Summary and Conclusion
I. Summary

*Ocimum sanctum* and *Evolvulus alsinoides* are well known in Ayurveda, the Indian System of medicine for their central and peripheral effects. These plants are rich in flavanoids and several other unknown compounds, which contribute to their observed pharmacological effects. Although because of its great therapeutic potentials and wide occurrence in India the practitioners of traditional systems of medicine have been using *Ocimum sanctum* L. for curing various ailments, a rational approach to this traditional medical practice with modern system of medicine however, is not much available. In view of the inadequacy of the work done till date in evaluating these plants, the bioassay-guided search was taken up in which, the chosen whole plants were extracted and fractionated. Subsequently, the important pure compounds were isolated and tested for anti-stress activity on peripheral stress markers and the pure compounds were studied for their effects on neurotransmitters and glucocorticoid receptor expression.

Stress has been postulated in etiopathogenesis of diseases and exacerbating various disease conditions. As stress is complex response involving both central and peripheral components, it is characterized by various biochemical and behavioral changes. In this study, stress models of varying duration and intensity were used.

Acute stress (AS), Chronic stress (CS) and Chronic unpredictable stress (CUS) is the three stress paradigms, which were considered to simulate stress mediated changes. In Acute stress (AS) model rats were immobilized for 150/min and were immediately sacrificed to observe the changes in stomach, blood and brain. This model mimics the typical fight or flight response and manifests the corresponding biochemical and behavioral changes. In chronic stress rats are exposed to single type of stressor (Immobilization) for seven days for /150min/day and sacrificed after the last stress session. This model provides us an insight into the adaptation response. Chronic unpredictable stress (CUS) is the most severe stress model in which rats were subjected to two different type of stressors every day for seven days and were sacrificed on 7th day immediately after immobilization. In all the stress models immobilization is the last stressor. Which, to some extent, minimizes the stressor variations.

The models are validated by observing changes in important stress markers in which damage of gastric mucosa was measured as ulcer index, adrenal gland weight and plasma is measured for changes in glucose, cretine kinase and corticosterone. Important brain regions frontal cortex, hippocampus and hypothalamus were observed for changes in
monoamines (Nor-adrenaline, Dopamine and 5-Hydroxy tryptamine) and also the glucocorticoids receptor expression by Western Blott analysis. Along with these, in view of the increasing attention for the role of free radicals in a variety of stress mediated pathologies like ageing, effect of stressors on free radical homeostasis was also studied by measuring the extent of lipid peroxidation and by measuring the activity of important defense enzymes like superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPX).

After the stress session animals were sacrificed by decapitation, adrenal gland and blood were collected and plasma was separated. Brain was dissected and frontal cortex (FC), Hippocampus (HP) and Hypothalamus (HT) were isolated. Brain regions are homogenated in 0.17M perchloric acid and analyzed for neurotransmitter changes by HPLC using electro chemical detector.

Plasma was analyzed for glucose and creatine kinase by auto analyzer using respective kits where as corticosterone and neurotransmitters were analyzed by HPLC with UV (240nm) and EC (+0.80V) detectors. Samples were prepared in TEGMED buffer for receptor expression studies by Western Blott analysis. In Western Blott analysis proteins were fractionated in 8%acrylamide gel and then transferred to a nitrocellulose membrane. The transferred proteins were treated with receptor specific primary anti-body and the primary specific secondary anti body tagged with biotin. Later the secondary was treated with avidin and developed using elcetrochemilumunisence (ECL) kit.

1. Effect of stress on central and peripheral markers

Stress was produced in rats by subjecting them to immobilization, footshock, swimming, fasting, day and night reversal and soil cage. According to the present study, acute stress (AS), chronic stress (CS) and chronic unpredictable stress (CUS) stressors are different in their effects. Peripheral effects of stressors in rats subjected to AS and CUS are similar in which there was a significant incidence of ulceration, adrenal hypertrophy, increased plasma creatine kinase and corticosterone levels. But, hyperglycemia observed in AS was not observed in CUS. In CS there was a decrease in ulcer incidence, Plasma creatine kinase levels, corticosterone levels and adrenal gland weight when compared to other models and hyperglycemia was not observed as observed AS conditions.

In AS, CS and CUS models considered in the present study, the extent of free radical load was tested. Out of the three models, oxidative stress was observed in CUS. There was a significant increase of lipid peroxidation, decreased total plasma anti-oxidant capacity and Plasma glutathione levels similar changes were also seen in frontal cortex
(FC), hippocampus (HP), hypothalamus (HT) and striatum (ST) regions of brain. Oxidative damage has marked effect on plasma anti-oxidant defense enzymes. Superoxide dismutase (SOD) and Catalase (CAT) were decreased. There is a marked raise in Glutathione peroxidase (GPX) levels with the depletion of glutathione.

Similar enzymatic changes are observed in the brain regions. However in AS and CS models, there was no marked difference from the control animals. This study conclusively states and support the concept that severe stressful conditions imposes a considerable “allostatic load” (wear and tear) which although not visible but is on longer duration may decrease the organisms efficacy to combat diseases.

Stress is a psychosomatic phenomenon and therefore the effect of stressors on open field behavior was studied. AS, CS and CUS decreased the open field activity. When monitored for 15 min after the last stress session there was a decrease in total distance traveled, stereotypy counts and horizontal distance traveled by the animals. This behavior was later normalized when observed after 24hrs. The behavioral depression observed in the study is not consistent and cannot serve as a marker for bioactivity testing. However behavioral changes are generally observed in severe stress models extending to several month duration.

With this data we further studied the neurochemical changes in brain of rats subjected to AS, CS and CUS. Acute stress exposure resulted in depletion of norepinephrine in cortex, hippocampus and hypothalamus where as the quantity of 5-HT (5-Hydroxy tryptamine) was elevated in these brain regions. Dopamine levels were increased in cortex and hypothalamus and decreased in hippocampus.

In CS there was a significant increase of 5-HT in hippocampus and a decrease of NA and DA in was observed in cortex.

In CUS there was a complete depletion of monoamines in all the above said brain regions. This study conclusively explains for most of the transient behavioral changes observed in several psychiatric disorders like depression, anxiety, addiction etc.

The study was further continued to evaluate the effect of stressors on glucocorticoid receptor (GR) expression in brain. There was a significant decrease of GR expression in cortex, hippocampus and hypothalamus regions of brain in AS model whereas in CUS it was observed only in hippocampus. However chronic stress did not show influence on GR density.

The crude extracts, fractions and pure compounds of Ocimum sanctum and Evolvulus alsinoides were screened for their central and peripheral effects in the
standardized stress models and compared with standard drug *Panax quinqufolium*.

2. **Anti-stress effect of crude extracts and fractions.**

Fresh juice of *ocimum sanctum* is commonly used for various minor ailments among the Indian population. So, fresh juice when screened it has shown good hypoglycemic activity Crude extracts of *Ocimum sanctum* was prepared in Ethanol (EtOH/W), Dichloromethane (DCM), Ethanol (EtOH) and fractionated in butanol (BuOH) and Water (Aq). All the extracts and fractions were preliminary screened for anti-stress activity in a dose dependent manner for their effects in acute stress model and found effective at a dose of 200mg/kg body weight. This dose was further continued in acute and chronic unpredictable stress models. In our study all the crude extracts and fractions except aqueous extract are effective in normalizing stress-induced increase in ulceration, adrenal gland weight, hyperglycemia, plasma creatine kinase and corticosterone levels. The anti-stress effects are comparable with standard drug *Panax quinqufolium*.

Crude extracts of *Evolvulus alsinoides* in ethanol (EtOH) and Water (Aq) were tested for their anti-stress activity in Acute stress (AS) and chronic unpredictable stress (CUS) models. EtOH extract was effective in AS and CUS induced changes whereas, aqueous extract was effective in AS model in all the parameters tested and ineffective in CUS model. Crude ethanolic extract fractions in Butanol (BuOH), Dichloromethane (DCM) and Water (Aq) were tested for their anti-stress activity out of which the BuOH fraction was found to be effective.

3. **Anti-oxidant Activity.**

Crude Ethanolic and aqueous extracts of *Ocimum sanctum* (OS) and *Evolvulus alsinoides* (EA) were tested at a dose of 200mg/kg body weight orally for *in-vivo* anti-oxidant activity during chronic unpredictable conditions in rats. In this study the crude ethanolic extracts of OS and EA shown a significant anti-oxidant activity in plasma and cortex, hippocampus, hypothalamus and striatum regions of brain as marked by reduced lipid per oxidation, increased plasma glutathione and toatal anti-oxidant capacity (TAC). The Superoxide dismutase, catalase and glutathione peroxidase levels were also normalized indicting their potent anti-oxidant activity during stressful conditions. However aqueous extracts have shown relatively poor anti-oxidant capacity.

4. **Effect of Ocimum sanctum and Evolvulus alsinoides on learning behavior**

In view of the potential implication of memory deficit during stressful conditions all the extracts and fractions of *Ocimum sanctum* (OS) and *Evolvulus alsinoides* (EA) were
evaluated for memory enhancement and anti-amnesic properties in Morris water maze and scopolamine induced dementia test.

Crude ethanolic extract of EA at a dose of 100mg/kg body weight has shown learning and anti-amnesic properties. However aqueous extract and fractions are ineffective.

OS is ineffective in exhibiting any learning enhancement properties.

5. **Isolation of pure compounds from active extracts**

The fractions were subjected to column chromatography (silica gel 60-180) and the pure compounds were isolated by monitoring TLC and there after they were characterized by IR UV, Mass and NMR studies.

6. **Anti-stress effects of pure compounds isolated from Ocimum sanctum**

Ursolic acid, 4-allyl-1-O-β-D-glucopyranosyl-2-hydroxy benzene, Apigenin -7-O-methylglucuronide and a sphingolipid were isolated from *Ocimum sanctum* and tested at a dose of 40mg/kg body weight orally for their effects on central and peripheral stress markers.

- **K-099 OS/A Ursolic acid**

OS/A was effective in all the tested parameters in acute stress conditions. But was ineffective in normalizing CUS elevated plasma corticosterone and creatine kinase levels. OS/A was effective in normalizing the acute stress induced decrease of 5-HT levels in cortex, hippocampus and hypothalamus regions of brain. It was effective in reducing the dopamine levels in cortex and hypothalamus but was ineffective in hippocampus. However OS/A did not show any effect on Noradrenaline levels in both acute and chronic unpredictable conditions. The glucocorticoid receptor density was not increased when compared to chronic unpredictable stressed rats. These effects mostly conform the dexamethasone like properties of ursolic acid.

- **K-116 (OS/B) [4-allyl-1-O-β-D-glucopyranosyl-2-hydroxy benzene]**

OS/B at a dose of 40mg/kg body weight was effective in AS and CUS models on all the peripheral markers. In our study this compound showed anti-stress effect at the neurotransmitter level regulating the Dopamine and 5-HT monoamines in AS and CUS models, OS/B was effective in decreasing the AS induced elevated levels of DA in frontal cortex and Hypothalamus and was ineffective in increasing the decreased dopamine levels in Hippocampus. Whereas effective in decreasing the AS induced elevated 5-HT levels in all the brain regions. In CUS, eugenol was effective in restoring the depleted dopamine and 5-HT levels in hippocampus and Nor-adrenaline, DA and 5-HT levels in
Hypothalamus, where as, ineffective in the frontal cortex. OS/B has not shown any effect on the NA in all the brain regions in AS and in CUS, and was effective in restoring the nor-adrenaline levels in hypothalamus only. The decreased plasma concentration of corticosterone was well correlated with the restored Glucocorticoid receptor levels in hippocampus of rats subjected to CUS.

- **K-171-OS/C**  
  \[3,4,5-trihydroxy-6-[5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-7-yloxy]-tetrahydro-pyran-2-carboxylic acid.\]
  
  In our study OS/C was administered orally at a dose of 40mg/kg body weight. OS/C was effective in reducing AS induced hyperglycemia, and adrenal hypertrophy. It was effective in decreasing ulcer incidence, elevated plasma creatine kinase and corticosterone levels. But was ineffective in reducing CUS induced adrenal gland hypertrophy.

  OS/C was effective at 40mg/kg dose in normalizing the acute stress induced increase 5-HT in frontal cortex, hippocampus and hypothalamus. It was also effective in reducing the elevated dopamine levels in frontal cortex and Hypothalamus but was ineffective in normalizing the depleted dopamine levels in hippocampus. However OS/C did not show any effect on AS depleted Nor-adrenaline levels.

  In CUS model, OS/C failed to produce any effect on depleted nor-adrenaline levels in all the brain regions. But, was effective in restoring dopamine and 5-HT significantly in frontal cortex and hippocampus but did not show any effect on Hypothalamus. From the peripheral and neurochemical data obtained in our study, the anti-stress effects of, are mainly due to its serotonin and dopamine modulation in cortex and hippocampus. In western blott analysis OS/C was effective in restoring the depleted glucocorticoid receptor density. The neurochemical profile and glucocorticoid receptor data supports the role of OS/C for the anti-stress and anxiolytic effects of *Ocimum sanctum*.

- **K-117 OS/D**  
  \[[Sphingo lipid]\]
  
  It is a long chain fatty acid ester first time isolated from *Ocimum sanctum*. In our study this component was not effective in any of the stress parameters considered.

7. **Anti-stress effects of pure compounds isolated from *Evolvulus alsinoides***

Caffeic acid, erythritol, and a flavone glycoside are the compounds in their pure form isolated from *Evolvulus alsinoides* (EA).

- **K-014- EA/A **  
  \[[Caffeic acid]\]
  
  Caffeic acid was tested for anti-stress effect at a dose of 40mg/kg body weight. It was effective in normalizing the AS and CUS induced peripheral changes. But, has not
shown any effect on stress induced adrenal hypertrophy. It was effective in reducing the AS induced increase in dopamine levels in cortex and hypothalamus and restored the depleted levels of dopamine in hippocampus. Caffeic acid normalized the stress elevated levels of 5-HT in all the brain regions. In CUS caffeic acid was ineffective in normalizing the depleted levels of neurotransmitters where as It has not shown any effect on nor-adrenaline levels in the entire brain region in both AS and CUS models.

- **K-015 EA/B 2-[4,5-dihydroxy-2-(4-hydroxy-phenyl)-4-oxo-4H-chromen-7-] glucose.**

  EA/B was tested at 40mg/kg body weight for its anti-stress properties. This compound was effective in reducing stress-induced hyperglycemia, mean ulcer severity, and plasma creatine kinase levels. But was found to be ineffective in reducing adrenal hypertrophy in AS and CUS models and normalizing the CUS induced elevated levels of corticosterone.

  EA/B glucose in AS model was effective in normalizing the stress induced elevated levels of Dopamine in cortex, hypothalamus and increased the depleted levels of dopamine in hippocampus. It was also effective in decreasing the elevated levels of 5-HT in all the brain regions. How ever in CUS model EA/B glucose was ineffective in producing any changes in the depleted monoamine levels and also has no effect on NA in all the brain regions in AS and CUS models.

- **K-019 EA/C Erythritol.**

  This compound at a dose of 40mg/kg has not shown any central or peripheral effects in all the parameters considered in our study.

## II. Conclusion

*Ocimum sanctum* is well known for its anti-oxidant and anti-stress effects. Since most of the studies carried out with this plant are on crude extracts, the important bioactive substances that constitute for its diverse functions were not clearly demonstrated. In our search with bioassay guided approach we found that Ursolic acid (OS/A) Eugenol glycoside (OS/B) Apigenin glycoside (OS/C), which constitute the most important components have considerable central and peripheral effects during stressful conditions. When the three compounds were compared Ursolic acid is having dexamethasone like affects due to which may be the anti-inflammatory and immunomodulatory effects of *Ocimum sanctum* were observed. Eugenol glycoside and Apigeninglycoside purely worked as anti-stress compounds by normalizing the neurotransmitter and Glucocorticoid
receptor levels. But, the specific mechanism of action is still to be verified by delineating the corticosterone effects (adrenolecctomy) and study for their effects on adrenocorticotropic hormone (ACTH) a silent marker of stress response and enzymes like Catechol-O-methyl transferase, Monoamino oxidase etc which can give better understanding of their neuromodulation.

_Evolvulus alsinoides_ is well known for its memory enhancing and anti-epileptic properties in Ayurveda. In our study the memory enhancing properties are well demonstrated in crude ethanolic extract. The active constituents caffceic acid (EA/A) and flavone glycoside (EA/B) were effective in acute stressful conditions but failed to have effect in chronic unpredictable conditions. Our study provides scientific evidence for the memory enhancing properties, supports the anti-oxidant properties as demonstrated earlier and suggests the possible central effects of _Evolvulus alsinoides_.

From this bioassay guided study it can be concluded that _Ocimum sanctum_ and _Evolvulus alsinoides_ are potential sources for centrally active substances of which few potential substances were identified.