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BENIFICIAL EFFECT OF *CENTELLA ASIATICA* AND ASIATIC ACID IN IMMOBILIZATION INDUCED STRESS IN RATS: A FTIR STUDY

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ABSTRACT

Stress can be defined as any stimulus that creates an imbalance in the internal environment. Exposure to stressful situations is among the most common human experiences. It is reported that exposure to stress can stimulate many pathways leading to increased production of the oxygen free radicals. It produces changes in the molecular structure and chemical boning in brain. Fourier transform infrared micro spectroscopy is considered an ideal tool for comparative studies. Very small alterations in bond lengths and angles can be detected by this technique, and so FTIR has emerged as a powerful tool to investigate the structural changes of molecules in detail. Present study was designed to evaluate the structural changes in brain during stress condition. Stress was induced using immobilization stress procedure (placing the animals in 20 cm × 7 cm plastic tubes for 2 h/day for 21days) upon treatment with *Centella asiatica* (200 mg/kg body weight) and its compound Asiatic acid (10mg/kg body weight) reduce the changes in brain of immobilization stress induced rats. This action may be due to its antioxidant properties of *Centella asiatica* and Asiatic acid.

Keywords Stress, Fourier transform infrared spectroscopy, *Centella asiatica,* Asiatic acid, immobilization stress.

1. INTRODUCTION

Stress is defined as a minor hassles and a life events lasting for hours or days, is associated with subsequent increases in activities (1) Exposure to stressful situations is among the most common human experiences. It is reported that exposure to stress can stimulate many pathways leading to increased production of the oxygen free radicals (2, 3). These are formed in human body both in physiological and pathological conditions in cytosol, mitochondria, lysosomes, peroxisomes and plasma membrane (4). Psychological stress is associated with increased reactive oxygen species (ROS) production and oxidative damage and long term exposure to psychological stressors may enhance the risk of many diseases like atherosclerosis, diabetes, rheumatoid arthritis and liver diseases (5,6,7). Stress plays a potential role in producing changes in molecular structure, which is already proved in thermal stress condition in animal cells (8).

Fourier transform infrared (FTIR) spectroscopy is considered as an ideal tool for comparative studies. Infrared spectroscopy is a widely used method in biology and pharmacology for analyzing molecular structure and structural interactions. It measures the absorption of vibrating molecules, which result from the energy transitions of the vibrating dipoles. Very small alterations in bond lengths and angles can be detected by this technique, and so FTIR has emerged as a powerful tool to investigate the structural changes of molecules in detail. With the development of sophisticated FTIR; there have been very rapid advances in the applications of IR spectroscopy to the study of biological molecules. Although the same

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information can be obtained from spectra recorded with dispersive instruments (IR) and interferometers, the FTIR technique enables the rapid and reproducible recording of high resolution, low-noise spectra, even in aqueous media. The data acquisition process is automated. The data obtained is stored in digitally encoded formats that facilitate spectral interpretation with the aid of post-aquisation data manipulation algorithms. This property of the technique provides the accurate detection of small changes even in weak absorption bands (9). Moreover, infrared spectroscopy has been used as a powerful method for the study of molecular structures and intermolecular interactions in biological tissues and cells (10).

FTIR spectrometry is based on a simple mathematical technique to resolve a complex wave into its frequency components. In frequency domain spectroscopy the radiant power G (ω) is recorded as a function of frequency (ω). On the other hand, the change in the radiant power f (t) is recorded as a function of time (t) in the case of Time domain spectroscopy. The conventional spectroscopy is based on the former technique while FTIR converts the Time domain plot into a Frequency domain spectrum. Data in time domain are converted into frequency domain by Fourier Transform technique. The actual calculation of Fourier Transform of usual system is done by means of high-speed computers.

There is a need for identifying alternative natural and safer sources of antioxidant. Therefore, search for natural antioxidants, especially of plant origin, has notably increased in recent years (11). And, none has attempted evaluating therapeutic intervention with the natural antioxidant like *Centella asiatica* (C.A) in immobilization stress conditions. Phytochemicals have long been recognized to possess many properties including antioxidant, antiallergic, antiinflammatory, antiviral, antiproliferative and anticarcinogenic effects (12) improve memory, general mental ability of mentally retarded children (13).

C.A (L) urban, synonym *Hydrocotyle asiatica*, belongs to the family Apiaceae and is found almost all over the world. In Ayurveda, an Indian system of medicine, this is used in the management of central nervous system, skin and gastrointestinal disorder. The major principles in the plant are the polyphenols (14) and triterpenes (15). Asiatic acid (AA), a pentacyclic triterpene derivative from C.A has been shown to display neuroprotective properties both in vitro and in vivo (16). AA exhibits numerous pharmacological activities that might be beneficial to the ischemic brain, and given that no significant toxicity was observed following subcutaneous or oral administration of AA in rodents (17).

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Eight-week-old adult male albino rats of Wistar strain, weighing approximately 160 to 180 g, were acclimatized for 7 days at room temperature (25 ± 3°C) and relative humidity (55%) in a 12-hour light/dark cycle in a room under hygienic condition. The animals reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Male animals were used throughout the investigation to avoid complications due to the estrous cycle. The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India). Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 808/166/1999/CPCSEA) and animals were cared in accordance with the guidelines by the "Committee for the purpose of control and supervision on experimental animals" (CPCSEA, 2004).

2.2 PLANT COLLECTION AND IDENTIFICATION

The fresh leaves of C.A leaves were collected in Chidambaram, Tamilnadu, INDIA. The accuracy of plant selection was proved and authenticated by Department of Botany, Annamalai University, and Tamilnadu, India.

2.3 EXTRACTION

The fresh leaves of the plant were air-dried at 40°C and ground to powder, which was then subjected to exhaustive extraction using water in a Soxhlet apparatus.

The dark green liquid extract was concentrated under vacuum and the resulting dried extract was lyophilized and preserved in a refrigerator at 4°C until the use in the experiments. Asiatic acid was purchased from Sigma-Aldrich Co, USA. It was suspended in DMSO and physiological saline and administered post-orally,

2.4 INDUCTION OF STRESS

Stress was induced in rats by placing the animals in 20 cm × 7 cm plastic tubes for 2 h/day for 21days. There are several 3mm holes at the far end of the tubes for breathing that allows ample air but animals will be unable to move (18).

2.5 TISSUE PREPARATION AND FTIR ANALYSIS

The brain samples from each group were isolated and homogenized with a 0.2 M phosphate buffer, pH 7.4, and centrifuged at 100,000 g for 10 min. The membrane-rich parts of these homogenates were lyophilized and made into a fine powder to be used for FT-IR analysis (19). 5 mg of membrane-rich sample was mixed with 100 mg of dried potassium bromide, and again lyophilized in order to remove bound water, which might interfere with the measurement of the amide band. This was then subjected to a pressure of 5 x 10^6 pa, and made into a clear pellet of 13 mm diameter and 1 mm thickness. The absorbance spectra were recorded using the Spectrum RX I FT-IR System (Nicolet Instrument Corporation, Madison, USA). For each spectrum, 8 scans were recorded, at a spectral resolution of 4 cm^{-1}. The frequencies for all the sharp bands were accurate to 0.01cm^{-1}. The spectrometer was continuously purged with dry nitrogen. The absorption intensity of the peak was calculated using the baseline method. Each observation was confirmed by taking at least three replicates. The spectra were recorded in the range 4000-400 cm^{-1} (20). Peak normalization was done with respect to 1654 cm^{-1}.

2.6 EXPERIMENTAL DESIGN

The rats were randomly divided into six groups with six rats each. The test extract (C.A) was completely dissolved in water and AA was dissolved in DMSO and normal saline.

Group I: Control rats
Group II: Stress
Group III: Control+ C.A (200 mg/kg bw, for 21 days)
Group IV: Control+ A.A (10 mg/kg BW, for 21 days)
Group V: Stress + C.A (200 mg/kg bw, for 21 days)
Group VI: Stress + A.A (10 mg/kg BW, for 21 days)

The total duration of the study was 21 days. On 21st day, the rats were anasthesised sacrificed by cervical dislocation.

3. RESULTS AND DISCUSSION

FTIR is one of the most important metabolamic tools which show the molecular changes that occur during a pathological condition. It is widely accepted that FTIR spectroscopy is a highly sensitive tool capable of providing strong insight on structural and functional alterations of biomolecules in tissues induced by various factors. The frequency shifts shows the molecular alteration of macromolecules such as protein, lipid, carbohydrate, and nucleic acid which can be considered for analysis (19). Previously, the effect of diabetes on rat liver and heart has been investigated using FTIR spectroscopy (21).

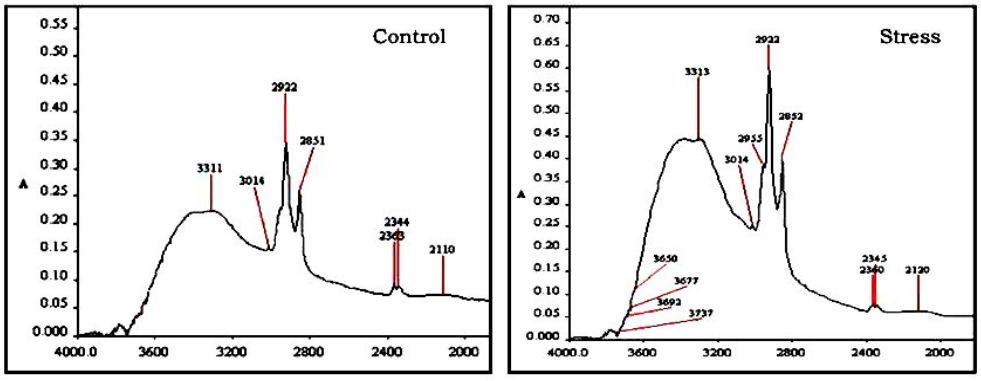
The FTIR analysis explores the vibration of functional groups present in macromolecules and shows the molecular structural change through shifts in wave numbers. In this study, the decrease in wave number of protein at amide are observed in stress loaded animal, it demonstrates the disorder of hydrogen bonding and alteration of secondary structures in the stress group. This change may reflect the overall changes in structure and synthesis of protein in brain during stress. The C.A and AA treatment might prevent the changes of protein in brain through reducing the structural modification. The decreased protein content in stress induced rats brain was identified by the increase in band area values of amide I (1653-1657 cm^{-1}) and amide II (1539-1543 cm^{-1}) bands. Amide I consists of functional vibrations of many secondary structures. According to findings of a previous study all the constituent amino acid side-chains in proteins are susceptible to free radicals, but some are more vulnerable than others. Thus, exposure of

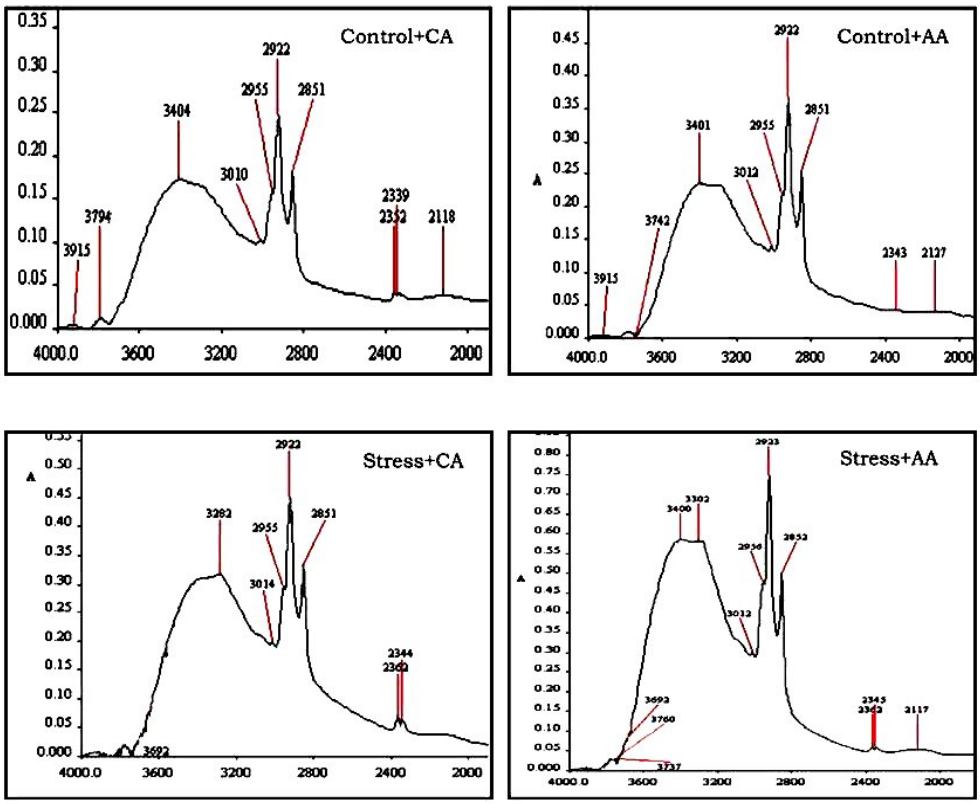
proteins to free radical-generating systems may induce secondary structural changes. Secondary structure is stabilized by hydrogen bonding of the peptide backbone and interference with the functional groups of the peptide bonds may cause structural modifications. Further the shifts in amide I and II regions correspond to the alpha-helix protein conformational change (22). Above evidence indicates that the alteration of protein secondary structure in stress treated brain tissues was due to oxidative stress. Protection shown by C.A and AA against changes in frequency and intensity of amide I, intensity alone in amide II is probably due to the prevention of free radical dependent protein modifications by antioxidant of C.A and AA.

The lipid region shows the indicator of polyunsaturated functional group, olefinic=CH stretching at the wavenumber of 3,014 cm^{-1} in the spectrum. The intensity of olefinic band can be used as an index of relative concentration of double bonds in the structure of unsaturated lipids (23). Appearance of the olefinic band in stress induced rat brain indicates increased lipid peroxidation. The absence of this functional group in C.A and AA treated groups may be due to the free radical scavenging and antioxidant properties of C.A and AA, playing a vital role in protecting cellular lipids from peroxidation and unsaturation. CH_2 asymmetric and CH_2 symmetric stretching of lipids shows no alterations in frequency. The frequency of the CH_2 bands of acyl chains depends on the degree of conformational disorder and level of flexibility. The position of these bands provides information about the lipid acyl chain flexibility (order/disorder state of lipids) (24). However, the increase in quantity of fatty acids shows that there is increase in total fat content in the stress induced groups. Previous reports have demonstrated that during stress condition lipid level in brain was increased (25). Structure of triglycerides in brain tissues shows alteration in the pattern of ester packaging (2857-2844 cm^{-1}). In addition, the dramatic shifting of this band to lower frequency values indicates a difference in packing of ester groups within the tissue in stress. Increase in wave number provides evidence for reduction in structural modification of triglycerides shown the protective effect of C.A and AA through its antioxidant potential.

The brain contains glycogen but at low concentration compared with liver and muscle. In the adult brain, glycogen is found predominately in astrocytes. Astrocyte glycogen content is modulated by a number of factors including some neurotransmitters and ambient glucose concentration. Compelling evidence indicates that astrocyte glycogen breaks down during hypoglycemia to lactate that is transferred to adjacent neurons or axons where it is used aerobically as fuel. In the case of CNS white matter, this source of energy can extend axon function for 20 min or longer. The alteration in the wave number (2341 cm^{-1}) representing glycogen content was significantly reverted back while co –administrated with C.A and AA to stressed animals.

Figure 1: Spectral lines observed in the brain of control and experimental animals by FTIR





4. CONCLUSIONS

Stress induced the changes in molecular structure and structural interactions of rat brain observed by FTIR. And the changes were reverted back to near normal in stress loaded animals treated with C.A and AA when compared to stressed rats. This action may be due to the high antioxidant potential and free radical quenching properties of C.A and AA. This shown the beneficial effect of C.A and AA in immobilization induced stress in rats.

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