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Adaptogenic Activity of *Morus Alba* Extracts in Wistar Rats

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Keywords: *morus alba, stress, homovanillic acid, urinary vanillymandelic acid, 6-**β**-OH-cortisol, 5-hydroxyindo- leacetic acid, ascorbic acids.*

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Adaptogenic Activity of *Morus Alba* Extracts in Wistar Rats

Dr. Somayeh Afsah Vakili [α] & Syed Fayazuddin [σ]

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I. Introducton

Stress can be defined as the sum total of all the reaction of the body, which disorganise the normal physiological condition and result in a state of threatened homeostasis. Stress is an internationally conceded phenomenon fortified by advancement of industrialization in a demanding civilization. Thus every individual is likely to face stressful situation in day to day life (Selye, 1998). Stress is a stimulus that activates the hypothalamic pituitary adrenal (HPA) axis and Sympathetic Nervous System (SNS) and begets a physiological change. Physiological responses to stressful stimuli, including the increases in blood pressure, heart rate, body temperature and plasma concentration of adreno-corticotrophic hormone (ACTH), can be related to the stress induced activation of the SNS. Stress prompts synthesis and release glucocorticoids (corticosterone and cortisol) and monoamines such as epinephrine, dopamine, norepinephrine and serotonin which are characteristic stress hormones (Carrasco et al., 2003). Adaptogens are the substances that help organisms to adapt to unfavourable stressful conditions, which could be physical, chemical, biological or mental conditions (Rege et al., 1999). The prevalent objective of adaptogenic therapy is due to diminish stress reactions during the alarm phase of the stress response, inhibit or retard the state of exhaustion and consequently issue a certain level of protection against long-term stress (Wagner et al., 1994). *Morus alba* belongs to family *Moraceae* commonly called as white mulberry. This plant has been used traditionally as anti-asthma, antidiabetic (Singab et al., 2005), hypotensive (Fukai et al., 1985) and neuroprotective (Kang et al., 2006). The current investigation was carried out to assess the anti-stress activity of aqueous and ethanol extracts of *Morus alba*.

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II. Materials and Methods

a) Plant material and Preparation of extracts

The fruits of *Morus alba* were collected from Chennai, Tamil Nadu, India and authenticated by Green Chem, Bangalore, Karnataka, India, a voucher specimen (MAT-SIP-501) were preserved for future references. The fruits materials (1kg) was dried, powdered and extracted with water and ethanol (60-80°C) using soxhlet methods. The filtrate was evaporated at 70 °C in a vaccum dryer to give final yield 40.5g.

b) Chemicals

Homovanillic acid (CAS 306-08-1), urinary vanillymandelic acid (VMA), 6-β-OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid was purchased from Sigma, ST Louis, MO, USA. Acetonitrile and methanol HPLC grade were supplied from Qualigens, Fischer Scientific, Mumbai. All other chemicals were analytical grade and obtained from local store of Visveswarapura Institute of Pharmaceutical Sciences.

c) Animals

Albino Wistar rats (150-200gm) of either sex obtained from the NIMHANS animal house, Bengaluru and were housed at room temperature in a well-ventilated animal house under 12 hrs light / dark cycle in polypropylene cages (29"x22"x14") with stainless steel grill top, bedded with paddy husk. The animals were maintained under standard conditions in an animal

house as per the guidelines of "Committee for the Purpose of Control and Supervision on Experiments on Animals" (CPCSEA) for at least one week prior to use. The rats had free access to standard rat chow and water *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore. (Registration No: 152/1999, renewed in 2012).

d) Calibration curves of HVA, VMA, 6-β-OH cortisol and 5-HIAA in urine by simultaneous HPLC determination

Simultaneous HPLC determination of HVA, VMA, 6-β-OH cortisol and 5-HIAA in urine were determined to assess their standard value (Sreemantula et al., 2004) . Different dilutions 25 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml, 1000 ng/ml, 2500 ng/ml, 5000 ng/ml of the HVA, VMA, 6-β-OH cortisol and 5-HIAA were made with mobile phase from the working standards (1mg/ml) which consist of 250 ng/ml internal standard (Ethyl-3-hydroxy-4-methoxy-mandelate). These dilutions were spiked into the HPLC and calibration curves were plotted as peak area ratio vs. concentration. The peak area ratios of HVA, VMA, 6-β-OH cortisol and 5-HIAA to that of internal standard were calculated and substituted in the respective regression equations to estimate the amount of the metabolite present in the sample.

e) Calibration curve for ascorbic acid

Calibration curve for ascorbic acid was done according method of (Roe and Kuether, 1943). Standard solution of ascorbic acid (1mg/ml) was prepared by dissolving 10 mg of ascorbic acid in 10 ml of distilled water. By using this different concentration of ascorbic acid 5, 10, 20, 30, 40 μg/ml were prepared with 4% Trichloro Acetic acid (TCA). 10 ml of each of these were taken and mixed well with 0.375 gm of activated charcoal and filtered. 4% TCA was used for the blank. From the filtrate 1 ml was taken in to the test tube and a drop of thiourea was added to that. Then to those test tubes 1 ml of 2,4-Dinitro Phenyl Hydrazine (2,4-DNPH) was added and kept in incubator for about 3 hrs, maintained at 37^{0}C. The tubes were then placed in the beaker containing ice and 5 ml of 95% sulphuric acid was added drop by drop within 1 min interval with intermittent mixing. Finally they were shaken and kept aside for 30 min. Then optical density was measured at 550 nm using spectrophotometer [Shimadzu (UV-1601)]. Standard curve was plotted by taking concentration of ascorbic acid on X-axis and optical density on Y-axis.

f) Evaluation of anti-stress activity

Rats of either sex weighing between 180–220 gm were divided into five groups (I, II, III, IV & V) each containing six animals. The 24 hr urine sample from each group were collected into two different beakers, one containing 5 ml of 10% oxalic acid for the spectrophotometric determination of ascorbic acid at 550 nm and the other containing 0.5 ml of 6N hydrochloric acid for the determination of stress metabolites. The experimental protocol was divided into four phases: In the first phase of the experiment, 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the normal values were recorded for four consecutive days. In the second phase, after a recovery period of one week, the animals in each group were subjected to fresh water swimming stress individually. In this method, rats are forced to swim until exhausted in a cylindrical vessel of 60 cm height and 45 cm diameter containing water at room temperature (28°C). Water depth was always maintained at 40 cm. The 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the values were recorded for four consecutive days. In the third phase of the experiment, after a recovery period of one week, the experimental animals were administered as follows for four consecutive days. Group 1 rats served as normal control and received 2ml/kg distilled water, group 2,3 rats were administrated orally with aqueous extracts of *Morus alba* at dose of 200 mg/kg and 400 mg/kg respectively. Group 4,5 rats received ethanol extracts *Morus alba* orally at dose of 200 mg/kg and 400 mg/kg respectively. In the final phase of the experiment, after a recovery period of one week, the administration of *Morus alba* extract were done as mentioned in the third phase, one hour prior to the daily induction of stress for four consecutive days while group I serving as control. The 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the values were recorded for four consecutive days to study the influence of the aqueous and ethanol extracts of *Morus alba* on the stress induced biochemical changes (Sreemantula et al., 2004).

g) Statistical analysis

The data were expressed as mean ± S.E.M. Statistical analysis was performed by using student's paired t-test, where the difference was considered significant if $p < 0.05$.

III. Results

a) Calibration curves of biomarkers for determination of standard value

A typical chromatogram was manifested in Figure 1. The retention times of VMA, IS (Ethyl-3-hydroxy-4-methoxy-mandelate), 5-HIAA, 6-β-OH cortisol and HVA were found to be 4.46, 5.210, 9.31, 10.84 and 12.15 respectively. The standard graphs of HVA, VMA, 6-β-OH cortisol and 5-HIAA were displayed in Figure

2,3,4 and 5. Calibration curve for ascorbic acid was exhibited to estimate standard value in Figure 6.

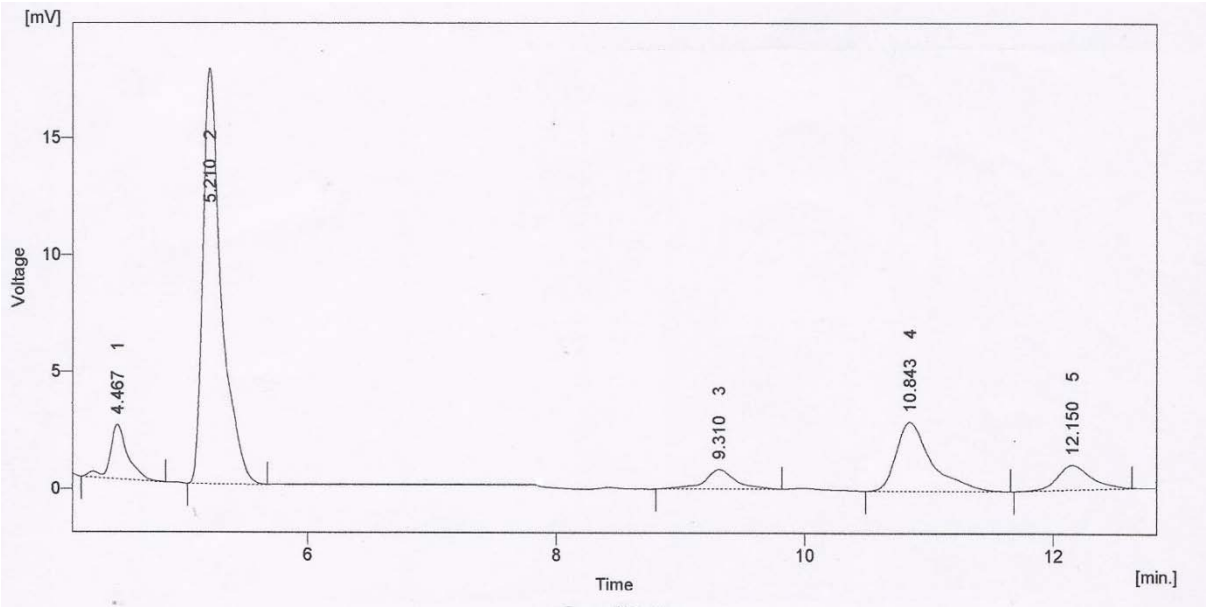

Figure 1: Chromatogram representing simultaneous method for the determination of standardHVA, VMA, 6-β-OH cortisol and 5-HIAA

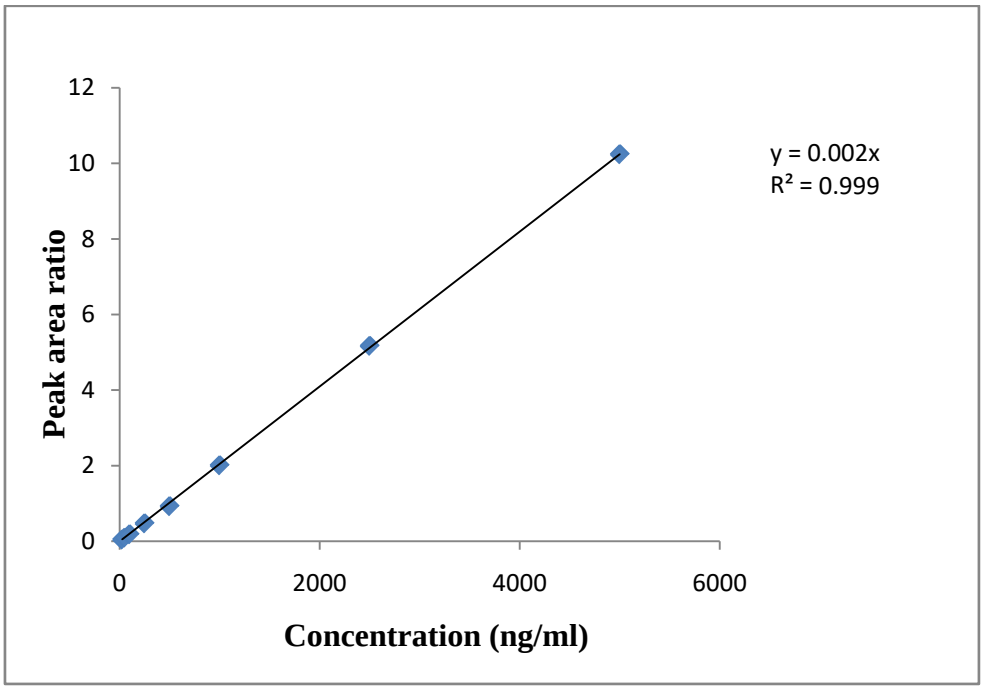

Figure 2: Standard graph of Homovanillic acid (HVA)

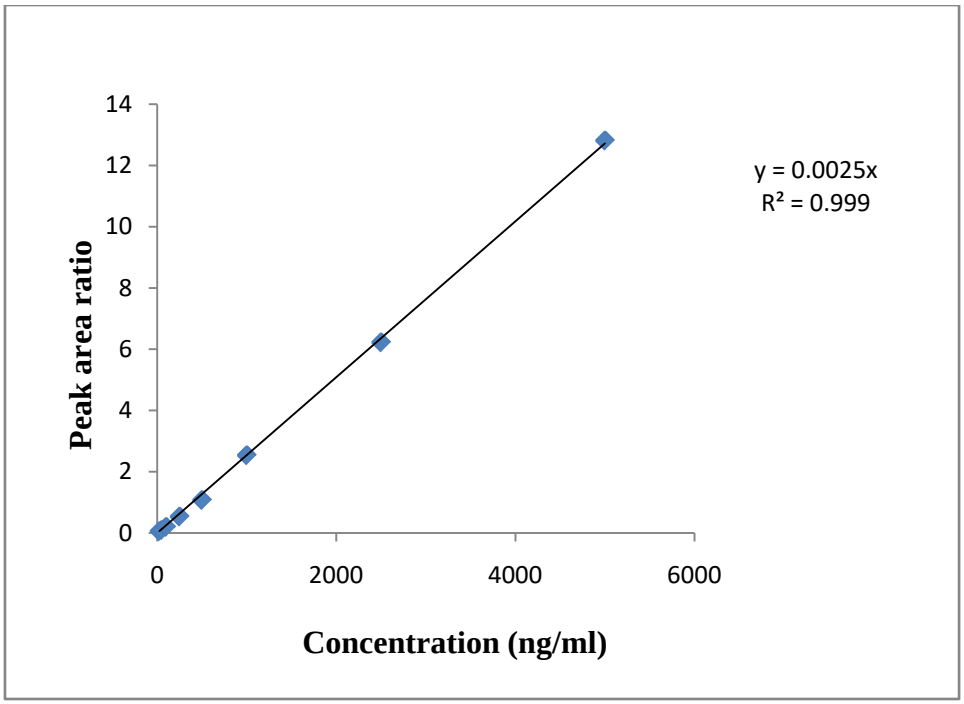

Figure 3: Standard graph ofVanillylmandelic Acid (VMA)

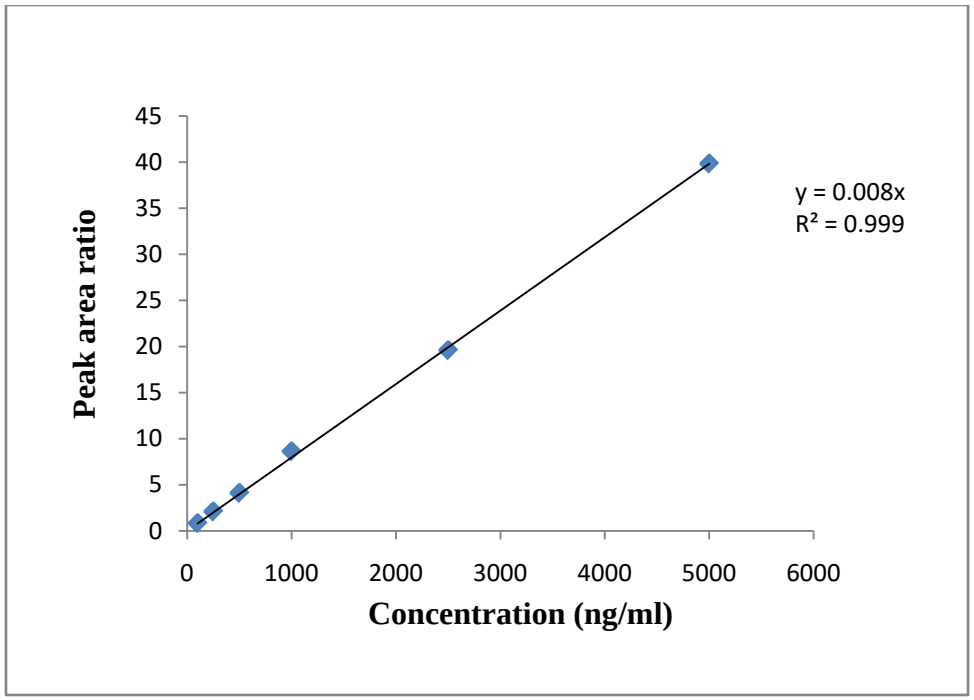

Figure 4: Standard graph of 6-β-Hydroxy Cortisol (6-β-OH cortisol)

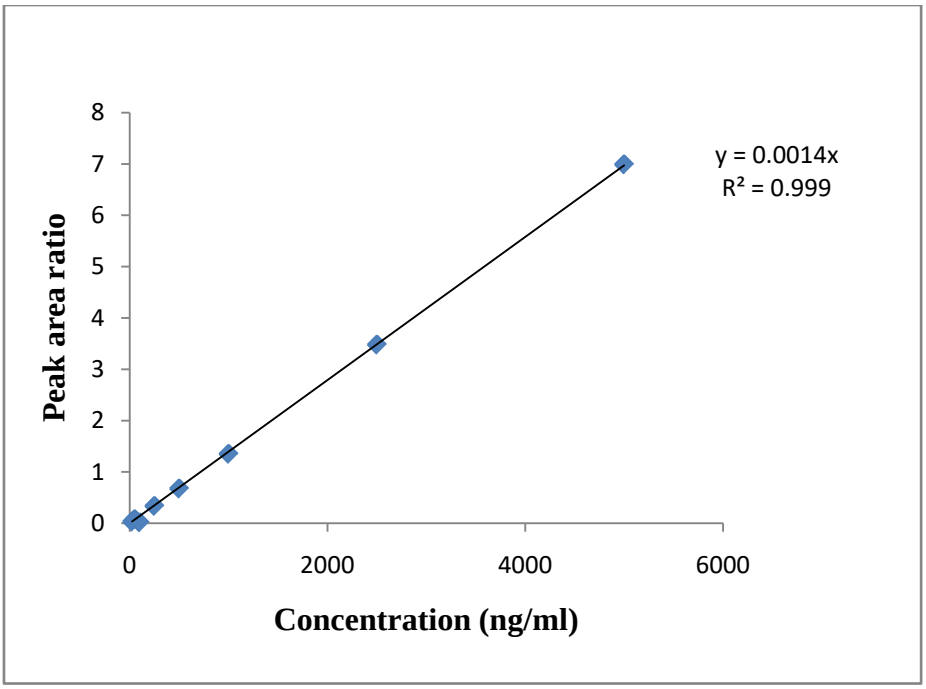

Figure 5: Standard graph of 5-Hydroxy Indole Acetic Acid (5-HIAA)

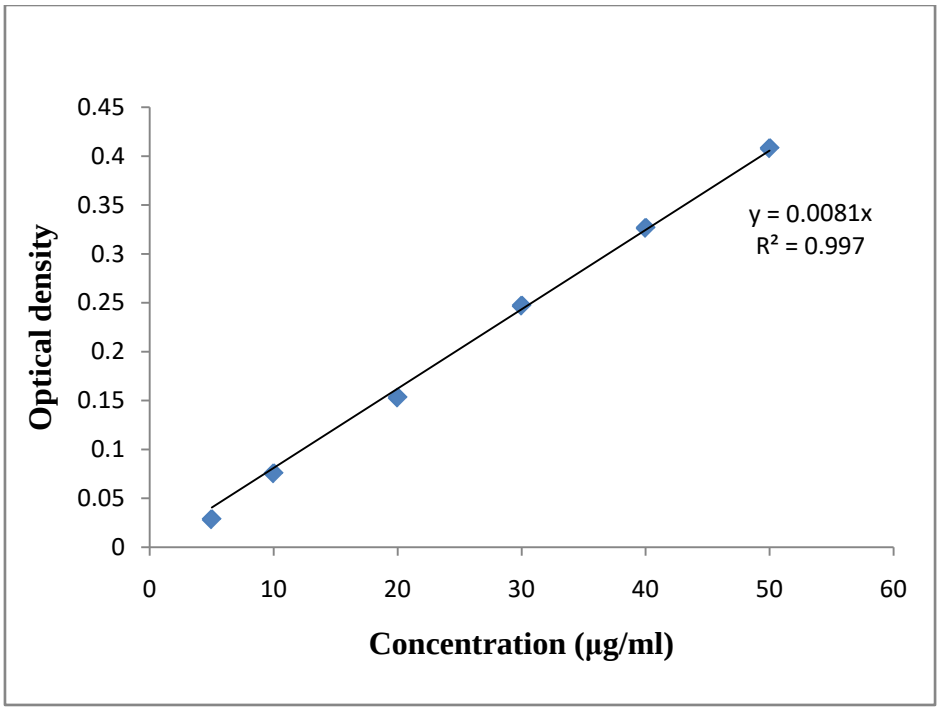

Figure 6: Standard graph of Ascorbic acid

b) Evaluation of anti-stress activity

Figures 7, 8, 9, 10 and 11 revealed the effect of aqueous and ethanol extract of *Morus alba* on urinary levels of biomarkers in normal and stress condition. There was variation in each biomarkers from group to group in normal state. The amount of VMA (0.343 µg ± 0.019), 5-HIAA (0.348 µg ± 0.013) and HVA (0.113 µg ± 0.009) were low in group I in normal state and high amount of VMA (0.431µg ± 0.017), 5-HIAA (0.463 µg ± 0.022) and HVA (0.176 µg ± 0.006) were found in group IV in normal state. The level of 6-β-OH cortisol was low in group V (0.424 µg ± 0.032) and high in group I (0.557 µg ± 0.010), also the amount of ascorbic acid was low in group IV (43.92 µg ± 2.33) and high in group I (52.64 µg ± 5.64) in normal condition. Significant increase ($P<0.05$) in urinary levels of VMA, 5-HIAA, 6-β-OH cortisol and HVA was noted in group I to V. Significant decrease ($P<0.05$) in urinary levels of

ascorbic acid was observed in group I to V in stress condition. There were slight changes in VMA, 5-HIAA, 6-β-OH cortisol, HVA and ascorbic acid levels in urine of animals treated with aqueous and ethanol extracts of *Morus alba* in the normal state. There was variation from day to day and the variation is different from group to group. However observed the changes in the levels of the urinary metabolites when compared to normal basal levels were found to be non-significant. Aqueous and ethanol extracts of *Morus alba* significantly ($P<0.05$) diminished urinary levels of VMA, 5-HIAA, 6-β-OH cortisol and HVA in group II, III, IV and V, also significant increased ($P<0.05$) in urinary ascorbic acid levels was perceived in group II, III, IV and V compared to their respective stress condition.

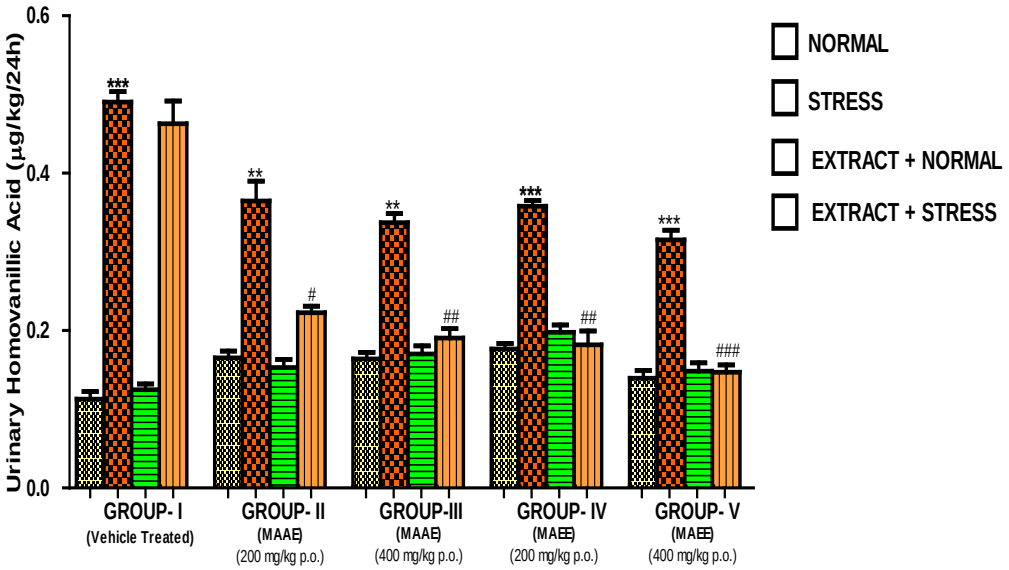

Figure 7: Influence of aqueous and ethanol extracts of Morusalbaon 24h urinary levels of HVA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. ***P<0.0001, **P<0.001 significant difference from normal condition of the corresponding groups; ###P<0.0001, ##P<0.001, #P<0.01 significant difference from stress condition of the corresponding groups.

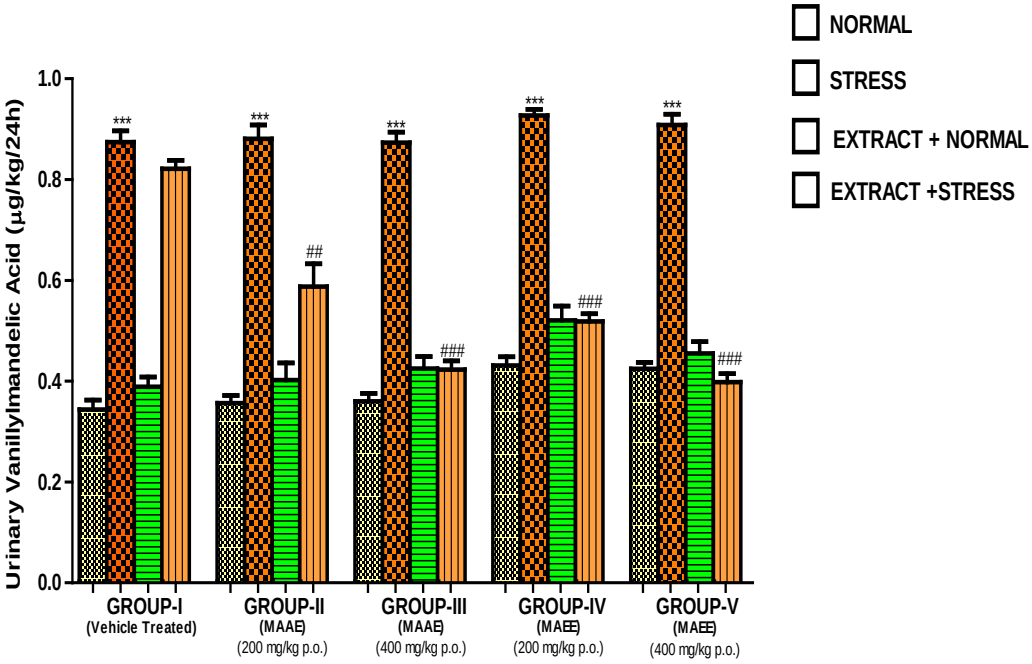

Figure 8: Influence of aqueous and ethanol extracts of Morusalba on 24h urinary levels of VMA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. ***P<0.0001 significant difference from normal condition of the corresponding groups; ###P<0.0001, ##P<0.001 significant difference from stress condition of the corresponding groups.

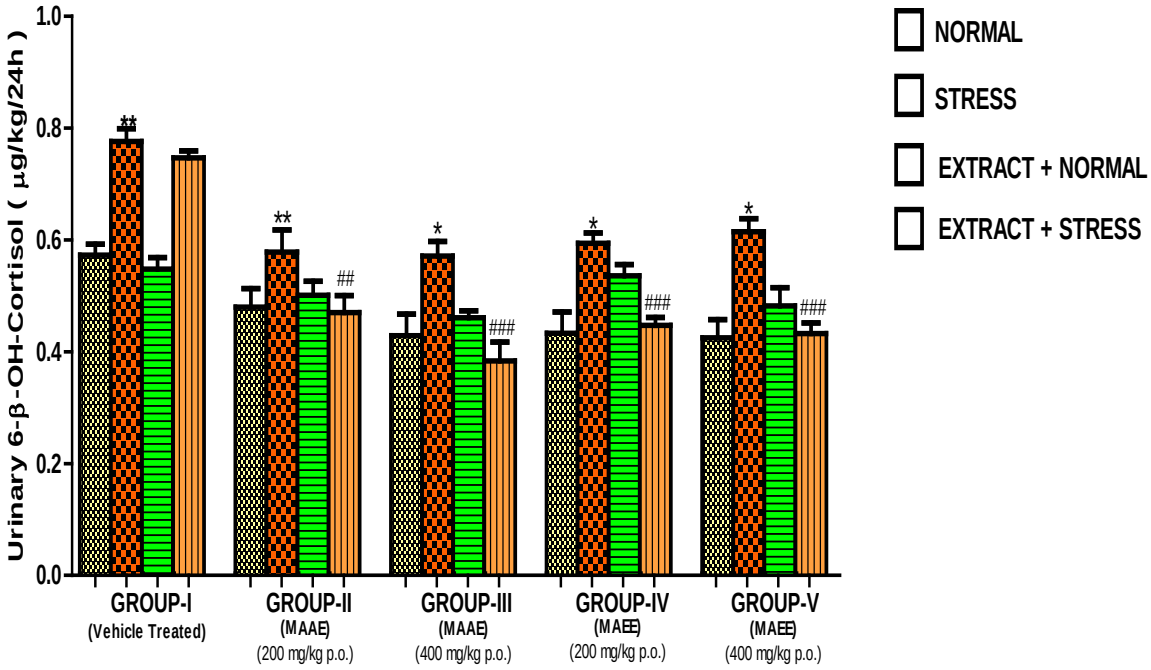

Figure 9: Influence of aqueous and ethanol extract of Morusalba on 24h urinary levels of 6-β-OH-Cortisol in normal and stress induced rats. Each bar indicates the mean excretion of six animals. **P<0.001, *P<0.01 significant difference from normal condition of the corresponding groups; ###P<0.0001, ##P<0.001 significant difference from stress condition of the corresponding groups.

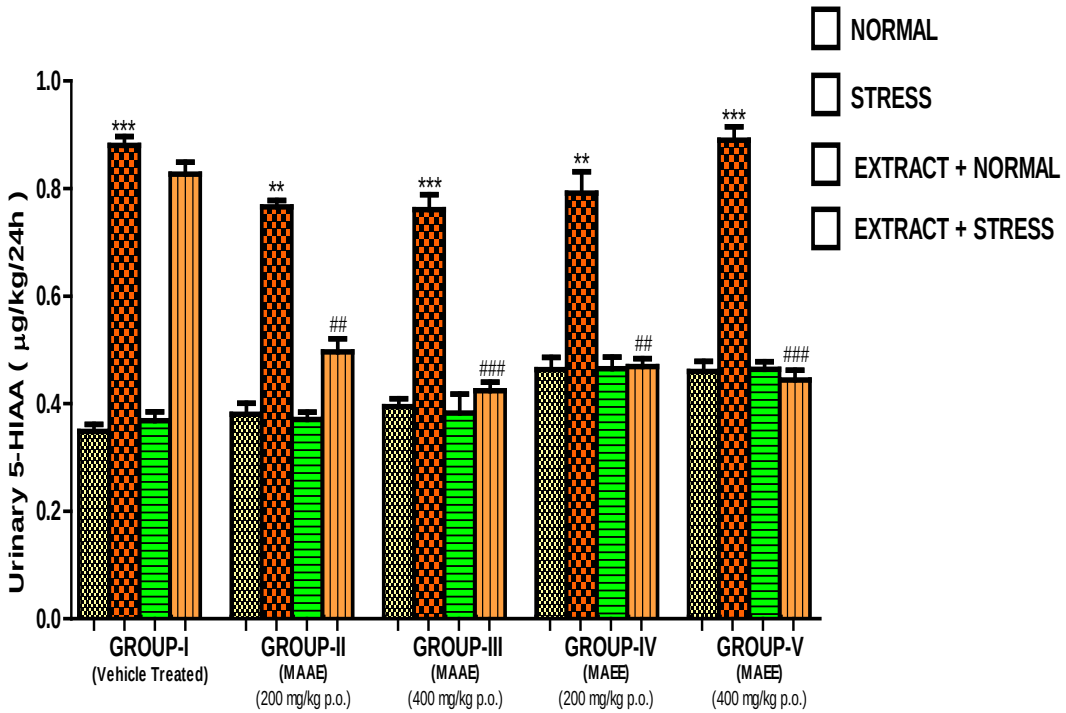

Figure 10: Influence of aqueous and ethanol extracts of Morusalba on 24h urinary levels of 5-HIAA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. ***P<0.0001, **P<0.001 significant difference from normal condition of the corresponding groups; ###P<0.0001, ##P<0.001 significant difference from stress condition of the corresponding groups.

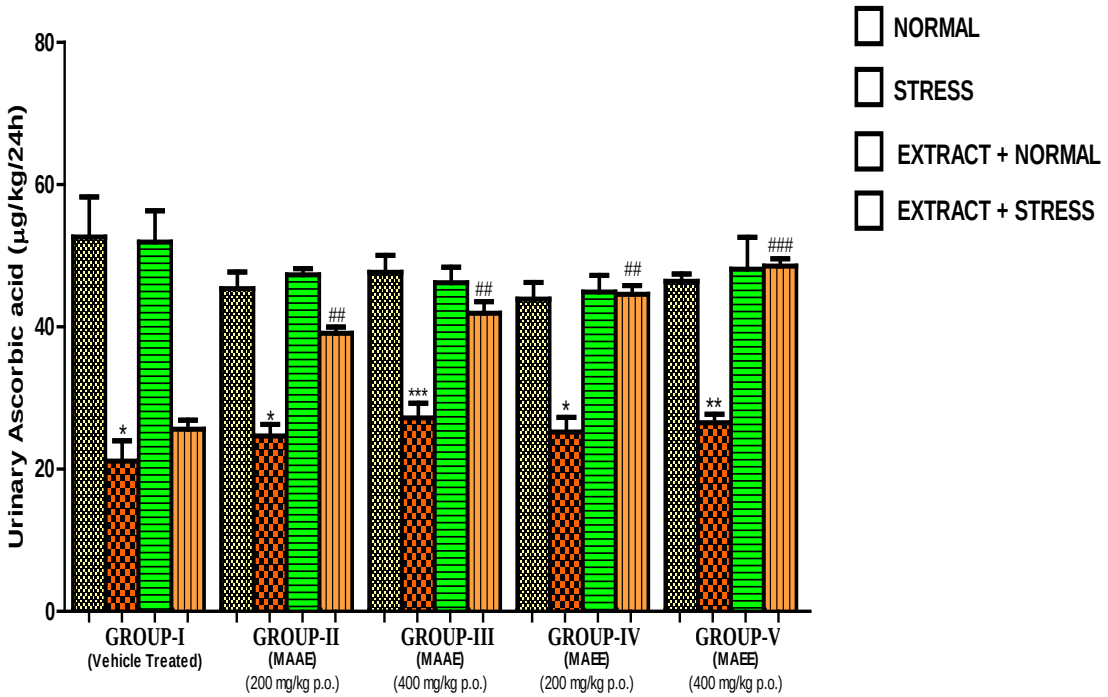

Figure 11: Influence of aqueous and ethanol extracts of Morusalba on 24h urinary levels of Ascorbic acid in normal and stress induced rats. Each bar indicates the mean excretion of six animals. ***P<0.0001, **P<0.001, *P<0.01 significant difference from normal condition of the corresponding groups; ###P<0.0001, ##P<0.001 significant difference from stress condition of the corresponding groups.

IV. Discussion

Stress represents reactions of the body to a stimulus that tends to modify homestasis (Selye, 1998). Stress hormones are synthesised during stress condition for example the catecholamines (epinephrine and norepinephrine) produced by the SNS, and corticosteroids, produced by the ACTH stimulated adrenal cortex and glucocorticoid stimulated increase in serotonin are the major stress hormones (Uresin et al., 2004). In the current investigation, VMA as the peripheral metabolite of NA, 5-HIAA as the main metabolite of serotonin, 6-β-OH cortisol as metabolite of cortisol, HVA as the predominant metabolite of dopamine and ascorbic acid as a metabolite of glucose (in rats) were taken as an non-invasive biomarkers to display the increase in peripheral sympathetic activity during stress to assess the anti-stress activity of aqueous and ethanol extracts of *Morus alba*. The data indicated that VMA, 5-HIAA, 6-β-OH cortisol, HVA and ascorbic acid were excreted in urine daily at certain levels (basal values) as metabolites of NA, 5-HT, cortisol, DA and glucose respectively. The stress affected on the neurotransmitter levels and increased VMA, 5-HIAA, 6-β-OH cortisol, HVA and diminished ascorbic acid excretion. When aqueous and ethanol extracts of *Morus alba* administered to normal animals did not change VMA, 5-HIAA, 6-β-OH cortisol, HVA and ascorbic acid in comparison with basal values but prior administration of aqueous and ethanol extracts of *Morus alba* to stress induced rats exhibited the reduction in urinary VMA, 5-HIAA, 6-β-OH cortisol, HVA and increased the ascorbic acid levels in dose dependent manner. The previous phytochemical evaluation of *Morus alba* divulged the presence of phenolic compounds such as flavonoids (Quercetin, rutin), tannin which could be expected to be responsible for anti-stress activity (Ayoola et al., 2011). As VMA is a metabolite of norepinephrine (NE) and NE is synthesized by dopamine. Previous reports exhibited that these phytochemicals can bind to the $GABA_A$-BZDS complex, consequently, enhance GABA level and decline dopamine and decrease plasma corticosterone level that lead to reduce level of VMA and 6-β-OH cortisol repectively (Patil et al., 2006). Phenolic compounds such as flavonoids showed the affinity towards D_2 receptor; hence they can block the dopamine receptor and decrease the serotonin which causes to decrease level of HVA (Samson et al., 2006). These active compounds can prevent activity of tryptophan hydroxylase enzyme which is involved in the biosynthesis of 5-HT, thus, they can reduce level of 5HIAA ultimately (Singh et al., 1990). Ascorbic acid is also utilized as a co-factor in the biosynthesis of NE from DA may also attribute to low concentration of ascorbic acid in urine. Hence, effect of these bioactive compounds on reduction of dopamine synthesis that can influence on the increase of urinary level of ascorbic acid.

V. Conclusion

The present investigation manifest that aqueous and ethanol extract of *Morus alba* have anti-stress activity due to normalization of monoamines and glucocorticoids homeostasis by acting on HPA axis and SNS.

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