CHAPTER-5

DISCUSSION

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5.1. Pilot study

Adaptogens are well tolerated, bioactive substances capable of counteracting or preventing homeostatic disturbances triggered by metabolic, environmental and mental stress. Adaptogens improve the response to stress and help the body to adapt by normalizing physiological processes during increased stress condition. A. Panossian and G. Wikman, 2009 states that adaptogens appear to exert their anti-stress effects by regulating homeostasis via the hypothalamic pituitary adrenal axis and inhibiting or decreasing circulating levels of nitric oxide and corticosterone. Results of pilot experiments reveal that amongst all parameters quantified in the bioassay using foot shock stressed mice, the one most effective was elevation of their core temperature which was antagonized by CLE-3R or metformin after their repeated daily dose. Although their acute oral dose had no significant effects on basal core temperatures of male or female mice, even the lowest tested daily oral dose of CLE-3R (5 mg/kg) administered for 5 consecutive days afforded complete protection against foot shock stress triggered elevation of basal core temperature than observed in stressed control animals on that observational day. Although the basal rectal temperatures of vehicle treated control animals increased further on subsequent test days. Basal core temperature of treated animals remained physiologically normal on all test days, even after the administration of highest (320 mg/kg/day) dose of CLE-3R. It has since long been well recognized that core temperature measurements is a feasible and reliable means for assessing physical fitness [E.F.J. Ring 1998; D.S. Moran and L. Mendel 2002]. Extensive oral bioavailability studies conducted with curcumin and other curcuminoids [P. Anand et al., 2007; H.F. Ji and L Shen 2014] have revealed that even after their very high doses (up to 1g/kg) their blood levels are undetectable in mice after 6 hours of their oral doses. Although,

most reports dealing with therapeutically interesting bioactivities of orally administered curcumin and curcuminoids have dealt mainly with their fairly high acute or repeated daily oral doses, a few others have revealed diverse bioactivities of their oral doses lower than 5mg/kg/day. Except for their observed efficacies in tail-suspension test for antidepressants and some other bioassays [Y. Xu, et al., 2005a; Y. Xu et al., 2005b] most other low dose efficacies of curcumin and curcuminoids have been observed after their repeated daily doses only. The observations reported in a recent and other more systematic study reconfirm this inference for their orally administered doses [X. Zhao et al., 2014]. Since like other edible phytochemicals, curcuminoids also modulates diverse cellular pathways involved in adaptive stress responses [H. Lee and G. Ko 2014]. It seems reasonable to assume that the low dose efficacies of curcuminoids observed after their repeated daily oral doses are due to their adaptogenic or stress response suppressing effects, and that some of their pharmacological sites of actions lie within the gastrointestinal tract. In any case, results obtained from pilot study revealed that CLE-3R is a metformin like stress response suppressing agent, and that in both male and female animals their efficacies increased with increasing number of daily oral doses. In general, efficacies of 20 mg/kg CLE-3R on all quantified parameters in the bioassay were similar in magnitude to those observed after 100 mg/kg daily oral metformin doses. Hereupon, their relative efficacies in male and female mice were not always equal in magnitude. After 10 daily oral doses, efficacy of 100 mg/kg/day metformin in antagonizing stress triggered body weight losses in females were somewhat higher than that observed in males, and on this day the efficacy of 5 mg/kg CLE-3R in both male and female mice were similar in magnitude. Such was not the case in stress induced hyperthermia test, where efficacy of 10 daily doses of CLE-3R 5mg/kg/day in females was somewhat lower than that observed for the tested dose of metformin. The observation revealed that the dose dependent

efficacies of 10 or 11 daily doses of CLE-3R in stress induced hyperthermia, tail suspension, and pentobarbital induced sleep tests increased further after its 80 and 320 mg/kg/day doses in both males and females. Observation indicates that its efficacies in these tests are most probably independent of its observed protective effects against intermittent foot shock triggered body weight losses and slight elevation in core temperature. Observation revealed that repeated daily oral doses of even 5 mg/kg CLE-3R is high enough as a stress response modulating agent, and strongly suggested that traditionally known medicinal and dietary uses of curcuminoids containing turmeric preparations are mainly due to their allostatic load regulating properties. It could be possible that observed stress response modulating efficacies of metformin and CLE-3R are mainly due to their antimicrobial activities, whereupon their high chemical reactivity and metabolic instability inside the gastrointestinal tract play crucial roles to obtain its therapeutic effect [N.R. Shin et al., 2014; Y.J. Wang et al., 1997].

Results obtained from comparative pilot study with different *Curcuma longa* extracts revealed that oral efficacy of CLEs are not only depends on the concentration of curcuminoids. It could also be due to some other moieties co-administered with it. We found CLE-3R 5 mg/kg shown more pronounced stress desensitizing activity as compare to other taken extracts. Solvent system used for the extraction procedure of *Curcuma longa* extracts (viz. CLE-1H, CLE-2B, CLE-3R and CLE-4M) could also be a responsible factor which affects their oral efficacy. CLE-1H and CLE-2B are water soluble extracts and contain lower concentration of curcuminoids shown always lower stress response counteracting effect than pure curcumin and CLE-3R which was enriched with curcuminoids. It has also been reported that ethanol solvent is more stable and efficacious for the curcuminoids extraction because it inhibits the decomposition of curcuminoids and curcumin [S. Revathy et al., 2011]. Some reports revealed that methanol or ethanol solvent is more appropriate for curcuminoids

separation although hexane solvent is mainly use for separation of sesquiterpenes or its essential oil. Therefore it could be possible that all four extracts have shown different oral efficacy due to different solvents system taken for their extraction procedure.

Reported observations reconfirm that 5 mg/kg daily oral doses of curcumin, or of turmeric curcuminoids in general, are high enough for protecting mice against chronic and unpredictable foot shock stress triggered alternations in body weight and thermoregulatory processes. This observation reveals that phytochemicals present in turmeric oils devoid of curcuminoids also possess analogous protective effects. However, CLE-3R like dose dependant protective effects of turmeric oil against stress triggered body weight losses were observed after its 3 mg/kg and other higher daily oral doses, its efficacy to afford protection against stress triggered elevations in basal core temperature, or foot shock stress triggered transient hyperthermic responses were observed only after its 30 mg/kg and higher daily doses. Moreover, unlike curcuminoids, even 100 mg/kg daily turmeric oil doses had no significant effects in the pentobarbital-induced sleep test. In the tail suspension test significant effects of the oil were observed only after its highest tested daily dose. Therefore it seems reasonable to assume that the biological processes and mechanisms involved in observed stress response suppressing activity profile of turmeric oil is not identical to those of turmeric curcuminoids and that effectiveness of turmeric curcuminoids as potential antidepressants are much higher than that of turmeric oil. Turmeric oil was devoid of curcuminoids, and was enriched in volatile secondary metabolite of the plant like turmerone, α-turmerone, curlone, and diverse other volatile ones. However, CLE-3R is highly enriched (95.4% w/w) in structurally analogous diarylheptanoids commonly referred to as curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) and devoid of turmeric volatiles. Since differences between bioactivity profiles of turmeric curcuminoids

and some known constituents of turmeric oil has also been observed in other laboratories [S.K. Sandur et al., 2007] it is apparent that the observed differences between stress resistance increasing activity profiles of turmeric oil and CLE-3R is due to the presence of structurally and functionally diverse bioactive constituents in them.

It was interesting to note that, protective effects of 5 mg/kg daily oral doses of purified curcumin (devoid of demethoxycurcumin and bisdesmethoxycurcumin, but containing some other minor impurities) against stress triggered body weight losses were observed after its 6 or more daily doses, whereas effect of the same dose of CLE-3R (5mg/kg) was apparent after its 4 daily doses. Analogous difference between the effects of CLE-3R and purified curcumin were also observed in foot shock stress induced hyperthermia test, and mean values of basal rectal temperature of the CLE-3R or the vehicle treated groups on the first treatment day were always lower than the purified curcumin treated group. Although the observed effects of 5 mg/kg daily dose of purified curcumin and CLE-3R in tail suspension test were quantitatively similar, such were not the observations obtained in the pentobarbital-induced hypnosis. These observed differences could as well be due to pharmacological interactions between curcumin and other turmeric constituents present in the CLE-3R. Hereupon, differences in oral efficacy or bio-accessibility or metabolic profiles of structurally diverse curcuminoids as well as differences in their bioactivity profiles [T. Ahmed and A.H. Gilani 2014] could play important roles in their respective action. In any case, it remains certain that apart from curcumin and other curcuminoids, *Curcuma longa* roots and rhizomes also possess other stress response suppressing constituents, and that traditionally known medicinal uses of diverse types of turmeric preparations must not be necessarily due to their curcumin or curcuminoids contents only. Our observations strongly suggest that regular intake of even fairly low daily oral doses of crude turmeric preparations can afford protection against

chronic mild stress triggered alterations in physiological processes and mechanisms regulating body weight and temperature.

Earlier observations in our laboratories have revealed that curcuminoids or turmeric oil like low dose stress response modulating effects of several other phytochemicals ubiquitously present in numerous edible or medicinal plants [S.A. Khan et al., 2015; A.J. Langstieh et al., 2014; N. Shivavedi et al., 2014; N. Shrivastava et al., 2014]. Salicylic and 4-hydroxybenzoic acids are just two examples of such phytochemicals also encountered in turmeric, and it has been recently suggested that salicylic acid could also be another health promoting bioactive constituent of turmeric [G.G. Duthie and A.D. Wood 2011; J.R. Paterson et al., 2006]. Therefore, it is apparent that traditionally known medicinal uses of turmeric preparations, or for any herbal preparation, do not solely depend on one or a few of their bioactive constituents and that proper understanding of biological interactions between them is essential for more rational understanding of their medicinal values or for obtaining novel therapeutic leads urgently needed for prevention and treatment of environmental or metabolic stress triggered pathologies. Since numerous bioactive constituents of turmeric are structurally and functionally analogous to those encountered in diverse other traditionally known medicinal plants [W Sun et al., 2016; A. Amalraj et al., 2016], observations made during such efforts could also be helpful for better understanding of therapeutic potentials of other plants containing such phytochemicals.

It is now well recognized that altered homeostasis in stress response regulating systems leads to diabesity and other metabolic disorders associated with mental health problems and that modulators of psychological and physiological stress responses could be useful for prevention of such and numerous other environmental stress related medical conditions [A. Bystritsky et al., 2014; G.P. Chrousos 2009; L.V. Guarner et al., 2011] including Alzheimer's

disease and cancer [S.M.T González et al., 1999; Y. Kim and B.A. Given 2008]. Demographic and epidemiological studies have often pointed out that turmeric could be a food ingredient involved in preventive effects on meals against diverse such medical conditions [B.B. Aggarwal et al., 2007; L. Azmi et al., 2015] and it has recently been estimated that turmeric curcuminoids consumed with curry products is less than 0.5 mg/person/day and that their contents vary considerably in different such currently consumed products [Y.J. Kim et al., 2015]. However, the questions whether other bioactive food phytochemicals consumed with meals also contribute to the health benefits of turmeric curcuminoids, or whether curcuminoids potentiates the effects of stress resistance promoting and other effects of edible phytochemicals are still remain open. Answering such questions is not only essential for obtaining optimal medicinal benefits from turmeric, but also for designing more rational dietary therapies needed for prevention of diabesity and other lifestyle associated medical conditions spreading like epidemics in the 21st century.

It is now well recognized that like numerous other food phytochemicals, curcuminoids are also extensively metabolized inside the gastrointestinal tract, and that their broad spectrums bioactivity profiles is mainly due to their high chemical reactivity to diverse biological targets [M. Heger et al., 2014] or might be due to their metabolic products like ferulic acid and vanillin. Observed low dose effects of turmeric derive product in our earlier study [S. Verma et al., 2015] strongly suggest that alteration in gut microbial ecology and functions are involved in their stress resistance promoting effects in mice could also play a keen role in its observed therapeutic effect. That such is indeed the case is further supported by a recent report demonstrating modulatory role of turmeric meals on gut microbiota and gut motility [N. Dey et al., 2015]. Since disturbances in the physiological functions of gut microbiota affects visceral pain, i.e. a hall mark of functional gastrointestinal disorders [S.M. O'Mahony

et al., 2014]. Further efforts were made during this research work to experimentally verify the possibility whether low dose curcuminoids could also be used for prevention and treatment of such disorders or for avoiding side effects of anti-inflammatory drugs and other currently available analgesics or pain killers. Ultimate goal of these efforts were to obtain an analytically as well as pharmacologically well standardized turmeric extract that could be further developed as a herbal therapeutic option for prevention and treatment of central sensitivity syndromes accompanying almost all chronic inflammatory disorders.

Observations reported in this study strongly suggested that the described bioassay system could be versatile for better pharmacological standardization of medicinally used herbal extracts, for identifying their bioactive constituents. Moreover, this bioassay could be used for estimating the pharmacologically interesting dose ranges of edible and other plant extracts, and for better understanding of the pharmacological interactions between their bioactive and other constituents. Such efforts could not only enable more rational medicinal uses of such medicinal plants, but also for better understanding of Ayurvedic pharmacology and systems biology dealing with thermoregulation, metabolism and body weight regulating physiological processes.

5.2. Analgesic activity

Effects of CLE-3R (10 mg/kg) and metformin (50 mg/kg) in hot plate test suggesting that their regular oral intake could be useful for suppressing central pain triggered by repeated exposures of peripheral noxious stimuli. Animals used in this study were four times pre-exposed to 55°C hot plate test for pre-selection purposes and the rate of alterations in the body weights and basal core temperature of animals of both the control groups (Subjected to hot plate test control group and not subjected to hot plate test control group) observed during the course of the experiment were similar. The observations revealed that 5 mg/kg daily oral

doses of CLE-3R is its maximally effective ones in affording protection against repeated exposures to hot plate (noxious stimuli) triggered alterations in body weight and core temperature. This study strongly suggests that turmeric curcuminoids are more effective modulators of energy metabolism than metformin. These observations taken together with available information on oral bioavailability and metabolic stability of curcuminoids [P. Anand et al., 2007] suggest that either the pharmacological site(s) of action involved in these effects of curcuminoids resides inside the gastrointestinal tract, or some of its bioactive intra-gastric metabolites are involved in its observed effects. In any case, it remains certain that like metformin and numerous other food phytochemical [A. Langstieh et al., 2014; N. Shrivastava et al., 2014; N. Shivavedi et al., 2014] low dose curcuminoids are also modulators of brain functions and homeostatic processes regulating body weight and core temperature changes triggered by environmental stimuli. Efforts to point out the mode of actions of curcuminoids have revealed that curcuminoids increase phosphorylation of AMP-activated protein kinase (AMPK) in hepatic cells and that curcuminoids are hundred folds more effective than metformin in activating the downstream targets of the kinase [T. Kim et al., 2009]. AMPK is a nutrient and energy sensor involved in maintenance of energy homeostasis regulating metabolic responses to cellular stress [D.G. Hardie et al., 2012; D.G. Hardie, 2015; M.F. Calabrese et al., 2014; M. Pelosse et al., 2015]. Therefore, it seems reasonable to assume that observed high efficacy of CLE-3R in protecting stress triggered alterations in body weight and core temperature is might be due to its ability to stimulate AMPK inside the gastrointestinal tract.

Our observations clearly reveal, that analgesics efficacies of CLE-3R observed after its 5 or more daily doses increased with its increasing daily doses, whereas its maximal possible protective effects against stress triggered changes in body weight and core temperature were

apparent even after its lowest tested daily doses (5 mg/kg). Curcuminoids are extensively metabolized inside the gut [K. Wang and F. Qiu 2013] and regular oral intake of curcuminoids alters gut microbial ecology and functions [N. Dey et al., 2015]. Moreover, they also possess antibacterial, antiviral, and antifungal activities [S.Z. Moghadamtousi et al., 2014]. Therefore, observed dose dependant anti-stress and analgesic activities observed after their fairly low oral doses are possibly due to slowly evolving and longer lasting effects of curcuminoids and their metabolites on gut microbial ecology and functions. Although, crucial role of gut microbiota in regulating physical and mental health status is now well recognized [R.D. Moloney et al., 2015; A.I. Petra et al., 2015] as yet only very little concentrated efforts have been made to translate available information of numerous food phytochemicals with bactericidal activities in terms of their therapeutic potentials. Such efforts will not only be helpful for better understanding of pharmacological principles behind traditionally known dietary and herbal therapies, but also could eventually lead to obtain novel therapeutic leads and pharmacological novel drug with preventive effects against central hypersensitivity to pain.

5.3. Antidepressant activity

Behaviour despair/forced swim, is most commonly used validated models of rodent to assess anti-depressant drugs. R.D. Porsolt et al., 1978, developed behavioural despair model and 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, stressed rats treated with CLE-3R (10 mg/kg) and metformin (50 mg/kg) were showed significant reduction in immobility period, which indicates their potential antidepressant activity. However such effect was less pronounced in metformin treated stressed group.

Results of the experiments strongly suggest that, effectiveness of 10 mg/kg daily doses of CLE-3R was observed in forced swimming test, was higher than metformin (50 mg/kg/day) in stressed and stressed diabetic rats. Although, both of them suppressed stress triggered elevation of plasma corticosterone levels, but only metformin showed significant protective effects against hyperglycemia observed in stressed as well as diabetic stressed animals. These observations could indicate that homeostatic processes and mechanisms regulate the circulating glucose levels are not involved in the mode of action for antidepressant activity of low dose curcuminoids. Observation also suggests that prevention of pre-diabetic and post diabetic depression is possibly could attenuate with higher doses of metformin.

It is now well recognized that like numerous other phytochemicals, curcuminoids are multi-targeted bioactive molecules and that blood levels observed after their oral intake do not correlated with their observed effectiveness in experimental animals or in humans. It has also been reported that curcuminoids are several hundred folds more potent than metformin in activating the downstream targets of the kinase [T. Kim et al., 2009]. Therefore, we speculate that low dose effects of CLE-3R on central nervous system functions observed after its repeated daily oral doses, is might be due its modulating effects on AMPK dependant digestive functions of the gastrointestinal tract regulated by the density and activity of gut microbiota. In any case, it remains certain that even fairly low daily oral doses of curcuminoids are effective in increasing stress resistance and that their slightly higher dose could be used for prevention of depression and other mental health problems in stressed as well as diabetic persons with abnormal allostatic load. Observation suggested that supplementation of even fairly low doses of curcuminoids with metformin could be a realistic and safe combination or reasonable therapeutic alternative for diabetic patients, also suffering from mental health problems. Proper understanding of pharmacological interactions between

metformin and plant derived food components like curcuminoids and their metabolites are essential prerequisites for achieving such goal. Such combination could also be used to prevention and treatment of several diabetes associated psychopathologies. For such purposes, the bioassay procedure used in the present and our previous study [S. Verma et al., 2015] seems to be well suited. Such efforts will also be useful for better understanding of biological processes and mechanisms involved in mode of actions of curcuminoids and other bio-similar phytochemicals as well as for more rational uses of their combinations with metformin, i.e. the only drug of first choice for prevention and treatment of diabesity and associated co-morbidities.

5.4. Anti-inflammatory activity

Formalin induced edema and nociception model is commonly used animal model for identifying anti-inflammatory agents with their central analgesic activities [V. Kumar et al., 2001; A.R. Campos et al., 2002; S. Trongsakul et al., 2003]. Qualitatively, the observed effects of repeated daily dose of CLE-3R (10 mg/kg) in the animal model used were quite analogous to those of the metformin (50 mg/kg). In any case, it remains certain that inflammatory mechanisms involved in swelling, edema, and pain behaviour are suppressed by repeated minimum dose of CLE-3R (10 mg/kg) and metformin (50 mg/kg). This test is widely used for identifying peripherally as well as central acting analgesics and anti-inflammatory agents. It has been reported that formalin test is also a model of hyperalgesia. There are two phases of this model, during the first phase peripheral sensitization occurs and in second phase central sensitization takes place [M. Dorazil-Dudzik et al., 2003]. Its first phase is acute phase and second is chronic phase. In acute phase hyperalgesic responses observed after two hour of formalin administration while in chronic phase this hyperalgesic condition enhanced from one to three days and persist till three to four weeks. CLE-3R and

metformin showed protection against both acute and chronic hyperalgesic condition. Therefore, it could be speculate that observed effects in this and other model of analgesia could be due to its modulating effects on CNS functions.

This model is well known for their predictive validity and for identifying peripheral anti-inflammatory and centrally acting analgesics. Other studies reported in this thesis have revealed that after repeated daily doses of CLE-3R or metformin, altered oxidative status 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). It is now well recognized that oxidative processes and cytokines are intrinsically involved in the pathogenesis of inflammatory swelling. The fact that CLE-3R and metformin having beneficial effect as analgesic and anti-inflammatory activity in rodents together with restore elevated glucose level. However, it cannot be ignored that CLE-3R contains three major bioactive constituents with pharmacological activity profiles. Therefore, it could be possible that observed efficacy of CLE-3R more than metformin might be due to modulating actions of three components of the curcuminoids or their degraded products [S. Liang et al., 2016].

Observations reported in this thesis add further experimental evidences to the conviction that the broad spectrum of psychopharmacological activity profile of CLE-3R 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 dose of CLE-3R (10mg/kg) is necessary for observing its anxiolytic as well as antidepressant-like activities. Thus, it seems possible that modulation of biological processes, or mechanisms, involved in anti-inflammatory effects of CLE-3R and metformin leads to adaptive responses in the central control mechanisms involved in exaggerated depression and central stress responses. Therefore, CLE-3R could be a starting point for discovering novel therapeutic leads and

pharmacological targets urgently needed for combating inflammation associated psychopathologies.

5.5. Food and water intake behaviour

Fructose feeding induces moderate obesity and several adverse metabolic effects, including weight gain hypertriglyceridemia and hyperinsulinemia, in rodents [S.S. Elliott et al., 2002; I. Zavaroni et al., 1980; M.K. Lee et al., 1994]. 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 [G.M. Husain et al., 2011a]. However, body weight gain and circulating insulin level in fructose fed animals were higher, whereas such increased weight gain and insulin level were counteracted by CLE-3R and metformin treated fructose fed rats. These observations strongly suggest that the effects of CLE-3R treatments on insulin levels and body weight depend largely on metabolic status of animals. Further, it indicates that modulating effects of the CLE-3R and metformin on biological mechanisms and processes regulating insulin secretion and metabolism could be involved in its modes of actions. Since CLE-3R and several of its components modulates the productions of diverse mediators of inflammation [V.B. Liju et al., 2011; Z. Meng 2013; R.S. Yadav et al., 2011], it might be possible that observed therapeutically interesting antihyperglycemia and anti-hypertriglyceridemia effects of CLE-3R and metformin are due to its antioxidant and anti-inflammatory activities. It has been reported that curcumin ameliorated insulin resistance by increasing oxidation of fatty acids and glucose [L.X. Na et al., 2011]. It has also been reported that curcumin attenuated high fat diet induced fat deposition by regulating hepatic lipid metabolism via AMPK activation. The observed hypolipidemic effect of CLE-3R and metformin in our study could be due to the down regulation of these lipogenic enzymes.

Oxidative stress and inflammation are the major cause, which can worsen this condition. In this study CLE-3R and metformin treatment significantly prevented the fructose induced hypertriglyceridemia, oxidative stress and inflammation [P. Faure et al., 1999; Y. He et al., 2015]. Therefore, it has been suggested that CLE-3R alleviated cardiovascular complications through restoring endothelial function, abrogating fatty liver development through its antioxidant and anti-inflammatory effects [G. Kapakos et al., 2012; W. Wongcharoen et al., 2009]. Hence, CLE-3R could be used as a potential adjuvant for the alleviation of diabetes associated cardiovascular and renal complications. It could also be used as therapeutic possibility for combating life threatening metabolic disorders commonly associated with, or caused by disturbances of glucose and lipid metabolism. Thus, CLE-3R seems to be a pharmacologically novel type of regulator of glucose homeostasis with the therapeutically used anti-hyperglycemic or anti-hyperlipidemic drugs.

5.6. Other pharmacological activities

Although during recent years a few reports revealing anti-hyperglycemic activity of *Curcuma longa* extract and curcumin in animal models have appeared [S.M. El-Bahr et al., 2013; M. Kuroda, 2015], as yet little attention has been paid to their therapeutic potentials for combating diverse other pathologies commonly encountered in diabetic patients. In view of the situation, it was of interest to test whether CLE-3R by virtue of its anti-stress and antidepressant activities could as well be a starting point for obtaining a therapeutic lead, or a phyto-pharmaceutical, potentially useful for combating diverse spectrums of diabetes associated pathologies.

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 [H. 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 against stress response and facilitate certain level of protection against long term stress [H. 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 [B. Tannenbaum et al., 2002]. However, if the stress is applied for protracted period of time, the body fails to acclimatize and expressed in term of stress related illnesses like gastric ulcerations, behavioural perturbations together with biochemical and endocrine imbalances [G.P. Chrousos and P.W. Gold 1992].

In view of to evaluate the comparative adaptogenic activity of CLE-3R and metformin in diabetes associated co-morbidity, we induces the diabetes in rats and subjected them to foot shock induced stress on different days. Daily treatments with CLE-3R afford protection against all chronic foot-shock stress-triggered pathologies studied, and that their efficacies are qualitatively quite analogous to that of standard antidiabetic drug metformin. Induction of diabetes is associated with the characteristic loss of body weight, which is due to increased muscle wasting [S.K Swanston-Flatt, et al., 1990; R. Mastrocola et al., 2005] and loss of muscle proteins due to persistent hyperglycemia [S.T. Russell et al., 2009]. Rats treated with CLE-3R or metformin counteracting such loss in body weight as compared to the stressed 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 ten oral daily doses of CLE-3R (10 mg/kg) significantly reduced the hyperglycemia in diabetic rats, but its effect is less pronounced than standard anti-diabetic drug metformin. The control in blood glucose by CLE-3R might be due to the β-cell protecting activity [K.I. Seo et al., 2008] and ability to decrease the insulin resistance [E.M. Jang et al., 2008] by inhibiting the oxidation of glucose

and fatty acid. Stress and diabetes both conditions cause the elevation in basal rectal temperature in stressed diabetic and stressed nondiabetic animals due to alteration of thermoregulatory homeostasis. Although, such effect was significantly antagonizes by CLE-3R (10 mg/kg) and metformin (50 mg/kg) in stressed diabetic rats. Observation reconfirms the adaptogenic activity of CLE-3R and metformin in co-morbid condition as well might be due to their thermoregulatory effect [S. Verma et al., 2015].

The results of the present study indicate that STZ-induced diabetes in rats tends to increased anxiety, as assessed by the various paradigms used viz. spontaneous locomotor activity, elevated plus maze test and marble burring test. All these behavioural paradigms have been validated for evaluation of anxiolytic agents [S.K. Bhattacharya and S.K. Mitra 1991; M. Ramanathan 1998; V. Kumar et al., 2000]. Locomotor activity is considered as an index of wakefulness or alertness of mental activity and a decrease may lead to calming and anti-anxiety like effect, and CNS acting drugs have been identified to influence the locomotor activity in animals [N. Singh et al., 2011]. As CLE-3R and metformin attenuated motor activity in stressed diabetic rats might be due their anti-anxiety effect. 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 [A.A. Walf and C.A Frye, 2007]. During elevated plus maze test, total time spent in open arms was less in stressed diabetic and stressed non diabetic control rats showing exaggerated anxiety might be due to novelty and risk/fear. Some earlier investigations have also reported that anxiety get increased in STZ diabetic rats in the plus maze test due to elevation of oxidative stress [M. Ramanathan et al., 1989]. A study suggests that hyperglycemia leads to increased oxidative stress, which in turn diminishes antioxidant defense system and short-term supplementation of ascorbic acid is safe and beneficial for

reducing anxiety levels in diabetic patients through alleviating oxidative damage [Z. Mazloom et al., 2013]. Anti-oxidant therapy has proved to be remarkably beneficial as remedy for reactive oxygen species induced injury in the CNS [G.V. Tsakanova et al., 2011]. In our finding, CLE-3R and metformin treatment significantly reversed hyperglycemic condition and oxidation of several enzymes in diabetic animals, which reconfirms that anxiolytic activity of CLE-3R and metformin possibly due to their anti-oxidant and glycemic control mechanism.

In marble burring test CLE-3R was found more effective in modulation of digging and burring behaviour than metformin. It indicates that there is other pharmacological site or mode of action by which CLE-3R shown significant attenuation of anxiety like behaviour in marble burring test as well. These observations also indicate that prolonged treatments with CLE-3R could be useful for helping patients with exaggerated anxiety, and other stress-triggered mental health problems commonly associated with numerous chronic diseases. Due to glucose toxicity and oxidative stress, hypotrophy of spleen and liver were found in stressed diabetic animals but daily treatment with CLE-3R and metformin antagonizes such hypertrophy in stressed diabetic rats. Adrenal gland hypertrophy in diabetic rats were also counteracted by CLE-3R and metformin, such effect confirmed their anti oxidative and adaptogenic activity in stressed diabetic animals.

5.7. Biochemical estimations

Observations made from co-morbid model (stress and diabetes) revealed that 10 mg/kg treatment of CLE-3R is effective in reducing all metabolic alterations observed in vehicle treated hyperglycemic and hypo-insulinemic animals and that these observed effects of the extract found after its repeated dose. All observed effects of CLE-3R on every metabolic parameter assayed were qualitatively analogous to observe effect in the metformin treated

stressed diabetic animals except their hyperglycemic control which was found less pronounced in CLE-3R treated stressed diabetic animals. It has been demonstrated that hypercorticosteronemia is observed in both, type 2 diabetes mellitus and stress conditions and also in their co morbid situation [T.Y. Wang et al., 2004; C. de Oliveira et al., 2011]. Similar to earlier observations, we found significant increase in the level of corticosterone in the plasma of stressed diabetic rats. In addition, this co-morbid condition also exhibited ulcers in the stomach of the stressed diabetic animals. CLE-3R and metformin equally reducing the plasma corticosterone and gastric ulceration in stressed diabetic animals. Similar to our findings, metformin regulated plasma corticosterone in diabetic patients [E. Carrizo et al., 2009]. It has also been reported that metformin exhibits the antidiabetic effect by down regulating the glucocorticoid receptors in the brain tissues of rats with hypercorticosteronemia [M.E. Cleasby et al., 2003]. CLE-3R protected against the elevation of corticosterone and ulceration index in stressed diabetic rats reconfirms its stress antagonizing and stress resistant activity.

Transaminase activity increases in insulin deficiency due to more availability of amino acid in blood of diabetes mellitus. In this present study we observed that glutamate oxaloacetate transaminase and glutamate pyruvate transaminase level get increased in diabetic rats although treatment with CLE-3R or metformin alleviates the levels of GOT and GPT as compared to stressed diabetic control rats. This could be due to protecting or adaptogenic activity of CLE-3R and metformin which significantly inhibited the disturbance in metabolic process in diabetic rats. GOT and GPT level are also indicator of liver function hence restoration of these enzymes indicates normal functioning of liver during diabetic condition. Observations reveal that, metabolic, physical or emotional stress produces long lasting psychological disturbances in diabetic rats that accompany with reduced SOD and CAT

enzyme activities. In the present study, CLE-3R and metformin significantly increased the levels of reduced catalase and also improved SOD activity in stressed diabetic animals. In addition, both lipid peroxidation and nitric oxide production were attenuated by metformin as well as CLE-3R. These findings confirm beneficial actions of CLE-3R and metformin on oxidative stress parameters in an animal model of type 2 diabetes [C. Sonia et al., 2008]. In addition to oxidative stress, another consequence of hyperglycaemia is non-enzymatic glycation of proteins. The first reaction was the formation of a Schiff base by the direct addition of open-chain glucose to lysine groups on proteins. This Schiff base undergoes a slow, spontaneous rearrangement to form a stable advanced glycation end-product and methylglyoxal, via non-enzymatic glycation reactions in patients with diabetic mellitus. Methylglyoxal derived glycated products accompanied with diabetic vascular complications and also with an increase in oxidative stress. Glyoxalase I is an enzyme, shown to protect against dicarbonyl glycation and the formation of advanced glycation end products. Glyoxalase I is the rate-limiting enzyme for detoxification of methylglyoxal, a side product of glycolysis which is able to induce apoptosis. Glyoxalase I enzyme mainly detoxifies the potent and cytotoxic methylglyoxal and eventually inhibits the accumulation of glycated products and further oxidation process [K.M. Kim el., 2012; O. Brouwers et al., 2011]. It has been found in this study chronic hyperglycaemia decreases the level of Glyoxalase I enzyme in stressed diabetic rats. However, repeated daily oral treatments with CLE-3R and metformin significantly reversed the reduced level of Glyoxalase I enzyme in diabetic rats. It show their protective action against the further oxidative stress induce consequences and pertubations.

PON 1 exhibits antioxidant properties and thereby helps in combating the oxidative stress in type 2 diabetes mellitus. PON 1 has unique antioxidant properties and it inhibited the

oxidation of HDL and LDL and reduces the cardiovascular complications generally associated to diabetes [S.A. Karabina et al., 2005]. The enzyme level goes down in type 2 diabetes. In our study it was found that CLE-3R and metformin significantly elevate PON1 activity in liver of diabetic rats. PON1 plays a protective role against the oxidative modification of plasma lipoproteins and hydrolyzes lipid peroxides in diabetes mellitus [E. Ozgun et al., 2016] although PON1 is a major defense barrier against lipid peroxides [K.G. Samani and E. Farrokhi, 2014]. Hence, our observations strongly suggest that daily treatment with CLE-3R improves the PON1 enzyme activity and protects against homeostatic imbalance of oxidative stress in diabetes mellitus. Analogous results obtained from metformin treated stressed diabetic animals and confirms its adaptogenic activity.

The present results demonstrated that CLE-3R is beneficial in management of worsened oxidative status and effectively reduced MAO activities in brain regions of diabetic animals. CLE-3R and metformin antagonized the lower hippocampal levels of all three monoamines viz. noradrenalin, serotonin and dopamine quantified in the diabetic control rats. Although qualitatively these observed effects of CLE-3R were quite analogous to those of the metformin. Thus, it seems reasonable to assume that its observed antidepressant-like efficacy in animal models are due to its modulating effects on central monoaminergic neurotransmitter systems. Such effects of the extract might be due its suppressive effects on brain mitochondrial monoamine oxidase activities. The observed bio- and neurochemical alterations reported in this thesis revealed that even the lowest oral dose of CLE-3R (10 mg/kg) 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. This dose of CLE-3R was also effective in partially reversing the lower hippocampal levels of the three quantified monoamines (NE, DA and 5-

HT) in stressed diabetic rats. Taken together with other reports on therapeutically interesting bioactivities of CLE-3R, our observations strongly suggest that the behavioural effects of CLE-3R in diabetic animals is due to its beneficial effects against oxidative damages caused by hyperglycemia and insulin deficiency.

Hyperglycemia triggers a series of events leading to the overproduction of free radicals that damage neuronal tissues and the consequent free-radical damage in brain affects the activity of two forms of acetylcholinesterase viz. AChE and BChE [K.M. Ramkumar et al., 2005]. Diabetes and stress mediated oxidative stress leads to vulnerable subsequent pathological events [N. Kawai et al., 1998]. It is clear from the previous reports that physical stress as well as metabolic or oxidative stress increases the level of cholinesterase activities in different brain regions [A.K. Thakur et al., 2013; M.I. Mohamed et al., 2007]. Observations reported in this study revealed that daily treatments with 10 mg/kg oral doses of CLE-3R significantly inhibited the AChE as well as BChE activity in different regions of brain and blood. Therefore, we hypothesize that inhibition of AChE and BChE activities by CLE-3R and metformin in the stressed diabetic rat brain reflects their anti-oxidative potential. It could also be due to prevention of homeostasis imbalance of glucose and insulin in diabetic animals. Taken together with the observed anti-oxidative and anti-inflammatory status in the brains of CLE-3R and metformin treated diabetic rats, indicate that cellular oxidative mechanisms involved in regulation of cholinesterase activity. Therefore, CLE-3R could be beneficial to treat neurological complications that commonly associated with diabetes mellitus.

In diabetes mellitus, hyperglycemia triggers a series of events leading to the overproduction of inducible nitric oxide synthase (iNOS) that causes further production of nitric oxide (NO) and cause neuronal tissues injury [R. Mastrocola et al., 2005]. NO is an important biological mediator in the living organism that is synthesized from L-arginine using molecular oxygen

and NADPH. Overproduction of NO by iNOS, is important in inflammation and its related processes. High levels of NO are markers for the treatment of inflammatory disorders. NO at its physiological concentration serve as a vasodilator, and neurotransmitter in different living tissues, however high levels of NO in presence of superoxides or peroxide radicals leads to production of peroxynitrite and NO free radicals, which ultimately aggravated the oxidative stress and inflammatory situations in stress and diabetes mellitus [F. Aktan 2004]. Inhibition of NF-κB activation is also considered important, because it is the main regulatory step for iNOS expression [J.L. Madrigal et al., 2001; F. Aktan 2004]. NF-κB is an important upstream modulator of pro-inflammatory cytokine, and the inhibition of activation of NF-κB can suppress expression of pro-inflammatory cytokines in blood and brain regions. Results shown that level of iNOS and NO significantly increases in stressed diabetic rats as compared to nondiabetic rats, however daily treatments with 10 mg/kg CLE-3R or 50 mg/kg metformin significantly reversed the elevated levels of both iNOS and NO in diabetic animals. Reported results have also revealed that, repeated daily oral administration of either CLE-3R or metformin significantly reduced the over expression of NF-κB in diabetic rats. This study indicates that an anti-inflammatory property of CLE-3R seems to be mediated by modulation of NF-κB signaling pathway [C. Buhrmann et al., 2011]. Therefore, obtained results verified that such observed effect of CLE-3R and metformin is due to their antioxidant, anti-inflammatory and adaptogenic activity.

On the basis of observation it could be suggested that both curcuminoids and metformin can be used for prevention and treatment of diverse spectrums of mental health problems and other comorbidities commonly associated with lifestyle disorders and aging [A.M. Ying et al., 2014; S. Hu et al., 2015; P. Dulbecco and V. Savarino 2013] and that turmeric could be an adjuvant diabetic therapy with metformin to more efficiently counteracted such conditions

[N.M.K. Selvi et al., 2014; V. Kumar et al., 2015]. However, as yet no reports on possible pharmacological interactions between curcuminoids and metformin have been appeared. Results of these efforts will not only be useful for more rational development of polypills with metformin and curcuminoids, but also for better understanding of pharmacological principles behind traditionally known medicinal and healthcare uses of turmeric.