicinal and food plants often used in Ayurvedic formulations for prevention and cure of diabetes associated co-morbidities [V. Kumar et al., 2015b] have revealed and reaffirmed that its 50 mg/kg daily oral doses in mice is high enough for its antidepressant or anxiolytics like activities in tail suspension test conducted in occasionally foot shock stressed mice [S. Verma et al., 2017; V. Tiwari et al., 2016] Therefore, in these comparative experiments, effectiveness of 10 mg/kg daily WSR and WFWS doses were compared with this daily dose of metformin for ascertaining reproducibility and predictive validity of the bioassay systems used in these rat experiments.

Results of these experiments reaffirm that the foot shock stress paradigm could also be used to identify and compare metformin like effectiveness of adaptogenic plant extracts differing in their chemical constituents for prevention and cure of depression, anxiety and other mental health problems commonly associated with diabetes and almost all other metabolic disorders. They reaffirm also that like diabetic patients, diabetic rats are also hyper-responsive to unpredictable adverse stimuli and that the bioassay procedures used in these experiments are well suited for quantifying the effects of drugs and potential drug leads on diverse biomarkers currently often used in clinical trials for diagnostic and drug development purposes and epidemiological studies. As judged by the results of the more specific behavioural model for

anxiety state of rats, i.e. elevated plus maze test, stress triggered worsening of this state were more pronounced in stressed diabetic control group than in stressed non-diabetic control group. Somewhat similar, but much less pronounced, difference in the depressive states of these two groups were observed in the more specific model for depressive state, i.e. forced swimming test. In general, almost all stress response parameters quantified in these experiments were indicative of exaggerated stress responsiveness, or increased allostatic load, in diabetic rats. These observations made in stressed and diabetic and non-diabetic rats are in agreement with our current knowledge on the etiology, pathogenesis and progression of almost so called lifestyle disorders, including diabetes and depression [R.P. Juster et al., 2010; A. Steptoe, et al., 2014; M. Picard, et al., 2014; A.C. Logan, 2015].

Although the observed protective effects of low daily oral doses (10 mg/kg/day) of WFWS and WSR on all stress triggered responses quantified in diabetic rats (35 in numbers) were qualitatively similar to those of 50 mg/kg/day metformin, there were several quantitative differences between their observed effectiveness (see **Table 5.1**). A most notable qualitative difference between the activity profiles of the tested extracts and the antihyperglycemic drug metformin was that after metformin drug had no significant effects in the marble burying test. Otherwise, their observed activity profile against all quantified parameters was qualitatively similar to those of the tested *Withania somnifera* extracts. Since the marble burying test is more specific for drugs often used for treatment of obsessive compulsive disorders, it seems that unlike the *Withania somnifera* extracts, metformin is not well suited for treatments of such disorders accompanying diverse chronic diseases and illnesses. It remains certain, though that like all herbal adaptogens, metformin also possess anxiolytics and antidepressants like, and stress response suppressing activities in diabetic rat. Moreover, our observations reaffirm that like *Withania somnifera* extracts (devoid of or containing withanolides),

metformin is also effective as an oxidative stress resistance increasing agent after its repeated daily therapeutically interesting oral doses.

Table 5.1: Comparative protective effectiveness of daily treatments with the tested oral doses of withanolides containing (WSR), withanolides free (WFWS) *Withania somnifera* extracts and metformin against diverse quantified parameters in the diabetic rat experiments.

Quantified Parameters	**Comparative effectiveness**		
	WSR (10 mg/kg/day)	**WFWS (10 mg/kg/day)**	**Metformin (50 mg/kg/day)**
First experiment : Anti-stress activity			
Change in body weight	Highest	Intermediate	Lowest
Basal rectal temperatures	Almost similar and maximally possible effect		
Stress induced hyperthermia	Highest	Intermediate	Lowest
Blood glucose level	Intermediate	Lowest	Highest
Blood insulin level	Highest	Intermediate	Lowest
Blood corticosterone level	Highest	Lowest	Intermediate
Cholesterol	Intermediate	Lowest	Highest
Triglyceride	Intermediate	Lowest	Highest
LDL Cholesterol	Intermediate	Lowest	Highest
HDL Cholesterol	Intermediate	Lowest	Highest
Inducible nitric oxide synthase (iNOS)	Intermediate	Highest	Lowest
Nitric oxide (NO)	Intermediate	Highest	Lowest
Nuclear factor kappa beta (NF-κβ)	Intermediate	Highest	Lowest
Second experiment : Anti-depressant activity			
Change in body weight	Highest	Intermediate	Lowest
Basal rectal temperatures	Almost similar and maximally possible effect		
Stress induced hyperthermia	Highest	Intermediate	Lowest
Marble burying behaviouur	Highest	Intermediate	No effect
Immobility period (forced	Highest	Intermediate	Lowest

swimming test)			
Spleen weight	Almost similar effect		
Adrenals weight	Almost similar effects		
Blood glucose level	Intermediate	Lowest	Highest
Blood insulin level	Highest	Intermediate	Lowest
Blood corticosterone level	Almost similar effects		
Lipid peroxidation (LPO) in blood	Almost similar effects		
Superoxide dismutase (SOD) activity in blood	Highest	Intermediate	Lowest
Catalase (CAT) activity in blood	Intermediate	Lowest	Highest
Glyoxalase 1 activity	Intermediate	Lowest	Highest
Paraoxonase 1 activity	Intermediate	Lowest	Highest
Lipid peroxidation (LPO) in brain tissue	Highest	Intermediate	Lowest
Superoxide dismutase (SOD) activity in brain tissue	Highest	Intermediate	Lowest
Catalase (CAT) activity in brain tissue	Highest	Lowest	Intermediate
Norepinephrine	Almost similar effects		
Dopamine	Almost similar effects		
5-hydroxytryptamine	Almost similar effects		
Monoamine oxidase (MAO)	Highest	Intermediate	Lowest
Gastric ulceration	Almost similar effects		
Third experiment: <u>Anti-anxiety activity</u>			
Change in body weight	Highest	Intermediate	Lowest
Basal rectal temperatures	Almost similar and maximally possible effects		
Stress induced hyperthermia	Highest	Intermediate	Lowest
Locomotor activity test	Highest and almost similar effects		Lowest
Elevated plus maze test	Highest	Intermediate	Lowest

Spleen weight	Almost similar effects		
Adrenals weight	Almost similar effects		
Blood glucose level	Intermediate	Lowest	Highest
Blood insulin level	Highest	Intermediate	Lowest
Blood corticosterone level	Almost similar effects		
Lipid peroxidation (LPO) in blood	Highest	Almost similar effects	
Superoxide dismutase (SOD) activity in blood	Highest	Almost similar effects	
Catalase (CAT) activity in blood	Highest	Lowest	Almost similar to WSR
Glutamic oxaloacetic transaminase (GOT)	Almost similar effects		
Glutamic pyruvic transaminase (GTP)	Almost similar effects		
Glyoxalase 1 enzyme activity	Intermediate	Lowest	Highest
Cholinesterase activity in blood	Almost similar effects		Lowest
Cholinesterase activity in cortex	Highest	Intermediate	Lowest
Cholinesterase activity in hippocampus	Highest	Intermediate	Lowest
Gastric ulceration	Almost similar effects		Lowest

One major goal of the rat experiments was to identify clinically relevant biomarkers that could be used for reaffirming therapeutic potentials of *Withania somnifera* extracts against diabetes associated co-morbidities in appropriately controlled clinical trials. All circulating enzymatic activities and other biomarkers of oxidative stress quantified in the experiments in stressed diabetic rats were always indicative of exaggerated oxidative state of diabetic animals. Quantitatively, protective activity profiles of the same daily oral doses of both WSR

and WFWS against all quantified oxidative stress parameters were also very similar. Therefore, it is apparent that presence of absence of withanolides in *Withania somnifera* root extracts is not very relevant for judging their oxidative stress resistance increasing potentials or of their therapeutically interesting stress resistance increasing activities observed after their fairly low daily oral doses (only 10 mg/kg/day). Moreover, it can also be said that quantification of enzymatic activities of the peroxygenase PON 1, Glyoxalase-1, and inducible nitric oxide synthase (iNOS) in blood samples are some of the more easily and cost effectively quantifiable parameters for assessing oxidative stress resistance increasing potential of *Withania somnifera* extracts and metformin.

It is now well recognized that oxidative stress is an important contributor in the development of chronic complications in diabetes mellitus [S. de M Bandeira et al., 2013; D. Ziegler et al., 2015], and that oxidative stress plays a major role in diabetes as well in Alzheimer's disease and other related neurological diseases and mental health problems [V.P. Reddy et al., 2009; S.M. de la Monte and J.R. Wands, 2008; F. Ng et al., 2008]. A large number of publications studying the relationship between hormonal status and insulin resistance or diabetic states have shown a correlation between ghrelin and insulin resistance or diabetes mellitus [O. Ukkola, 2011]. In addition, ghrelin is linked to neuro-modulation, neuro-protection, and cognitive processes [W.N. Cong et al., 2010]. Role of serum levels of the butyrylcholinesterase in regulating ghrelin homeostasis and its physiological functions [C. De Vriese et al., 2004; E. Oda, 2016; E.W. Randell et al., 2005; T.D. Müller et al., 2015] has been well recognized, and estimation of serum level of this enzyme as a prognostic marker for diverse malnutrition triggered medical conditions has also been proposed [L. Santarpia et al., 2013, E.W. Randell et al., 2005; L. Shi et al., 2017; A.R. Allam et al., 2006]. Although inhibitory effects of withanolides, and diverse types of extracts obtained from *Withania*

somnifera and diverse other plant extracts on cholinesterase activity have often been reported [M.I. Choudhary et al., 2004; R. Tundis et al., 2016; H. Khan et al., 2011], by far a vast majority of them deal mainly with their inhibitory effects of acetyl-cholinesterase, and too often using *in vitro* bioassays and fairly high concentrations of test agents.

Results of the reported third experiment (see **Figure 4.52**) revealed that fairly low daily oral doses of *Withania somnifera* extracts containing or devoid of withanolides on circulating as well as brain levels of enzymatic activities of both acetyl- and butyryl- cholinesterase. In this experiment, the estimated activity levels of both the choline ester hydrolyzing enzymes in stressed diabetic rats were much higher than those observed in similarly stressed non-diabetic animals. Enzymatic activities of both acetyl- and butyryl-cholinesterase in the brain or circulating blood in stressed diabetic groups treated with WSR or WFWS were always almost similar to those of estimated in the non-stressed non-diabetic one, and analogous (but somewhat lower) effects of metformin were also observed in the experiments. However, as anti-hyperglycemic or anti-hypoinsulinemic agents, metformin was more effective than WSR or WFWS. These observations strongly suggest that modulation of the physiological functions and biological processes regulated by circulating acetylcholine is involved in the modes of actions *Withania somnifera* extracts and metformin, and that both the tested extracts are several folds more effective as allostatic load modulators than as anti-hyperglycemic or anti-hypoinsulinemic agents.

It is now well recognized that oxidative processes and cholinergic mechanisms are involved in the etiology, pathogenesis, and progression of almost all slowly progressing metabolic disorders and stress triggered pathologies, including Alzheimer's disease and other neurological diseases associated with caused by metabolic disorders [M. Ilenia et al., 2017; A.R. Allam et al., 2006; B. Benyamin et al., 2011]. Butyrylcholinesterase (BChE) plays a

pivotal role in physiological regulating of bodyweights, and thermoregulatory metabolic processes by modulating the hormonal and signaling functions of the orexigenic peptide ghrelin [S. Brimijoin et al., 2016; V.P. Chen et al., 2016; 2017]. The observations that Almost complete preventive effects of metformin and of the tested *Withania somnifera* extracts against oxidative stress triggered elevation of BChE activities in both blood serum and brain strongly suggest that either the biosynthesis and of the enzyme or other physiologic processes regulating BChE activity in different bodily compartments are involved in their broad spectrums of therapeutically interesting bioactivity observed after their therapeutically relevant daily oral doses. In any case, it can safely be said that quantification of serum BChE activity is also another feasible means for assessing allostatic load or stress resistance increasing potentials of *Withania somnifera* extracts and metformin.

Several earlier reports have often speculated that modulation of the functions of central cholinergic processes are also involved in the systems poly-pharmacology of metformin and other antidiabetic drugs and antioxidants, and a more a report have revealed indeed that metformin is a fairly potent inhibitor of AChE than of BChE [M. Markowicz-Piasecka et al., 2017a; 2017b; D.K. Mostafa et al., 2016; J.A. Saliu et al., 2016; N. Ashokkumar et al., 2007]. However, in some such reports, no significant effects of 100 or 300 mg/kg daily oral dose of metformin on AChE activities in diabetic or scopolamine treated rats were observed. On the contrary, daily oral doses of only 50 mg/kg of the antidiabetic drug almost completely suppressed also the elevated circulating as well as brain levels of both AChE and BChE in stressed diabetic rats. Since the brain permeability of low dose metformin is almost negligible, these observations strongly suggest that observed effects of metformin on brain biochemistry and functions are not due to its ability to inhibit effects on AChE, and that like *Withania somnifera* extracts and numerous other plant derived products and phytochemicals

with ant-oxidative and stress response suppressing activities, metformin is also a modulator of physiological processes regulating allostatic load.

Stress triggered alterations in brain functions are often reversible in healthy individuals and which, therefore, do cause longer lasting effects on brain and metabolic functions [B.S. McEwen, 2007; 2013]. However, patients suffering from or at risk to diabetes and other metabolic disorders are more susceptible to stress triggered psychopathologies which can be quantified by appropriate uses of diverse combinations of stress biomarkers [R.P. Juster et al., 2010; B.S. McEwen, 2015]. Observations reported in this thesis reaffirm that such is indeed the case also for quantifying allostatic load in diabetic rodents, and reveal that together with quantification of stress triggered longer lasting alterations in body weight and thermoregulatory processes, quantification of the several enzymatic activities in serum could also be a reliable means for quantifying the allostatic load modulating effects of Withania *somnifera* and metformin. Moreover it can also be said that withanolides are not the only bioactivities metabolites of the plant, and that observations made in cellular and other in vitro systems are not very reliable predictors of therapeutic potentials of *Withania somnifera* extracts and their already known or unknown bioactive constituents.

5.3 Lessons for quantitative systems pharmacology

Despite considerable efforts of medicinal phytochemists and pharmacologists during the past few decades, many questions concerning the bioactive constituents and modes of actions of medicinally used *Withania somnifera* extracts and almost all other herbal remedies often used in Ayurvedic and other traditionally known systems of medicine still remain open. Answering this question is necessary not only for obtaining reproducible and reliable therapeutic benefits from them, but also for better understanding of their modes of actions and therapeutic possibilities offered by them. Availability of pharmacologically well

validated bioassays and biomarkers and well standardized experimental procedures (necessary for better understanding of systems pharmacology of multi-targeted phytochemicals and their combinations) are essential prerequisites for rationally answering such questions.

Observation in our laboratories and elsewhere have revealed and reaffirmed that repeated oral dose studies are necessary for such purposes [V. Kumar and SS. Chatterjee, 2014] and that *Withania somnifera* is one of the many plants potentially useful for obtaining novel therapeutic leads for prevention and cure of diabetes associated co-morbidities [A. Dey et al., 2017]. It is apparent from the reported and discussed observations that the foot shock stress paradigm used in the experiments is well suited for not only better understanding of quantitative systems poly-pharmacology of *Withania somnifera* extracts, their subtractions, and many other currently commercialized drugs. Amongst all reference drugs used in these experiments, the observed pharmacological activity profile of metformin was quite similar or analogous to those of diverse types of *Withania somnifera* extracts and their subtractions tested. These observation justify the classification of *Withania somnifera* as an anti-diabetic herb, and suggest that appropriate combination of a pharmacologically well standardizes *Withania somnifera* extract and metformin could as well be a better alternative than diverse combinations of the antidiabetic drug, aspirin, statins, and other drugs currently often commercialized or prescribed for prevention and cure of diabetes and other metabolic disorders associated of physical and mental health problems.

For such purposes, the extraction and bioassay procedure used for the initial dose finding study for estimating the therapeutically interesting and safe dose ranges of WSR can be warranted. The observation that activity profiles of the extracts obtained from parts of the plant other than roots are almost identical in both male and female animals, suggest that

stems and areal parts of *Withania somnifera* are also well suited as alternatives for traditionally known medicinal uses of its roots. These and other reported observation reaffirm also that analytical standardization of *Withania somnifera* extracts on their contents in withanolides (i.e. the chemotaxonomic markers of the plant) is appropriate only for ascertaining the parts of the plant used in preclinical and clinical studies, but are not very useful for predicting their therapeutic potentials and medicinal values, or for better understanding their quantitative systems pharmacology. Taken together with our current knowledge on medicinal phytochemistry and pharmacology of the plant, reaffirm only that bioactivity profiles of *Withania somnifera* extracts devoid of withanolides are qualitatively very similar. But as yet no definitive statements on the bioactive constituents of WFWS can be made. For better understanding of quantitative systems pharmacology of the extractives of the plant, further collaborative efforts between medicinal phytochemists and experimental pharmacologists will still be necessary.

Observations made to-date with TEG and numerous other bacteriostatic constituents of diverse types of extracts of the plant in our laboratories and elsewhere strongly suggest that that almost all of them are non-systemically acting drugs like substances and that physiological processes and mechanism regulating stress response and allostatic load are involved in their modes of actions. Although oral bioavailability of metformin is much higher than those any other known metabolites of the plant, it is now well recognized that its some major pharmacological targets also reside inside the gastrointestinal tract and that its modulating effects on physiological functions of gut microbiota are also involved in its modes of actions [R. Burcelin, 2013]. Observations made in diabetic rat experiments reaffirm this inference, and strongly suggest that estimation of serum BChE could be used for judging the therapeutic effectiveness of metformin and diverse types of *Withania somnifera* extracts

for prevention and cure of diabetes and associated co-morbidities, or for better understanding of quantitative systems pharmacology of such extracts and their bioactive constituents. Knowledge and knowhow evolving from efforts necessary for such purposes will be useful not only for translating Ayurvedic pharmacology in terms of modern medical sciences, but also for obtaining therapeutic leads from traditionally known medicinal plants and other drug leads designed according to the post modern concepts of systems biology and evidence based medicine.