DISCUSSION

Antistress drugs are known to enhance the non-specific resistance of individual against a stressful conditions. The conventionally used drugs to treat stress include benzodiazepine, anxiolytics and antidepressants which are capable of exerting effective antistress activity against acute models of stress only and have not proved effective against chronic stress induced adverse effects. Furthermore, these drugs are associated with many side effects and adverse reactions. At the same time many herbs reported in ancient literature have potent antistress activity. The number of medicinal plants like *Panax ginseng*, *Withania somnifera*, *Asparagus racemosus*, *Panax ginseng* etc. have been screened by using various animal models involving exposure to various types of stress. In the present study we have tried to evaluate antistress potential of *Murraya koenigii* and *Ocimum sanctum* leaves extracts followed with development of the formulation for biologically active extract of MK and OS and evaluating their invivo efficacy for antistress activity. Efforts had been also directed in exploring possible mode of action of extracts by observing their effects on bioamine levels and acetyl choline levels in brain and their effect on cognition and memory and various biochemical metabolism correlates and biomarkers of stress. Further the efforts had been made to isolate a phyto-constituent from the plant responsible for the claimed antistress effect. The isolated pure phytoconstituent was also tested *In vitro* to predict the efficacy of compound.

Exposure to the chronic stress is reported to cause oxidative stress in brain leading to various adverse physiological consequences which on prolonged exposure to stress leads to the genesis of many disorders associated with stress. Thus in present study antioxidant potential of plant extracts of OS and MK was evaluated by calculating their IC50 values by performing *Invitro* DPPH radical scavenging activity and nitric oxide radical scavenging activity. Extracts of MK and OS were found to exhibit an excellent antioxidant potential. The potency of MK extracts to scavenge DPPH and Nitric oxide radicals was found to be in order of MKM>MKHA>MKAQ. Thus MKM was found to exhibit maximum radical scavenging activity with lowest IC50 value. As Carbazole alkaloids, flavonoids and terpenes were found to be present in plant MK on phytochemical evaluation [350-361], these phytoconstituents present within a plant might be conferring free radical scavenging ability to MK extracts. Among OS extracts degree of DPPH and Nitric oxide radical scavenging activity was found to be of order OSHA>OSM>OSAQ demonstrating that OSHA was bearing maximum antioxidant potential. Various studies had already reported about rich phenolic content of
compounds present in *Ocimum sanctum* [451]. Many of its active principles such as eugenol, methyleugenol, urosolic acid, and β-caryophyllene, methylchavicol, linalool, 1,8-cineole and flavonoids such as orientin, vicenin etc may act as antioxidant. This observed antioxidant property of test extracts provided a direction for further invivo evaluation of antistress effect of extracts of MK and OS invivo by using various types of stress models, however before evaluating antistress potential the plants were screened preliminary to find out their neuro pharmacological effects. Acto photometer was used to evaluate effect of test extracts on spontaneous motor activity. On treatment with test extracts of MK no significant change in activity score was observed as compared to vehicle control group. OS test extracts also on treatment did not produce any significant alteration in spontaneous motor activity. Thus it was found that MK and OS extracts did not alter the motor tone and did not exhibit skeletal muscle relaxant activity.

Anxiety is another important aspect of stress. Effect of extracts on anxiety was tested by using Elevated plus maze model in which two parameters e i.e. effect on number of entries in open arm and total time spent in open arm were measured to judge effect of extracts on exploratory behaviour of animals. Except MKAQ MKM and MKHA were found to be producing significant increase in the number of entries and time spent in open arm of EPM thus revealing anxiolytic potential of plant. OSHA, OSAQ and OSM were also found to a similar effect. Hole board was another model to assess anti anxiety effect of drugs. In this model significantly effective extracts namely MKM and MKHA were found to be producing increase in number of dips, MKM was observed to be demonstrating highest percentage increase in number of dips. Thus extracts were again found to be significantly exerting antianxiety activity by increasing exploratory behaviour of animals. Though MKAQ was observed to be significantly increasing number of dips, the extent of increase was almost 50% less than that of produced by MKM. OSHA, OSAQ and OSM were also effective in increasing number of dips where later two extracts were effective only at higher dose of 200mg/kg.p.o. Highest percentage increase in number of dips and time spent in them was observed on treatment with standard Diazepam. All three extracts of MK (MKM MKHA and MKAQ) showed significant increase in duration of time spent in dips so as OSHA OSAQ and OSM. The extent of increase in exploratory behaviour produced by OSHA and MKM were found to be almost nearly equal at dose 200 mg/kg.p.o thus from these observations MK and OS were found to exhibit significant anti anxiety potential.
Discussion

Forced swim test was designed by Porsolt as a primary screening test for antidepressants. It is still one of the best models for this procedure. This is a low-cost, fast and reliable model to test potential antidepressant treatments with a strong predictive validity.[591] Introduction of an animal into water which is not their natural habitat forces the animal to swim so as to keep its head above the surface of water and to escape from the water. After initial struggling when animal fails to escape from the water it gives up the efforts due to depression which is presented in form of immobility, thus duration of immobility (DOI) is an indication of depression produced in animal which was used as an evaluation parameter for this study. FSW was performed to assess antidepressant effect of test extracts of OS and MK. Imipramine which was used as a standard showed a significant decrease in DOI on 7th day of treatment. DOI was found to be decreased significantly on treatment with all three test extracts of MK. However the degree of significant fall in DOI was found to be exerted by MKM and MKHA at doses 100 and 200mg/kg p.o. The decrease in DOI were found to be comparable with the standard Imipramine. On treatment with OSHA and OSAQ significant fall in DOI was observed at doses 100 and 200 mg/kg p.o. Whereas OSM was found to be effective only at higher dose 200 mg/kg. p.o. But on comparison of percentage decrease in DOI produced by test extracts of MK and OS, it was observed that MK was able to demonstrate the higher degree of antidepressant effect as compared to OS. From all these observations it can be concluded that MK exhibited a greater antidepressant effect than OS.

Another model which was used for assessing antidepressant effect was tail suspension model. In the case of the TST the stressful situation involves the haemodynamic stress of being hung in an uncontrollable fashion by their tail which induces a state of despair in animals similar to FST. This immobility, referred to as behavioural despair in animals is claimed to produce a condition similar to human depression[592,593] in which immobility behaviours represent the psychological concept of “entrapment” described in clinical depression[594,595].

Thus, the development of immobility disengages the animal from active forms of coping with stressful stimuli. Further, immobility in the TST is due to inability or reluctance to maintain effort rather than a generalized hypoactivity, as evidenced by the fact that animals can adopt this posture quickly and drugs which may suppress activity (as is the case of many antidepressants) counter the immobility response. As such, this immobility may be analogous to the clinical observations in depressed patients who often lack sustained expenditure of effort reflected in a pronounced psychomotor impairments [596]. In this model also MKM and MKHA were found to produce an excellent antidepressant effect at doses 100 and
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200mg/kg p.o. Similarly OSHA and OSAQ were also found to exert significant antidepressant effect by reducing the DOI thus supporting the findings of FSW model. The another modification of FSW used was Weight loaded forced swimming test. It is a strainous exercise leading to physical stress triggering the activation of pituitary adrenocortical activity and consequently, the production and secretion of corticosterone which was found to be elevated in stress control group as compared to vehicle control group, not subjected to weight loaded forced swimming [597-600]. Where as on treatment with OS and MK extracts stress induced rise in levels of serum corticosterone were found to be prevented indicating less degree of stress and/or fatigue experienced by animals. Hence treatment with extracts of MK and OS were found to enhance the ability of mice to continue the exercise for a longer period as compared to stress control group.

This rise in levels of corticosterone in stress control group might have induced prompt increase in the level of glucose in the blood. But in weight loaded swimming exercise, to sustain in water mouse was forced to continue swimming exercise against the downward pulling force of load applied to its tail which might had lead to rapid utilisation of glucose as exercising muscles in hind limbs continuously needed energy for swimming. As a result, the available glucose in blood might had got used up rapidly by the muscle tissues as a source of energy leading to significant fall in blood glucose level in stress control group. However, it is possible that a decrease in blood glucose concentration (hypoglycaemia) might had contributed in part to the occurrence of fatigue in the stress control groups. Since blood glucose is a major source of energy to both muscle tissue and the CNS specially the only preferential energy substrate to CNS for its activity which might be compromised in a state of hypoglycemia [601]. It is also reported that suppression of the active functioning of the brain during exercise often leads to the inability to continue exercise [602]. Thus, the amount of blood glucose can illustrate the speed and degree of fatigue development [603]. One can observe from the Table (4.43 and 4.44) that in stress control group the observed decreased in duration of swimming and rapid induction of fatigue might have resulted from significant fall in blood glucose levels during WFS where as prolonged swimming endurance period in treatment groups at various dose levels of three extracts of OS and MK with relative increase in plasma glucose levels indicated the blood-glucose-regulating ability of plants in treatment groups by maintaining availability of energy substrate i.e. glucose in blood for a longer period. This homeostasis of blood glucose played an important role in prolonging swimming endurance period during exercise [604].
Discussions

Energy for exercise is derived initially from the breakdown of glycogen in muscle and, later, from circulating glucose released by the liver as a result of glycogenolysis [605]. Thus the amount of glycogen, which reflects the source of the energy, would be more suitable marker of physical fatigue. In general, glycogen is consumed in both muscle and liver during physical fatigue. The role of hepatic glycogen is to complement the consumption of blood glucose and maintain the blood glucose in the physiologic range. Fatigue will happen when the liver glycogen is mostly consumed [606] as a result of the depletion of hepatic glycogen stores plasma glucose concentration decreases during prolonged exercise.[607]. As a result ensuing hypoglycaemia could lead to impairment in CNS functionality. Thus, the maintenance of glycemia during physical activity by hepatic gluconeogenesis and glycogenolysis is of extreme importance for exercise continuation [608]. As shown in Table (4.51, 4.52), the liver glycogen levels of treatment mice were significantly higher than that of the stress control group, These results indicate that exercise induced depletion of glycogen stores in liver tissues was found to be prevented in groups treated with MK and OS extracts suggesting that the anti-fatigue activity of MK and OS may be due to the improvement in the metabolic control of exercise and the activation of energy metabolism [602]. Similarly muscle glycogen levels also demonstrated the important factor in determining endurance during exercise as depletion of muscle glycogen plays a key role in development of fatigue and exhaustion. Depletion of these deep carbohydrate stores should certainly prove limiting for any type of physical activity. Our findings indicated that the degree of glycogen depletion during exercise was reduced significantly in the MK and OS extracts treated mice. Thus treatment with MK and OS extracts could decrease carbohydrate utilization during exercise thus sparing muscle glycogen stores for a longer period .The possible reason may be that pre treatment with MK and OS extracts might had increased the content of liver and muscle glycogen in mice before subjecting to exercise by improving glycogen reserves, or by reducing the consumption of glycogen during exercise, or might be both. thus resulting enhanced swimming capacity and delayed onset of fatigue in mice.

On the other hand, TG levels in the blood were found to be declined in MK and OS treated mice which indicated that while the glycogen stores were conserved in both muscle and liver by MK and OS intake, the good performance during the swimming test was due to the energy supplied by TG degradation in the treated mice. In view of the lowered plasma level of triglyceride in the MK and OS extracts treated groups as compared to the stress control group, it would seem reasonable to suggest that lipid utilization may be increased in treatment groups as an alternate source of energy [609,610]. The present results suggest that
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enhancement of exercise capacity on treatment with MK and OS extracts could be due to increase fat utilization by mice during swimming, allowing glycogen sparing and therefore delay in the onset of fatigue [611,612].

The response to exercise in mammals begin with an increase in aerobic muscular activity, which switches over to anaerobic metabolism if the exercise is intense in which O₂ and pyruvic acid are reduced by lactate dehydrogenase (LDH) to Lactic acid (LA) and that of increase in lactic acid presents a fatigued condition. In this study the LDH levels of stress control group were observed to be increased significantly as compared to vehicle control group. Accumulation of LA decreases the pH, affecting the skeletal muscle system function. By decreasing the contractile strength of the muscle that eventually induces fatigue so the levels of LDH in blood were measured. In MK and OS treated mice serum LDH levels were found to be significantly lowered than that of the stress control group. The results suggested that MK and OS can reduce the production of blood lactic acid during exercise. Which was another confirmation that MK and OS treatments are capable to induce an anti-fatigue effect.

Urea is formed in the liver as the end product of protein metabolism. During digestion, protein is broken down into small peptides and amino acids. The amino acid nitrogen is removed as NH₄, while the rest of the molecule is used to produce energy or other substances needed by the cell [614]. Circulating ammonia is taken up by the liver and most of it is detoxified in this tissue through the urea cycle;[615,616]. Thus Blood urea nitrogen (BUN), which is a product of energy metabolism, is another sensitive index of fatigue status. BUN levels of the treatment mice were significantly lowered on treatment with MKM and MKHA as compared to the stress control group while the decrease in BUN levels observed in MKAQ treated group was not statistically significant . Whereas in OS treated mice serum BUN levels were found to be decreased significantly on treatment with OSAQ and OSHA. The reduced protein metabolism in the MK and OS treated group is an indication of enhanced endurance.

The data mentioned above revealed that the content of liver and muscle glycogen of mice in MK treated groups were higher than that of stress control group after swimming. However its detailed mechanism is not clear. The possible reason may be that MK and OS might had increased the content of liver and muscle glycogen of mice post exercise by improving glycogen reserves, or by reducing the consumption of glycogen during exercise, or both. The later effect has a greater probability as triglyceride levels of treatment groups were found to be lowered thus indicating shift in metabolism from glycogen to triglycerides thus conserving energy stores such as liver and muscle glycogen. Serum urea nitrogen and LDH levels are important blood biochemical parameters related to fatigue. In our experiment, the blood LDH
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levels in MK treated groups were lower than that of stress control group after weight loaded forced swimming. The content of serum BUN raises with the exercise load Our results showed that serum BUN levels in the MK and OS treated groups were lower than stress control group. In conclusion, treatment with MK and OS extracts induced anti-fatigue effects on mice and these effects were variably significant in all three extracts but the strongest effect of normalising fatigue induced perturbations on most of biomarkers was seen with MKM (200 mg/kg p.o.) and OSHA (200mg/kg p.o). Thus from all these onbservations one can say that the mechanism involved in observed anti-fatigue effect of MK and OS might be mediated through regulating central nervous system, and/or improving substance metabolism and aerobic capacity.

The cold restraint stress model (CRS) used involves combination of two stress stimuli, one is cold causing physical stress and the other is immobilisation leading to physical as well as unescapable psychological stress [617]. Thus advantage of selecting cold restraint stress model is that synergistic effects of both exposure to cold temperature and immobilisation leads to generation of intense stress leading to a stimulation of hypothalamic pituitary adrenal axis which might have produced marked rise in plasma corticosterone levels at the end of the CRS [618] in our study as significant rise in levels of serum corticosterone were observed in stress control group. Treatment with standard AS prevented this CRS induced rise in serum corticosterone to the highest degree. In this model treatment with standard Diazepam was also found to induce significant reversal of CRS induced rise in serum corticosterone levels. Thus explaining the role of stress induced anxiety in genesis of peptic ulcer. Treatment with test extracts MKM and MKHA (100 and 200 mg/kg p.o) were found to be effective significantly in lowering CRS induced elevated levels of serum corticosterone whereas MKAQ was significantly effective only at higher dose of 200mg/kg p.o. Among three extracts of *Murraya koenigii* MKM produced maximum degree of prevention in CRS induced rise in serum corticosterone levels. Where as on treatment with test extracts of *Ocimum sanctum* OSHA was the most effective extract causing reduction in CRS induced elevated levels of cortisol to the highest extent as compared to other two extracts. Increased demand of corticosterone raises the workload on adrenal gland for synthesis of more corticosterone. To keep a pace with the increasing demand of corticosterone due to stress the cells of adrenal gland undergo hyperplasia thereby causing significant increase in mean weight of adrenal glands as observed in stress control group. In treatment groups significant reversal of stress induced adrenomegaly was observed and was found to be parallel to the effects of test extracts on corticosterone levels. The highest degree of prevention in stress
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induced adrenal hypermegaly was observed on treatment with MKM and OSHA which were comparable with the effects produced by standard Ashwagandha.

The two main systems involved in stress response are the HPA axis and the sympathetic nervous system, triggered primarily by an area in the brain stem (lowest part of brain) called the locus coeruleus. The sympathetic nervous system on activation secretes catecholamines. The hypothalamus is a major integrating centre for receiving messages from divergent centres and converting them to hormonal signals, via the control of the pituitary gland and by neural pathways[619]. The activation of this HPA system results in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACTH), β-endorphin and glucocorticoids into the circulation. Release of ACTH in stress stimulates adrenals to increase production of hormones-epinephrine, norepinephrine and corticosteroids [620]. These hormones have profound effect on metabolic functions. Increased plasma cortisol influences the mobilisation of stored carbohydrate reserves [621] which in turn increase blood glucose. In our study significant hyperglycemia was observed in stress control group on 7th day as compared to vehicle control group. Test extracts of MK and OS significantly prevented this stress induced hyperglycemia. The hypoglycaemic effect demonstrated by extracts MKM and OSHA were found to be superior than that of effect exerted by standard Ashwagandha.

CRS is commonly used paradigm to evaluate stress induced gastric ulceration. In literature various theories have been proposed to explain CRS induced gastric ulceration. According to a widely accepted concept it was suggested that the development of restraint ulceration in the rat stomach is accompanied by an increase in Norepinephrine (NE) turnover in the glandular portion organ [622]. Since NE reduces gastric blood flow [623,624] and causes vascular insufficiency with resultant ischemia which may be involved in genesis of ulcer. NE also results in a general inhibition of the rate of cell renewal of several epithelial tissues including the glandular intestinal mucosa of the rat [625,626].Thus rendering the mucosa susceptible to ulcer primarily by cold stress, Another report suggested the role of paraventricular nucleus (PVN) in generation of ulcer. Stimulation of the PVN of hypothalamus processes the input stress signals exerting its influences through the autonomic nervous system and the neuroendocrine systems on to the target organ, the stomach enhancing the development of stress ulcers[627]. In our study on exposure to CRS of 4 hours/day successively for 4 days exhibited significant ulcers formation in stress control group. Standard Ashwagandha was observed to be producing maximum inhibition in occurrence of ulcer on treatment. Standard Diazepam was also found to offer significant protection against CRS induced ulcers,
indicating the role of neuronal involvement in generation CRS ulcers. MKM was found to exert significantly highest protection against stress induced ulcer at dose 200mg/kg p.o. which was comparable with the degree of protection offered by standard Ranitidine. Highest degree of protection was exhibited by extract MKM. These results suggested that plant MK bears a vital antiulcer potential. On treatment with OS extracts OSHA offered significant protection against ulcer at both the doses 100 and 200mg/kg p.o. whereas Extracts OSAQ and OSM were capable of restricting occurrence of CRS induced ulcers only at the higher dose of 200mg/kg p.o. Thus revealing antiulcer activity of OS These results thus suggested that MK as well as OS have a potential ability to prevent occurrence of stress induced ulcer by normalising the activation of two important systems governing stress responses which include HPA and sympathetic system.

Stress hampers the immunity adversely. To probe in to the effects of extracts on changes in immunological reactions occurring in response to antigenic challenge Milk induced Leukocytosis model was chosen. Raw cow milk was injected (S.C.) as an antigenic challenge in to rat body subcutaneously. Raw Cow milk contains mixture of proteins, 80% of which is mainly casein and these proteins belong to the strongest antigen in human diet [628]. Subcutaneous injection of cow milk (not pasteurised) thus produces an infection like condition in rats causing significant increase in leukocyte count and reduction in weight of thymus and spleen . Stress induces glucocorticoid production which exerts a major impact on metabolism, inflammation, the lymphatic organs and peripheral blood leukocytes [629]. Our study was found to support these observations as significant rise in levels of leukocyte count in blood was observed 24 hours after exposure to antigenic challenge (Raw cow milk) in stress control group in contrast to WBC count observed in vehicle control group which was not subjected to any antigenic challenge. Treatment with MK and OS extracts restricted rise in blood leukocyte count .However MKAQ was observed to be producing highest extent of inhibition of raised WBC count. Among three extracts of *ocimum sanctum* OSHA was observed to be offering maximum significant protection against rise in blood leukocyte count. 

Sir Hans Selye reported that acute stress causes thymic atrophy It has been also reported that this thymic atrophy results due to release of glucocorticoids during stress which cause apoptosis and necrosis in immature T and B cells resulting in the decline of thymus weight [630]. The death of thymocytes occurs due to activation of an endogeneous pathway within the cell (Ca2+ activated endonuclease) which leads to cell suicide and thymic involution [631]. On isolation of thymus gland after 24 hours of antigenic challenge it was observed that mean thymus weight in stress control group was decreased as compared to vehicle control.
Discussion

Treatment with MK and OS extracts significantly prevented decrease in mean thymus weight, where the maximum effect was produced by MKAQ. Other extracts like MKM and MKHA also restricted decrease in mean thymus weight at both the doses (100 and 200mg/kg p.o.) but degree of increase in weight of thymus gland was observed to be lesser than that exhibited by MKAQ. Among three extracts of *ocimum sanctum* OSAQ (100 and 200mg/kg p.o) was observed to be offering maximum significant protection against reduction in thymus weight. Similarly OSHA (100 and 200mg/kg) and OSM (200mg/kg p.o.) were also effective in preventing stress induced thymus atrophy. During stress nerve terminals accelerate recruitment of lymphocytes to blood from spleen which is a major storage pool of lymphocytes this results in squeezing of the spleen causing reduction in the weight [632]. Immediate recruitment of lymphocytes from spleen due to antigenic challenge to immunity lead to significant reduction in mean weight of spleen in stress control group. MK and OS extracts significantly and effectively prevented stress induced fall in mean spleen weight. Relatively maximum increase in weight of spleen was observed on treatment with MKAQ (100 and 200mg/kg p.o) as compared to MKM (200mg/kg p.o) and MKHA (200mg/kg) Thus from these results one can say that MK and OS leaves extract have an ability to modulate immune responses to antigenic challenge exhibited by cow milk. In present study MK and OS extracts had been shown to reverse all the alterations in organ weights and WBC count due to induction of antigenic challenge by raw cow milk, thus suggesting the ability of two plants to impart resistance against immunological stress.

Restraint stress is a very commonly used model to assess antistress effect of drugs. It is a combination of physical (immobilisation) and psychological stress. Stress in rats brings about transient activation of the HPA axis, as measured by increased adrenal gland weight with subsequent increase in plasma corticosterone level and other correlates of adrenal activation which prepares the organism for threatened homeostasis [633]. The important biochemical changes in plasma under stressful conditions, i.e. elevated corticosterone is necessary to maintain the energy balance which include increased plasma glucose, and decreased triglyceride and cholesterol levels [634]. Under stressful condition adrenal cortex secretes cortisol in man and corticosterone in rats. Hypersecretion of cortisol helps in maintenance of internal homeostasis through the process of gluconeogenesis and lipogenesis. In present study RS induced significant hyperglycaemia which was found to be inhibited by treatment with extracts of MK and OS. Diabetes mellitus is a well accepted consequence of continuous stress, indicating the close interrelations between stress and the endocrine and
autonomic nervous systems [635,636]. In accordance to literature survey MK and OS had been reported to possess hypoglycemic effect [399,477] and in present work also they were found to be effective in normalising stress-induced perturbation of glucose homeostasis. Especially effect of MK on stress induced hyperglycemia was not found to be reported elsewhere in literature but in our study treatment with MK extracts were found to be preventing stress induced hyperglycemia on both 7th and 14th days of treatment so as Os extracts.

In present study RS induced reduction in levels of plasma cholesterol as compared to vehicle control group (unstressed). It has been observed that the stress raises utilisation of serum cholesterol resulting in increased liberation of catecholamines and corticosteroids through enhanced activity of hypothalamohypophyseal axis [637] but this effect of stress on levels of cholesterol was ameliorated following 14 days treatment with leaf extracts of MK and OS. Specifically MKM was found to be effectively controlling serum cholesterol levels on 7th as well as on 14th day of treatment.

Effect of stress on TG levels is found to be variable probably due to mobilisation of lipids from adipose tissues by catecholamines released in high concentration during stress. Results of present study showed decline in levels of triglycerides in stress control group but on treatment with MK and OS extracts this effect of stress on TG levels was found to be prevented. MKM (200 mg/kg p.o.) and OSHA (200mg/kg p.o) were observed to be the most effective extracts in preventing this stress induced decrease in levels of triglycerides which might be due to suppression of stress induced lipolysis. Probably an effective MK and OS extracts are able to promote assimilation of glucose in tissues as an immediate source of energy as a result, breakdown of lipids as an alternative source of energy was reduced which might had prevented decline in levels of serum TG. These observed effects of extracts on biochemical parameters are interdependent and might be due to suppressant effect of MK and OS on hyperactivity of adrenals consequently preventing rise in serum corticosterone levels induced by restraint stress. This normalizing effect on plasma corticosterone is one of the possible reasons for their anti stress properties.

Extracts of MK and OS were further investigated to understand the mechanism underlying the anti stress effect exhibited by them. Estimation of bio amines levels in brain may indicate the effects of extracts at neuronal levels. Various research workers had given various different opinions about changes in the levels of brain monoamines, This may be due to the
nature of stressor, duration of stressor and the animal used in experiments. In our study immobilization for duration of 120 min for 14 days continuously resulted in significant decrease in levels of Noradrenalin and increase in 5-HT and Dopamine levels in brain. Stressful conditions activate monoaminergic system leading to an increase in the turnover of Noradrenalin in cortex and hippocampus regions of brain [638]. It has been suggested that dopamine levels in brain elevate as a compensatory mechanism. As it is a precursor for synthesis of norepinephrine to cope up with increased turn over rate of NE during RS Dopamine demand might be increasing [639] resulting in the observed increased levels of dopamine in brain. Whereas 5HT levels in brain of stress control group might had increased in response to physical and psychological stress due to immobilization in RS [640]. The extracts of MK and OS were effective at different extents in resisting these perturbations in levels of NA, 5-HT and Dopamine in rat brain but the most significant results were found on treatment with MKM and OSHA. These normalising effects of MK and OS extracts on bioamine levels probe in to the probable mechanism involved in preventing stress induced biochemical alterations on treatment.

During stressful conditions, changes in monoamines (NA, DA and 5-HT) are well associated with transient behavioural aberrations in memory learning and other mood disorders. Experimental stress was reported to have adverse effects on the memory engram in rats. The learning acquisition was minimally affected, the major action being disruption of retention of learned tasks [641]. MK was reported earlier to have significant nootropic activity [423] but its effect on stress induced amnesia is not yet established. In this study when tested for retention of learned task in elevated plus maze after exposing to RS, groups treated with MK showed decrease in TL on 7th and 14th day of test and similarly in step down inhibitory avoidance test, SDL was found to be increased suggesting the ability of extracts to avoid detrimental effects of stress on memory. The exact mechanism for these observations cannot be explained with our present data, however the role of corticosterone can be hypothesized, as effects of stress on memory are inhibited by CRF antagonist and adrenalectomy suggesting the possible mediation of corticosterone. The various physiological changes seen in response to stress are primarily due to increased hypothalmo pituitary action which in turn induces activation of pituitary adrenal system. Adrenaline stimulates β2 receptors on the pituitary gland causing greater release of ACTH which in turn can stimulate the adrenal medulla as well as cortex leading to adrenomegaly. In this study in treatment groups significant restoration of adrenal gland weight was observed which showed potential role of MK and OS.
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in attenuating uncontrolled activation of HPA axis. The carbazole alkaloids and terpenes found to be present in MK and Phenolic compounds found to be present in OS may be probably contributing to the antistress potential of plants. Thus from the observations one can conclude that plants MK and OS may provide a protection against stress probably by preventing biochemical and humoral perturbations during stress and their adverse implications on body physiology and thus can be a safe alternatives with antistress effect for therapy of stress related disorders.

Another model which was used to explore the effect of test extracts on stress induced depression was foot shock. Exposure to an inescapable or uncontrollable stressor (shock) leads to "learned helplessness," defined as the inability to escape or avoid one stressor when it is subsequently presented in a different context, such as a shuttle box task, from which the animal can escape by moving into the other chamber of the box [642]. In addition, during the application of inescapable shock (IS) the animal displays inactive behaviour correlated to escape failures in the subsequent shuttle box task [643]. Learned helplessness has a good predictable validity, useful in the treatment of human depression, because it may be reversed by administration of various antidepressant drugs [644,645]. This learned helplessness model resembles human clinical depression not only in the display of similar behavioural disturbances and responses to specific treatments but also in the alterations of hypothalamic-pituitary-adrenal axis. Such alterations have been observed in helpless animals with an impaired ability to suppress corticosterone in response to dexamethasone, an homologous response to that observed in a large number of depressive patients [646,647][60,61] it has been demonstrated that corticosterone plays a critical role on the behavioural strategies adopted by rats when they are forced to cope with an aversive and inescapable stressful situation [648].

In stress control group serum corticosterone levels appeared to be increased significantly after acute exposure to FS stress on first day and then on 7th and 14th day of study continued to remain elevated due to chronic exposure to foot shock stress. Prevention in stress induced rise in levels of serum corticosterone was observed after the treatment with both the standards such as Imipramine and Ashwagandha but extent to which they caused prevention varied and the effect produced by AS on levels of serum corticosterone was found to be statistically significant whereas that of Imipramine was not statistically significant. Treatment with MKM (100 and 200 mg/kg p.o.) was able to significantly prevent the stress induced rise in levels of serum corticosterone on both 7th as well as 14th day of treatment and the degree of reduction
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in serum corticosterone levels produced was found to be comparable with that of standard AS. MKHA (100 and 200 mg/kg p.o.) produced significant fall in stress induced elevated levels of serum corticosterone only after the treatment of 14 days. In groups treated with OS extracts it was observed that both OSHA (100 and 200mg/kg) and OSAQ (100 and 200mg/kg p.o.) were able to demonstrate significant fall in stress induced raised levels of serum corticosterone on both 7th as well a 14th day of treatments on chronic administration. OSM (200mg/kg p.o.) was also found to be effective in restricting rise in serum corticosterone levels. It has been reported that in Inescapable foot shock the inactivity in animal was reduced by corticosterone synthesis inhibitor, metyrapone, thus indicating that the inhibition of increased corticosterone secretion provoked by the stressful situation can revert the depression and deficit in motor activity induced by foot shock. And the test extracts might be exhibiting their antistress effects by following similar mode of action i.e reducing the stress induced elevated levels of corticosterone[649].

Similar to other stress models in this model also stress induced hyperglycemia was observed on 1st, 7th and 14th day of study. MKM (100 and 200 mg/kg p.o.) was found to be effective in lowering serum glucose levels at both the time intervals i.e. 7th and 14th day of treatment whereas MKHA was found to to be effective on 7th day only at higher dose of 200 mg/kg p.o however on continuation of treatment with MKHA for next 7 days significant hypoglycaemic effect was observed even at both the doses (100 and 200 mg/kg p.o) thus indicating dose dependent effect of extract. The other extracts MKAQ (200 mg/kg p.o) ,OSM (200 mg/kg p.o) and OSHA (100 and 200 mg/kg. p.o) were found to be effective in preventing stress induced hyperglycemia on both 7th and 14th day of treatment. OSAQ (200mg/kg p.o ) was also found to be significantly effective in preventing stress induced hyperglycemia on continuation of treatment for another 7 days hypoglycemic effect was observed even at dose 100mg/kg along with 200mg/kg p.o. Both the plants MK and OS were found to possess excellent ability to induce hypoglycaemic effect might be by regulating peripheral glucose homeostatic mechanism and also by preventing stress induced rise in serum glucocorticoid levels which is responsible for inducing stress induced hyperglycemia.

Anticholine esterase drugs have been proved to be effective nootropic drugs. Effect of extracts on Hydrolysed Ach concentration was estimated which indirectly indicates the degree of choline esterase inhibition caused by extracts in the brain: In stress control group the concentration of hydrolysed Ach was found to be significantly increased thus indicating the cause behind significant memory deficit observed in animals. Standard Piracetam was
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found to be the most effective in restricting the hydrolysis of Ach by showing the highest fall in hydrolysed Ach concentration. Standard AS was also significantly effective in restricting Ach hydrolysis but the extent of choline esterase inhibition was almost 44% less than that demonstrated by Piracetam. Treatment with MKM (100 and 200 mg/kg p.o.) was found to significantly inhibit hydrolysis of Ach. However the effect exerted by MKM was less than that exerted by Piracetam it was comparatively higher than that of AS thus indicating that MKM has a significant nootropic potential by restoring Ach levels by preventing its hydrolysis. This effect on levels of Ach might be responsible for observed retrieval of memory of learned task in presence of stress on treatment with test extracts. MKHA (200mg/kg) was also found to be effective in preventing hydrolysis of Ach whereas MKAQ was not found to be effective in preventing acetyl choline hydrolysis significantly. OSHA (100 and 200mg/kg p.o.) and OSAQ (100 and 200 mg/kg p.o.) demonstrated significant inhibition of choline esterase enzyme Whereas OSM treatment failed to exert significant inhibition in choline esterase enzyme. Thus extracts of MK and OS were able to restore the levels of ACH effectively thereby preventing the metabolism of principle neurotransmitter (Ach) involved in process of memory retention in hippocampus. An active avoidance test was carried out to evaluate effect of extracts on retention of memory by using shuttle box. Stress-induced behavioural depression might have occurred due to alteration of brain acetylcholine (ACh) levels in addition to NE[650]. Ach metabolising enzyme Acetyl choline esterase (AChE) in the brain is primarily localized to areas containing cholinergic neurons [651,652] where it inactivates synaptic acetylcholine. However, AChE is also present in areas not containing cholinergic neurons and seems to exist in certain regions beyond requirements. Frontal cortex, hippocampus and striatum are areas where the levels of AChE activity seem to be related to the amount of acetylcholine [650,653].These areas are of interest in the context of stress due to FC model because projections between frontal cortex and the hippocampal system seems to be involved in "learned helplessness" [654,655]. Striatum, which is an area extremely rich in acetylcholine and AChE activity, is involved in extrapyramidal control of motor activity and thus in escape performance [656]. It has been reported that uncontrollable foot shock induced changes in AChE activity showed escape behavioural deficit. In our study the effect on a learned escape performance was assessed by using active avoidance test in shuttle box. Where animals were trained to escape from noxious stimulus before subjecting them to stress and then after subjecting them to the foot shock for 14 days chronically were again tested for retrieval of learned task. It was observed that escape failures were increased significantly in stress control group which can be
explained on basis of observed increased metabolism of neurotransmitter Ach in brain of stress control group. Increased hydrolysis of Ach due to stress was found to be restricted on treatment with test extracts thus corresponding decrease in number of escape failures were observed at doses 100 and 200mg/kg of MKM and MKHA. However on treatment with MKAQ no significant fall in number of escape failures were found may be because of failure to decrease the rate of hydrolysis of Ach observed with it. OSHA and OSAQ were also found to be significantly effective in reducing the degree of escape failure and thus successfully retrieving memory of learned task as number of escape failures in treatment groups were found to be decreased at doses 100 and 200 mg/kg. OSM failed to produce any significant decrease in number of escape failures. Thus the effective test extracts were found to reduce escape failures due to chronic foot shock by improving the motor activity as well as by maintaining retention of the memory of learned task Depression of active behaviour has been reported to occur in animals following exposure to highly stressful conditions that the animals could not control.[650,653,657-659] [71- 75]. The best-known explanation for why this depression occurs states that being exposed to an uncontrollable stressor an animal learns that it is helpless, or ‘nothing I do matters’. This learning is said to then transfer to other situations, resulting in a depression of active behaviour. Another type of explanation which had emerged pointed that the behavioural depression in inescapable foot shock was brought about by a transient change in brain catecholamines produced by uncontrollable shock [660,661]. Thus in this study depressive behaviour of helplessness induced by foot shock was evaluated as decline in active behaviour in terms of ”behavioural despair” for which forced swim test model was used. In this test DOI was considered as a parameter to be measured to evaluate degree of depression. In stress control group DOI was found to be decreased significantly similar to that observed in earlier studies. Treatment with MKM and MKHA at doses 100 and 200 mg/kg p.o. were found to decrease DOI significantly Whereas MKAQ did not exert any significant effect on DOI. OSHA and OSAQ at doses 100 and 200mg/kg p.o. and OSM at dose 100mg/kg p.o. were found to be significantly reducing the duration of immobility. In this model occurrence of depression is due to exposure to highly stressful conditions which animal could not control and teaches an animal that it is helpless. This learning transfers to other situations resulting in depression of active behaviour due to disruption in normal catecholaminergic transmission in brain mainly that of Norepinephrine. In stress control group brain norepinephrine levels were found to be decreased significantly. Treatment with MKM (100 and 200mg/kg p.o.) and MKHA (200mg/kg p.o.) was found to prevent this stress induced fall in levels of NE whereas MKAQ was not observed to be
effective in restoring the levels of NE levels in brain significantly. Treatment with OSHA and OSAQ were found to be significantly effective only at dose 200 mg/kg OSM was not found to be significantly effective. As high levels of corticosterone leads to increased sympathetic stimulation and high release of NE on prolonged exposure to stress. It has been observed that turnover rate of NE increases on exposure to stress for a long period thus showing initial aggressive response followed with depression lasting for a longer time. Norepinephrine levels in brain were found to be restored significantly by treatment with extracts may be by preventing excess turnover of NE occurring during the stress. In one experiment [662] it has been reported that if animals were given the MAO inhibitor Pargyline just prior to uncontrollable shock in order to prevent intraneuronal oxidation of amines, the subsequent impairment of shuttle avoidance-escape produced by the uncontrollable shock session did not occur. On estimation of brain NE levels it was observed that treatment with extracts of MK and OS were found to be prevent stress induced fall in brain norepinephrine levels. Thus extracts might be conferring the observed antidepressant effects by reducing the rate of NE turn over during the stress.

Another model used to evaluate anti stress effects of test extracts was sleep deprivation model. Loss of sleep generates a state of stress which induces deleterious effects on HPA axis. there by producing profound sleep disorders. As HPA axis and glucocorticoids are responsible for exerting the effects on variety of physiological processes such as metabolism, immune function neuronal viability, learning and memory [663]. To correlate these effects of stress in man various animal models have been developed to induce stress in rodents by partial or total sleep loss including single platform method, multiple platform method modified multiple platform method, [664], pendulum method etc [665] [81] In the present study single platform method was used. During sleep deprivation by single platform method activation of HPA axis may not be related to sleep loss but rather to the arousal resulting from being kept awake. As in single platform sleep deprivation method, to avoid falling in to water surrounding the platform animals were forced to stand on a limited area of platform for which they had to maintain mental and physical activity, which requires activity of the neuroendocrine systems to support the brain and the body in dealing with the tasks at hand. Furthermore, by staying awake, animals were potentially exposed to stressful input or were exposed by themselves to stress by thinking and worrying about their problems. The latter may increase the activity of the neuroendocrine stress systems beyond the level of relaxed wakefulness. This may be one factor contributing to the elevated levels of corticosterone in
stress control group. It is also reported that high cortisol levels are often seen in chronic insomniacs[666,667]. In our study the sleep deprivation for 72 hours lead to the significant rise in levels of serum corticosterone in stress control group as compared to vehicle control group. Treatment with MKM and MKHA at both the doses 100 and 200 mg/kg p.o. significantly prevented stress induced increase in levels of serum corticosterone and the extent of reduction in levels of serum corticosterone at these two doses were found to be equivalent to the decrease in serum corticosterone levels observed on treatment with standard AS. Treatment with MKAQ was not found to be effective in preventing increase in the levels of serum corticosterone to the significant extent .Standard Diazepam was also found to prevent the rise in serum corticosterone to 35% but was not found to be statistically significant on comparison with stress control group. Treatment with OSHA (100 and 200mg/kg p.o ) conferred a significant reduction in stress induced raised levels of serum corticosterone where the extent of reduction obtained at dose 200mg/kg p.o. was nearly equal to the effect produced by standard AS on serum corticosterone levels . Though other two extracts significantly reverted stress induced elevation in levels of serum corticosterone only at higher dose of 200mg/kg only , the reductions observed in levels of serum corticosterone were found to be comparatively less than that produced by OSHA. 

Serum glucose levels in stress control group were found to be decreased significantly in stress control group thus sleep deprivation lead to a significant hypoglycemia in stress control animals. MKM (100 and 200 mg/kg p.o.) and MKHA (200mg/kg p.o.) treatment prevented stress induced hypoglycemia .The extent of prevention of stress induced hypoglycemia demonstrated by both the extracts MKM and MKHA were observed to be greater than that produced by standard AS. In animals treated with Diazepam no significant restoration in levels of serum glucose was observed. OSHA OSAQ and OSM at doses100 and 200mg/ kg p.o) were observed to be preventing stress induced hypoglycemia at lower doses but the extent of rise in serum glucose level observed at the dose of 200mg/kg p.o. was the highest. The other two extracts were able to prevent stress induced hypoglycemia significantly but the degree of rise in serum glucose shown was less than that produced by OSHA. However were comparable with the standard AS. These results revealed a vital ability of extracts of MK and OS to restrict stress induced variations in glucose metabolism which is an important aspect of drug to prevent adverse consequences of stress on glucose metabolism such as hypoglycemia. Thus test extracts might had increased the tolerance of animals to withstand with stress
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during sleep deprivation by accelerating utilisation of some alternative secondary source of energy and/or sparing the glucose and making it available to tissues for a longer period.

An intensity of stress due to sleep deprivation was might be so high that due to earlier rapid utilisation of glucose from tissues stores, glucose in body might have depleted exhaustively which could have directed the shift in the body metabolism. Thereby, accelerating utilization of triglycerides (TG) as an alternative source of energy generation. As a result TG might had got used up as a secondary source of energy consequently accelerating uptake of TG from blood thus when TG levels in stress control group of animals were tested they were found to be significantly lower than that of serum TG levels in vehicle control group animals. As observed in previous studies, the treatment with test extracts were able to restore glucose homeostatic metabolism, expenditure of triglyceride in treatment groups was found to be decreased. MKM and OSHA treatments were found to exert effective control over TG levels at lower (100mg/kg p.o) as well as higher dose (200 mg/kg p.o) where as the other extracts were effective only at the higher dose although effects produced by all extracts on serum TG levels were comparable with that of standard AS.

Continuous exposure of animals to an intense stress induced by SD along with restricted mobility of animal might had lead to persistent activation of HPA axis which was evident with significant rise in serum corticosterone levels. This rise might be responsible for increase in synthesis of corticosterone in adrenal glands. To facilitate this accelerated synthesis of hormone corticosterone its precursor, cholesterol might had got released in circulation but due to immediate and rapid utilisation of cholesterol by adrenal glands for synthesis of corticosterone in stress control group. This might be the reason for the observed significant decrease in serum cholesterol levels as compared to vehicle control group. Treatment with MKM, MKHA, OSAQ and OSHA were found to produce significant prevention in stress induced fall in cholesterol levels significantly at doses 100 and 200mg/kg. Where as extracts MKAQ and OSM failed to exert any significant restoration in levels of serum cholesterol. This observed effect of restoration in serum cholesterol levels produced on treatment with test extracts were found to be parallel to the decrease in serum corticosterone levels produced by test. Thus effects on serum cholesterol were found to be in accordance with the earlier mentioned hypothesis of inverse proportionality relation between corticosterone and cholesterol levels in blood. Thus one can say that ability of extracts to prevent uncontrolled rise in serum corticosterone levels might had decreased the rate of
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synthesis of corticosterone leading the decreased utilisation of cholesterol as it’s precursor thus ultimately preventing fall in levels of serum cholesterol

Another chief candidate for mediating gradual changes in neuroendocrine stress reactivity and sensitivity to stress-related diseases with chronic sleep restriction is the serotonergic system. The serotonergic system is one of the major neuromodulatory systems in the brain, and there is a long history of research suggesting its involvement in sleep regulation.[668,669]. Serotonin neurons from the raphe nuclei have traditionally been considered as an integral part of the brain stem’s sleep generating system. Although serotonin may not be essential for maintaining sleep, it is thought to prepare the organism for sleep by attenuating brain systems responsible for cortical activation and behavioural arousal [670,671]. However, as one would expect from a major modulatory system, the actions of serotonin in the brain are complex and diverse. While under normal conditions serotonin prepares an organism for sleep, under adverse conditions it plays an important role in mediating stress responses [672]. Serotonergic neurons from the raphe have projections to areas involved in the regulation of emotionality and stress. It has been observed that lesions in the Raphe nuclei produced suppression of sleep and a decrease of brain serotonin [673]. Another study had reported that an enhanced utilization of released 5-HT from terminals decreases the intra neuronal levels of the amine in Sleep-deprived animals [674] thus one can say that increased turnover of serotonin in Sleep deprived animals appears to be related to the impossibility of triggering sleep. Our results are also found to be in accordance with these recordings in literature as in response to SD mean brain 5-HT concentration in stressed animals were observed to be significantly decreased. Treatment with MK extracts was able to restrict rapid utilisation of 5HT in brain significantly thus indicating potential ability of extracts in preventing amine perturbations most effectively in stress In case of OS extracts significant effects on brain 5HT levels were observed on treatment with OSHA (100 and 200mg/kg p.o.) and OSAQ (200mg/kg p.o) thus the ability of test extracts to prevent fall in brain levels of 5HT levels indicated that extracts might be executing their effects in a mode similar to conventional antidepressants thus declining the turnover of 5HT during SD. This study of extracts also suggested the antidepressant property of MK and OS extracts.

With respect to memory and cognitive performance, there are numerous reports of impairments after SD. For example, SD by the single platform method had found to result in impaired retention of passive avoidance memory, a context-dependent fear memory task [675] as well as impaired performance of spatial memory in the Morris water maze [676] and
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a reduction in long-term potentiatiation in the CA1 region of the hippocampus [677]. Sleep has been reported to impair memory consolidation [678]. In SD when tested for retention of learned task by using active avoidance task on cooke’s pole apparatus after the exposure to 72 hours of SD number of successful attempts in climbing the pole were signifi-cantly lowered in stress control group than that of unstressed animals. It is possible that the deficit on the avoidance task might had occured by a dysfunction of the septo-hippocampal system. Evidence supporting this hypothesis comes from different lines of research [679] which have shown that hippocampal lesions performed one day after training has abolished contextual fear memory The task used in our experiments is also based on contextual fear memory, as well. More direct evidence comes from the observation that SD was found to induce sub sensitivity of postsynaptic cholinergic receptors in some brain structures, among them is the medial septal area [680] , origin of the septohippocampal cholinergic pathway. Lesions to these pathways or pharmacological cholinergic blockade are known to impair memory [681,682]. Moreover, Skinner et al (1976) have found that the SD-induced memory deficit on inhibitory avoidance is blocked by physostigmine administration [683] suggesting the role of Ach in memory retrieval ; However some studies have also found that pre and postsynaptic dopaminergic and postsynaptic noradrenergic receptors are also affected by SD [684.685] thuıs from all these observations reported in literature it is not possible to rule out that dysfunction of these neurotransmitter systems are responsible for the deficit in retention of memory [686,687,688] . Treatment with extracts was found to prevent this SD induced deficit in retention of memory task as MKM (100 and 200mg/kg p.o.) and MKHA (200mg/kg p.o) treated group showed signifi-cant increase in number of successful attempts of climbing the rod , Whereas MKAQ failed to prevent memory deficit due to SD. Similarly lag time to jump on rod was measured on exposure to SD to analyse the effect of stress on recollection of memory .It was observed that in stress control group lag time was significantly increased Whereas treatment with extracts of MK and OS produced significant restoration in memory by demonstrating significant decrease in lag period to climb on rod. Hence the test extracts were found to be effective in retention of memory of learned task even after exposure to stress. When total time spent by animals on pole after every climb was calculated it was observed that mean residing period on the pole was found to be decreased in stress control group as compared to vehicle control group This effect might be because of combined effect of stress induced depression and amnesia resulting in less no of successful attempts performed by the animals to climb on the pole in stress control group but at the same time the possibility of fatigue due to SD and restricted mobility contributing to the increased falls
from the pole can not be ruled out. Treatment with standards was found to increase total mean residing period on the rod. Treatment with MKM and MKHA (100 and 200mg/kg p.o) MKAQ (200 mg/kg p.o), OSHA, OSAQ (100 and 200mg/kg p.o) and OSM (200mg/kg p.o) were found to produce significant increase in the retention period. similarly in the other study like WFS also extracts had already demonstrated their anti fatigue activity thus in this model increase in retention period exhibited on treatment with extracts might be due to anti fatigue property of test extracts which might had imparted the ability to animals to remain holding the rod for long period. However after falling down attempts to climb back on the rod to avoid noxious stimuli had also suggested that the extracts were effective in maintaining memory retention of learned task thus the resultant increase in RP on rod may be a combined impact of anti fatigue and anti amnesia effects executed by test extracts. Thus this study further supports the ability of MK and OS to increase non-specifically the resistance of animals to the stress to avoid its adverse consequences.

Since in In vitro studies of test extracts of MK and OS were found to possess good antioxidant potential their ability to prevent oxidative stress was also evaluated. oxidative stress was induced by subjecting the animals to chronic electro convulsive shocks (ECS). It had been reported that hormonal changes along the HPA axis mainly the neuropeptide corticotropin releasing hormone (CRH) had been shown to change during or after ECS. In rats treated with ECS an increase in CRH mRNA was found in a subset of paraventricular neurons, followed by an increase of pituitary ACTH content and elevated plasma corticosterone[689]. In our study in stress control group significant rise in levels of serum corticosterone on 7th and 14th day of ECS were observed. Treatment with MKM produced prevention in stress induced uncontrolled rise in levels of serum corticosterone initially on 7th day only at higher dose (200mg/kg p.o) where as on 14th day of treatment significant reduction in serum corticosterone were observed even at dose 100 mg/kg p.o. along with dose of 200 mg/kg p.o. MKHA (200mg/kg.p.o.) and OSHA (100 and 200mg/kg p.o.) and OSAQ (200mg/kg p.o) significantly prevented stress induced increase in mean serum corticosterone levels on both 7th and 14th days of study. Where as OSM (200mg/kg p.o) was found to be significantly effective only on 14th day of study. Effect produced by OSHA (200mg/kg p.o.) was the highest and was equivalent to that observed with standard Ashwagandha. Thus indicating once again the significant abilities of test extracts to resist stress induced uncontrolled rise in levels of serum corticosterone.

In stress control group due to significant rise in mean serum corticosterone levels demand of cholesterol for it’s synthesis might had increased thus mean cholesterol levels in stress
control group were found to be increased significantly on 1\textsuperscript{st}, 7\textsuperscript{th} and 14\textsuperscript{th} day of study. Treatment with MKM and MKHA prevented this stress induced rise in levels of serum cholesterol on both the time intervals 7\textsuperscript{th} and 14\textsuperscript{th} day of treatment. Thus as mentioned in earlier studies decreased demand of precursor (cholesterol) along with decreased levels of serum corticosterone on treatment with extracts may not be the only reason for fall in serum cholesterol levels but in literature cholesterol lowering effects of plant MK have been also reported with the findings that saponins and saponin like compounds present in \textit{Murraya koenigii} are able to prevent cholesterol absorption, interfere with its entero-hepatic circulation and increase its faecal excretion thus lowering serum cholesterol levels [395,396]. Hence resultant fall in serum cholesterol levels may be due to combined effect of central and peripheral actions of MK extracts. On treatment with OS extracts, OSHA and OSAQ were also found to decrease stress induced rise in serum cholesterol levels significantly on 7\textsuperscript{th} as well as 14\textsuperscript{th} day of study whereas OSM was effective only on 14\textsuperscript{th} day of study .Treatment with MK extracts was found to lower cholesterol levels more effectively than that of OS extracts and were comparable with the effect shown by standard AS.

The pronounced effect of high levels of glucocorticoids in stress is hyperglycemia which was observed in stress control group after subjecting the animals to ECS. Mean glucose levels in stress control group were found to be increased significantly as compared to vehicle control group. Treatment with MKM, MKHA and MKAQ were found to be effective in decreasing stress induced hyperglycemia on 7\textsuperscript{th} and 14\textsuperscript{th} day of study, OSHA and OSAQ were also found to be effective in preventing stress induced on both 7\textsuperscript{th} and 14\textsuperscript{th} day of study whereas OSM was found to be effective only on 7\textsuperscript{th} day of treatment. Both OS and MK have been reported in literature with the claims of significant hypoglycaemic effect. Various mechanisms had been proposed for their hypoglycaemic effect including increase in sensitivity of insulin receptors, increase in activities of carbohydrate-metabolising enzymes such as hexokinase, glucose-6-phosphate dehydrogenase and glycogen synthase and decrease in lactate dehydrogenase, fructose-1,6-bisphosphatase, glucose-6-phosphatase and glycogen phosphorylase [690,402]. This scientifically proven peripheral control of glucose homeostasis exhibited by MK and OS might be also responsible along with prevention of stress induced rise in levels of glucocorticoids for the observed hypoglycaemic effect.

The electrical storm that the nervous system is subjected to by ECS makes it unlikely that any information uniquely encoded in neural electrochemical activity will survive. Retrograde amnesia (RA) in patients who have received Electro convulsive shock treatment (ECS) often has a temporal gradient, so that memory loss is most severe for the time period
closest to the ECT [691]. ECS induced amnesia affects both semantic memory (recalling facts) and episodic memory (recalling details) [692]. Several hypothesis have been proposed for the induction of amnesia by ECS such as massive release of excitatory neurotransmitters and activation of their receptors, decreased cholinergic transmission, and disruption of long term potentiation (LTP) [693]. It has also been suggested that hippocampal mossy fibre sprouting, could be a possible mechanism for ECS amnesic effects as well [694]. While the traditional view is that ECS-induced amnesia results from medial temporal lobe disruption, [695]. ECS induced RA is associated with increased theta activity in the left frontotemporal region and greater reduction of regional cerebral blood flow in prefrontal cortex and anterior temporal regions [696]. According to the widely accepted Long term potentiation (LTP) mechanism of memory consolidation, repeated activation of a neuron's NMDA-type glutamate receptors leads to synaptic strengthening. The NMDA receptor has a binding site for glutamate and another for its co-agonist glycine. Ca2+ and Na+ channels open effectively only when two criteria are satisfied simultaneously which include receptor binding of glutamate and glycine and depolarization of the neuron [697]. The dual requirements of the NMDA receptor, ligand-gating and voltage-gating, allow associative learning [698]. The influx of Ca2+ into the post-synaptic cell activates signalling pathways responsible for long term synaptic changes. It is plausible then that ECS-induced amnesia is due to ECS-induced activation of NMDA receptors in a widespread and indiscriminate manner, thereby disordering the pattern of strengthened synapses and erasing stored memories [699]. Support for this mechanism of ECS-induced RA comes from several lines, including an animal model of ECS involving repeated electroconvulsive shock (ECS) induce increasing amounts of LTP and reduce the amount of further LTP that can be induced, suggesting saturation of the strengthened synapses [700, 701]. In addition it was found that in electrically stimulating rats' hippocampus causes RA for spatial information, as tested by a water maze. It was observed that when NMDA receptor antagonist was given beforehand, blocking the activation of NMDA receptors during the electrical stimulation, the rats demonstrated no amnesia. It is plausible that ECS could activate NMDA receptors indiscriminately [702] leading to excitotoxicity resulting in oxidative stress. There has been a recent suggestion that ECS induced glucocorticoid release may underlie in occurrence of amnesia [703]. In humans, the glucocorticoid dexamethasone has been shown to significantly worsen ECS-induced amnesia [704]. It had been suggested that higher glucocorticoid levels may predispose to more severe ECS-induced amnesia because of the positive modulatory effect that glucocorticoids on NMDA receptors [705] and has been shown to induce loss of dendritic spines and synapses,
shrink the hippocampus and impair cognition in both animals and humans [706]. In our study we observed that intensity of development of amnesia went on increasing with increase in intensity and duration of ECS in stress control group. When tested on Water maze latency time to reach to the platform was found to be increased as compared to animals not subjected to ECS. This RA induced by ECS was found to be reversed significantly on treatment with standard Piracetam on 7th as well as 14th day of study. Treatment of standard AS was also found to be significantly effective in preventing ECS induced RA. But the extent of effect observed with standard Piracetam was almost double than that produced by AS in restricting occurrence of RA. On treatment with MKM and MKHA test extracts significant prevention in ECS induced RA was observed. However decrease in LPP produced by MKAQ was not found to be statistically significant. Similarly OSHA (200mg/kg p.o) OSAQ (200mg/kg p.o) were also found effective in preventing ECS induced RA. Where as OSM failed to prevent ECS induced RA. Thus it can be suggested that plants MK and OS were found to possess an excellent nootropic potential. Test extract by preventing stress induced rise in levels of glucocorticoid in brain might be able to prevent the detrimental pathological effects of excess of glucocorticoid on neurons in hippocampus and amygdala portion s of brain which are rich with glucocorticoid receptors .This protective effect of test extracts maintained the integrity of neurons and thus the memory of learned task might have found to be retained.

Number of studies have reported that repeatedly administered ECS results in excitotoxicity and increase in plasma glucocorticoid levels. As brain tissues are rich with polyunsaturated fatty acids and are metabolically active. Oxidative stress was found to be induced by subjecting rats to ECS of gradually increasing intensity every day for a period of 15 days. In this model oxidative damage caused by ECS was found to be indicated by raised levels of aggressive factors such as lipid peroxidation and decreased levels of defence factors such as reduced glutathione and catalase levels in brains of stress control group. Thus in this study the effect of extracts on lipid peroxides, glutathione and catalase were estimated in rat brains [707]. When brain is exposed to oxidative stress, it increases the activity and expression of antioxidant enzymes as a compensatory mechanism against oxidative damage. The increased activity of the antioxidant enzymes may be adequate to protect the brain against potential oxidative damage. MK and OS had been extensively studied for their antioxidant activity in literature. In *in vitro* testing by DPPH and nitrite radical scavenging assay of MK and OS test extracts , both the plants were found to exhibit excellent free radical scavenging activity there by indicating anti oxidant property of test extracts . This free radical scavenging activity of test extracts might be responsible for prevention of free radical induced lipid peroxidation in
brain on treatment with test extracts of MK and OS. TBARS levels in animals treated with MK and OS extracts were found to be lower than that of stress control group. Thus protecting the brain from oxidative stress and maintaining the integrity of neurons hence preventing the deterioration of memory. In stress control group due to oxidative stress caused by ECS protective antioxidant such as catalase and GSH were found to be exhausted but on treatment with test extracts of MK and OS these protective aliments were found to be spared as their levels were found to be increased as compared to stress control group. Thus one can say that MK and OS extracts possess a promising potential to prevent oxidative stress and the resultant amnesia generally observed in Alzheimer patients.

Forced swimming exercise is regarded as a form of stress to the animal [708] which when is combined with cold temperature (20-25⁰c) may enhance intensity of stress even further. In the present study, we investigated the duration of immobility in animals using the forced swimming test in cold water and estimated the blood biochemical parameters to evaluate the antidepressant-like effect of the test extracts of MK and OS. Estimation of plasma cortisol level is a valid and controlled way to study how physiology of body reacts to psychological and physical stressors in laboratory settings [709]. It is known that cold swimming stress increases plasma corticosterone level [710] [127] In our study it was observed that levels of corticosterone were increased significantly on 1st as well as on 7th day of study. Treatment with both the standards AS and Imipramine significantly prevented stress induced rise in levels of corticosterone. However the extent of effect exerted by standard Imipramine was found to be higher than that that produced by standard As. On treatment with extracts it was observed that MK and OS extracts significantly prevented fall in serum corticosterone levels.

The most sensitive behaviour to corticosterone (the principle glucocorticoid in the rat) is immobility in the Porsolt forced swim test. When rats are forced to swim in a confined space from which they cannot escape after an initial period of vigorous activity they stay floating in a characteristic immobile position [702,703]. It has been reported that presence of glucocorticoids is responsible for the retention of the acquired immobility response in the test as inhibition of the synthesis of corticosterone with Metyrapone had been found to reduce the immobility time [704]. In our study when animals were exposed to cold water (20⁰c-25⁰c) swimming duration of immobility was observed to be increased on first as well as on 7th day of study in stress control group. The body’s hypothalamic set-point for temperature regulation is about 37⁰C+/- 1⁰C. It has been reported that exposure to cold temperature signals the thermoregulatory centre in the posterior hypothalamus to set off a number of
mechanisms to increase heat production. These include shivering causing involuntary muscular contractions in response to cold. This can cause a 4 to 5 fold increase in heat production. At the same time shivering results in decreased muscular coordination and impairs motor performance [705] resulting decreased mobility. Another reason for decreased immobility may be due to decreased rate of ATP hydrolysis occurring on exposure to cold causing decreased energy production and leading to increased fatigue thereby producing consequent increase in duration of immobility [706]. On first day of treatment only MKM was found to be effective in preventing rise in duration of immobility (DOI) in the beginning as well as at the end of 1 hour swimming. Where as OSHA was found to be effective in decreasing DOI only at the end of 1 hr of swimming session. After the treatment with extracts for seven days it was observed that all three extracts of MK as well as OS were able to reduce the immobility at variable extents but the highest reversal of increase in DOI was observed with MKM (200mg/kg p.o.) and OSHA (200mg/kg p.o.).

To assess the effects of stress on body metabolism the levels of several blood biochemical parameters in rats were estimated as alterations in DOI might had occurred due to changes in body metabolism in response to threatened homeostasis during CST. Increased plasma cortisol also influences the mobilisation of stored fat and carbohydrate reserves [707]. On estimation of serum glucose levels at the end of one hour swimming significant hyperglycemia was observed on both first as well as on 7th day of stress. A reason which can be postulated for this effect is that chronic stress application had been reported to cause loss of sensitivity of insulin receptors leading to hyperglycemia. Our results are also supported by a finding which had reported that every day swimming of one hour in cold water for consecutively seven days ultimately lead to significant rise in serum glucose level on 7th day of study. This commenced significant hyperglycemia indicated unavailability of glucose as a source of energy to exercising tissues thereby resulting in on set of fatigue which might had lead to significant fall in DOI on the 7th day of study. Treatment with MK and OS extracts were found to be significantly preventing stress induced hyperglycemia. This effect of extracts on serum levels of glucose indicated that both the plants MK and OS have significant hypoglycaemic effect. Fall in corticosterone levels produced by test extracts may be one of the reason in preventing rise in serum glucose levels especially for extracts MKM and OSHA which had been found to decrease stress induced elevated levels of corticosterone significantly. However other extracts though not showing very significant effect on corticosterone levels hypoglycemic effects observed on treatment with them hints that the extracts might be possessing an ability to control peripheral glucose homeostasis in addition
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to their central effect of controlling corticosterone secretion. May be the extracts are able to prevent loss of insulin sensitivity due to stress thus facilitating the absorption of glucose in tissues thus significantly reversing the stress induced hyperglycemia. Consequently effect of test extracts on glucose levels increased the availability of energy substrate which helped the animals to sustain in water by continuing the swimming for a longer period. Thereby decreasing DOI and preventing the onset of depression.

Serum cholesterol levels were found to be declined in stress control group. Numerous investigations had shown that psychological factors alter the normal pituitary adrenal activity and results in increased amounts of ACTH and cortisol secretion [708]. Established actions of ACTH include the activation of cholesterol side chain cleavage [709] and an increase in the uptake of cholesterol-rich serum lipoproteins [710]. Since cholesterol is precursor to corticosterone, it is possible that elevated cortisol secretion during stress increases demands for supply of its precursor [711]. These combined effects of increased ACTH secretion and activation of cholesterol side-chain cleavage [712] and lipoprotein uptake in response to stress may be one of the reasons for decrease in cholesterol levels in stress control group. MKM and MKHA and OSHA prevented stress induced fall in levels of cholesterol significantly at both the doses 100 and 200mg/kg p.o. whereas MKAQ OSAQ and OSM produced the effect only at higher dose of 200mg/kg p.o. On comparison between two plants MK was observed to be exerting significantly highest reversal of stress induced fall in serum cholesterol which was found to be equivalent to the effect produced by standard AS. Increase in serum cholesterol levels observed on treatment with standard Imipramine was not found to be statistically significant when compared with cholesterol levels of stress control group. As the extracts were found to prevent rise in serum corticosterone levels it might had resulted in reduced demand of blood cholesterol for synthesis of corticosterone thus reducing the uptake of serum cholesterol in adrenal glands for synthesis of corticosterone resulting in sparing of serum cholesterol and thus preventing fall in levels of serum cholesterol in treatment groups.

It had been reported that exposure of rats to cold stress increases plasma ACTH [713]. Serum Triglyceride (TG) levels in stress control group were found to be decreased as compared to vehicle control group. As stress liberates catecholamines, which together with ACTH and the ensuing corticosteroid production cause lipid mobilization. This includes increased TG hydrolysis and concomitant increase in free fatty acid (FFA) in circulation [714] which may be the reason behind the observed significant decrease in serum TG levels in stress control group. Another explanation which can be postulated involves shift in body metabolism to the utilisation of triglycerides as secondary source of energy due to unavailability of glucose to
the tissues. This metabolic shift might had increased utilisation of TG causing significant fall in serum TG levels. It is observed that stress induced activation of the sympathetic nervous system releases epinephrine and nor-epinephrine in response to cold exposure, causing a release of free fatty acids from fat stores by increased break down of TG from adipose tissues[715]. This fall in serum TG was prevented significantly and most effectively by MKM and OSHA at dose 200mg/kg p.o. Thus from these observations it is evident that MK and OS extracts conferred an ability to animals to withstand with stress due to forced swimming and exposure to cold temperature may be by altering peripheral energy metabolism and substrate utilization to enhance cold thermogenesis and to produce warmer body temperatures. Hence preventing shivering and loss of muscle in coordination. Thereby increasing the ability of animals to continue swimming for longer periods. At the same time an already explored antifatigue potential of plant extracts might had enabled the animals to continue swimming exercise for longer time thereby delaying the onset of depression.

From the invivo pharmacological screening of MK and OS extracts for evaluation of antistress effect it was evident that MK and OS plants possess a good anti stress potential. Plants. MK is reported to contain mainly Carbazole alkaloids in addition to terpenes whereas OS is known to be rich with presence of phenolic compounds. These phyto -constituents present in plants might had contributed to the observed anti stress effect exerted by test extracts of MK and OS.

**Formulation development:**
Pharmacological screening of test extracts of MK and OS revealed that MKM and OSHA were the most active extracts, significantly preventing stress induced physiological perturbations. Hence these extracts were formulated in capsule dosage form as hard gelatine capsules which are the preferred dosage formulations for herbal preparations. Three trial batches were prepared by using the various excipients at different ratios and their in vivo efficacy was predicted from dissolution studies.

In first trial batch FM1-01, Avicel was used as a diluent and adsorbent for the extract MKM during wet granulation. Aerosil was added as a glidant and adsorbent. It also has a good suspending property thus was added to increase the rate of release of MKM from the formulation and to enhance the absorption. Magnesium stearate was added as a lubricant to get the good flow properties to ensure uniform filling of the capsules as well as in vivo uniform release of the drug. On dissolution studies at pH 1.2, 83% release was observed with FM1-01 within first 15 minutes which was increased gradually from 85% to 90% from
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sampling period of 30 to 60 minutes intervals. After 1 hour there was no further increase in
dissolution profile. Thus in trial batch FM1-02 quantity of diluent was increased which did
not show any improvement in dissolution profile instead percentage release was observed to
be decreased. From these observations formula of FM1-01 was considered again with
addition of some more excipients to improve dissolution and disintegration properties. Thus
to the basic formula of FM1-01 was reconsidered to which Tween 80 was added as it is a
very good solubilising agent and Sodium starch glycollate was used to improve disintegration
properties of granules. On dissolution studies it was observed that FM1-03 exhibited
maximum release of 94% at 1 hour sampling period. This increased dissolution profile might
had occurred on addition of Sodium starch glycollate as it might had facilitated disintegration
of granules and Tween 80 might had improved the solubility of extract leading to increased
percentage release of extract. It was observed that percentage release of MKM was maximum
at acidic pH (1.2) than at pH 6.8 and in water. Since MK is known to be a rich source of
carbazole alkaloids which are weakly basic in nature and hence in acidic media might had
formed a salt. There by demonstrating maximum solubility of extract at acidic pH. This
release profile of MKM revealed that in Invivo studies formulation FM1 will be absorbed
maximally at stomach where physiological pH is acidic.

In first trial batch of OS FO1-01 as the extract OSHA was sticky hence wet granulation
process was adopted by using Avicel which is a good capsule diluent and adsorbant. Aerosil
was added as it is a glidant and improves the flow properties of granules. Magnesium stearate
was added to lubricate the granules and to impart good flow properties to the granules On
observing dissolution profile at pH6.8 , FO1-01 was found to produce maximum release of
84% at the end of 45 minutes of sampling period thus in Trial batch FO1-02 quantity of
Avicel was increased as granules of FO1-01 appeared to be hard and sticky thus hindering the
release of extract. Sodium starch glycollate was added to improve the disintegration property
of granules. Dissolution profile of FO1-02 demonstrated maximum release of 91% at
sampling interval of 45 minutes .Then in trial batch FO1-03 quantity of diluent Avicel was
further increased by keeping remaining formula same and on dissolution studies it was
observed that maximum percentage release of 95% was produced at 45 minutes sampling
interval. It was also evident from dissolution studies that FO1-03 produced maximum release
of active extract at pH 6.8 as compared to release observed at pH 1.2 and in Water. Since
OSHA is rich with Phenolic compounds which are acidic in nature they might had formed a
soluble salts at pH 6.8 which might had improved the percentage release of an extract at pH
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6.8. thus one can say that maximum absorption of FO1 is expected at intestine in In vivo studies.

These formulae were used to manufacture stability batches which were found to be stable throughout the stability period of 12 months as disintegration rate, dissolution profiles and content uniformities were found to be within limits of USP.

Isolation, Structural elucidation and Evaluation:
During separation of alkaloid fraction from extract alkaloids being weakly basic in nature might had formed a salt on addition of gl. Acetic acid at pH=2.5. Thus alkaloid fraction was separated from extract by acidifying the extract. The alkaloids salts were dissolved in water thereby pooling out alkaline carbazole alkaloids. carbazole alkaloids being highly non polar in nature were separated from aqueous phase by liquid-liquid extraction with the most non polar solvent Methyl Isobutyl ketone (MIBK). On TLC of alkaloid fraction three different spots at Rf were observed which developed a brown colour on spraying with Dragendorff’s reagent thus confirming the presence of alkaloids. On performing a column chromatography of MKMF1, the collected chloroform elutes were then subjected for structural elucidation by using various physical methods such as UV, IR, proton and 13 Carbon NMR. From the results of these physical methods structure was found to be analogues to the carbazole alkaloid Mahanimbine. Hence to confirm it MS spectra of MK1 was carried out. MS confirmed the structure of MK1 as Mahanimbine as from the peak at M+ ion 332 m/z observed in MS spectra of the MK1 molecular weight of the Mahanimbine for structure C23H25NO was confirmed.

In vitro testing of MKM, MKM1F and MK1 IC50 values were recorded to evaluate their antioxidant potential. Effect of extracts on DPPH radical scavenging activity and on lipid peroxidation were observed. The degree of antioxidant potential were observed to be of order MK1>MKM>MKMF1. As an extract MKM is composed of mixture of many other phyto constituents in addition to carbazole alkaloids which include terpenes flavonoids, steroids thus it’s antioxidant activity may be due to synergestic effect of mixture of phyto-constituents present in it. Where as MKMF1 was a fraction of only alkaloids separated from extract MKM thus lacking the presence of other phyto-constituents contributing to the antioxidant potential of the plant extract Hence fraction was found to posses comparatively lower anti oxidant potential as IC50 value for fraction MKMF1 was found to be higher than that of extract MKM. Whereas MK1 was the pure isolated component with high potency due to exclusion of all other impurities while performing column chromatography, which might be responsible
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for the observed lowest IC₅₀ value as compared to MKM and MKMFI when tested *in vitro* for both DPPH radical scavenging and inhibition of Lipid peroxidation activity. Thus the test extracts of MK and OS were found to possess significant antistress activity. As the test extracts were able to prevent stress induced rise in levels of corticosteroids and thus preventing the detrimental effect of hypercorticosteroinism on various physiological processes and avoiding the occurrence of stress consequences such as hyperglycemia, amnesia and depression. On evaluating the ability of extracts to prevent increased turnover of catecholamines in brain due to sympathetic activation during stress made us to speculate it as a possible mode of action to reduce depression. Ach turnover rate was also found to be decreased on treatment with test extracts which also hinted the possible mode contributing to observed decreased amnesia in various memory paradigms. Test extracts were also found to exhibit protective effect against immunological reactions and occurrence of stress induced ulcers. Extracts of MK and OS were also found to be bearing significant antioxidant potential in both *in vitro* as well as *in vivo* studies thereby executing neuro protective effect by demonstrating significant radical scavenging activity to avoid oxidative stress. From all these observed effects MK and OS were found to posses a significant potential to resist the adverse consequences of various types of stress and thus to avoid the genesis of stress related diseases. The plants were found to be exerting their antistress effect by increasing the body’s resistance to the stress by not a one specific mechanism but non-specifically by acting via different modes of actions for ultimately preventing the stress induced perturbation in homeostasis and its adverse consequences (diseases) during stress. These studies also contributed to emergence of *Murraya koenigii* as a new antistress herb in very limited plethora of antistress medicinal plants. Further our efforts directed towards the isolation of a phytoconstituent from MK provided a novel, an economical and precise method of isolation of Mahanimbine which on invitro testing was found to possess excellent antioxidant potential. Observed significant antioxidant effect of Mahanimbine threw a light on directing the research efforts for scientifically venturing a novel molecule with antistress potential in future.