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FT-IR Study of the influence of *Tribulus terrestris* on Mercury intoxicated mice, *Mus musculus* liver

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Abstract. FT-IR spectra of liver tissue isolated from mice, *Mus musculus*, have been recorded in the region of 4000 – 400 cm^{-1} for normal, mercury treated and recovery phase. In this study, the total protein content was found to be decreased in the liver tissues after treatment with median-lethal dose of mercuric chloride. The marked fall in the level of bio-chemical constituent in the tissue due to metal exposure indicates the rapid initiation of the breakdown of the bio-chemical constituents to meet the energy demand during toxic stress. During the recovery phase, the decreased levels of bio-chemical constituents are restored to near normal level. Methanol fractions of *Tribulus terrestris* fruit extract was administrated on mercury intoxicated mice for 15 days. After the administration, the mercury-intoxicated animals slowly recovered from the adverse effect of mercury poisoning with the help of plant bio-formulations. The results are discussed in detail.

INTRODUCTION

Infrared spectroscopy is a powerful method for the study of molecular structure and intermolecular interaction in biological tissues and cells (Partrick *et al.*, 1993). Several authors have studied infrared spectroscopy on biological substance like muscle, liver etc. Galin *et al.* (2000) have also studied the changes in primary, secondary and tertiary structures of the nucleic acid of RNA in rats exposed to gamma radiations through FT-IR spectroscopic studies. Feride *et al.* (2000) studied the effect of sreptozotocin (STZ) induced diabetes on rat liver and heart tissues using FT-IR spectroscopy. Chiriboga *et al.* (2000) studied infrared spectra of normal and cancer liver tissues such as glycogen, DNA and RNA. Patrick *et al.* (1993) studied the human colon tissues at molecular level from normal epithelium to malignant tumor investigation by pressure tunning FT-IR spectroscopy.

The biological macromolecules are classified as: the proteins, lipids, nucleic acids and polysaccharides. One of the remarkable characteristics of living organisms is how many macromolecules they utilize for living and what different and important roles these macromolecules play in their physiological systems. The functioning of all biological macromolecules depends on their shapes or three-dimensional structures. The biological macromolecules provide us the clearest example and the most sensitive expression of the relationship between molecular structure and chemical and physical properties of a substance (Whitaker *et al.*, 1981).

Bio-chemicals are highly sensitive to heavy metals and are one of the earliest indicators of heavy metal poisoning (Margarat & Jagadeesan, 2000; Kavitha & Jagadeesan, 2004). The impairment in bio-chemical synthesis due to heavy metal stress has been reported by many investigators (Jacobs *et al.*, 1977; Margarat

& Jagadeesan, 2000). However, only few of them attempted to determine the toxic effect of heavy metal on animal tissues and its recovery phase. Medicinal plants commonly included in Ayurvedic recipes for liver ailments have drawn much attention, as no reliable hepatic-protective drug is available in modern medicine. Research investigations conducted on several natural plant products used as liver protection are well documented. With this point of view, the present experimental work has been designed to study the effect of mercuric chloride on the bio-chemical profile of the liver tissue of mice, *Mus musculus* and also the withdrawing effect of mercury using the Fourier Transform Infrared (FT-IR) Spectrometer.

MATERIALS AND METHODS

Experimental design

Thirty-six laboratories breed white mice, *M. musculus* (Linn), 45 days old and weighing 25 + 0.5 gram were procured from the animal house of Rajah Muthiah Medical College, Annamalai University. The animals were divided randomly into the following batches:

Batch a:	Control	– 6 mice
Batch b:	Hg Cl_2 treated alone	– 6 mice
Batch c:	*Tribulus terrestris* fruit extract alone	– 6 mice
Batch d:	*Tribulus terrestris* fruit extract followed by mercury treatment	– 6 mice
Batch e:	Mercury followed by *Tribulus terrestris* fruit extract	– 6 mice

Each batch of animals was housed separately in suitable cage and fed on standard laboratory diet, supplied by Hindustan Lever Limited, Mumbai and Tap water *ad-libitum*. The Mice **batch b** and **e** were oral dosed on $MgCl^{-}_2$ at sub-lethal dose every day for 30 days. After 30 days, the animals of **batch e** were again orally administrated with *T. terrestris* fruit extract each day for another 15 days. The mice of control (**batch a**) enjoyed the laboratory diet alone and tap water *ad-libitum*. **Batch c** mice were administrated with *T. terrestris* fruit extract alone and tap water *ad-libitum*. Methanolic fractions of *T. terrestris* fruit extract are very useful to eliminate the unwanted toxic heavy metal from the animal body through the urine. For that purpose this herbal plant fruit extract was administrated on mercury intoxicated animals. Total weight of diet was kept constant throughout the experimental period. After the scheduled treatment, the animals were sacrificed by cervical dislocation and the whole liver tissue was isolated immediately and then used for FT-IR study.

Plant procurement and Extraction

Fresh fruits of plant material were collected from October to December near paddy fields located in and around Chidambaram area (5 Km away from the University campus), Tamilnadu, India and identified by a Taxonomist and preserved in the Department of Botany, Annamalai University, Annamalainagar, India.

Taxonomy of the Plant

Class	–	Dicotyledons
Sub class	–	Polypetatae
Series	–	Thalamiflorae
Order	–	Geraniales
Family	–	Zygophyllaceae
Genus	–	*Tribulus*
Species	–	*terrestris*

Preparation of plant extract

Tribulus terrestris fresh fruits were collected and dried in shade at room temperature (25 ± 2°C) and powered in an electric blender. Then 250 g powder was kept in the soxhlet apparatus and soxhlation was done with the help of methanol solvent up to 24 hours for separating the contents, which were present in it (Shipping *et al.*, 1999).

Sample preparations

The whole liver tissue samples of each group of mice were isolated. The isolated whole liver tissue samples were lyophilized and made into fine powder. The tissue powder samples and KBr (all solid dry state) were again lyophilized in order to remove most bound water, which might interfere with the measurement of amide I, band. 5 mg of liver tissue sample was mixed with 100 mg of dried KBr and subjected to pressure of 5x106 pa and made into a clear pellet of 13 mm diameter and 1mm thickness. Absorbance spectra were recorded using Nicolet Avator-360 FT-IR spectrometer equipped with a KBr beam splitter and an air- cooled DTGS (Deuterated Triglycine Sulfate) installed in the CISL. For each spectrum 100 scans were recorded, at a spectral resolution of 4 cm^{-1}. The frequencies for all sharp bands were accurate to 0.01 cm^{-1}. The spectrometer was continuously purged with dry Nitrogen. The absorption intensity of the peak was calculated using the base line method. Each observation was confirmed by taking at least three replicates. Spectra were recorded in the range 4000 - 400 cm^{-1} using Nicolet Avatar-360 FT-IR spectrometer equipped with KBr beam splitter and a DTGS detector, at Centralized Instrumentation and Services Laboratory (CISL), Annamalai University.

RESULTS AND DISCUSSION

FT-IR spectra of normal liver tissues, Mercury treated liver tissues, *T. terrestris* fruit extract, *T. terrestris* fruit extract followed by Mercury treated liver tissues and Mercury followed by *T. terrestris* fruit extract treated liver tissues are shown in figure 1. The relative intensities (log I_o / I) and tentative assignments of fundamental Infrared absorption frequencies are shown in table 1.

FT-IR spectra revealed significant differences in band position and absorbance intensities between normal, recovery and treated tissues. Infrared spectra reflect the total chemical composition of cells and some of the spectral bands can be assigned to distinct functional groups or chemical substructures. The increasing use of FT-IR spectroscopy demonstrates that this technique is a valuable tool because of its ability to monitor simultaneously protein, lipid and poly-saccharide components.

The nutritional value of different animal depends on their biochemical contents like proteins, carbohydrates, amino acids, lipids and minerals. It is known that tissue proteins, carbohydrates and lipids play a major role as energy provider for animal exposed to stress conditions. Friberg *et al.* (1979) indicated that a majority of toxic substances initiate biochemical alterations acting at the molecular level by anyone of the following mechanisms:

- (i) Inhibition of the enzyme system,
- (ii) altering the level of enzyme and specificity or by
- (iii) altering the permeate properties of body membranes.

Proteins, the principal constituents of the protoplasm of all cells, are of high molecular weight and consist of alpha-amino acids joined by peptide linkage. Different amino acids are commonly found in protein, each protein has a unique, genetically defined amino-acids sequence, which determines its specific shape and function. They serve as enzymes, structural elements, hormones, immuno globulins, etc. and are involved in oxygen transport, muscle contraction, electron transport and other functions.

The infrared spectra of protein are characterized by a set of absorption regions known as the amide region and the C-H region. The most widely used modes in protein structure studies in the amide region are amide I, amide II and amide III. The amide I band arises principally from the C=O stretching vibration of the peptide group. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations. The amide III absorption is normally weak and arises

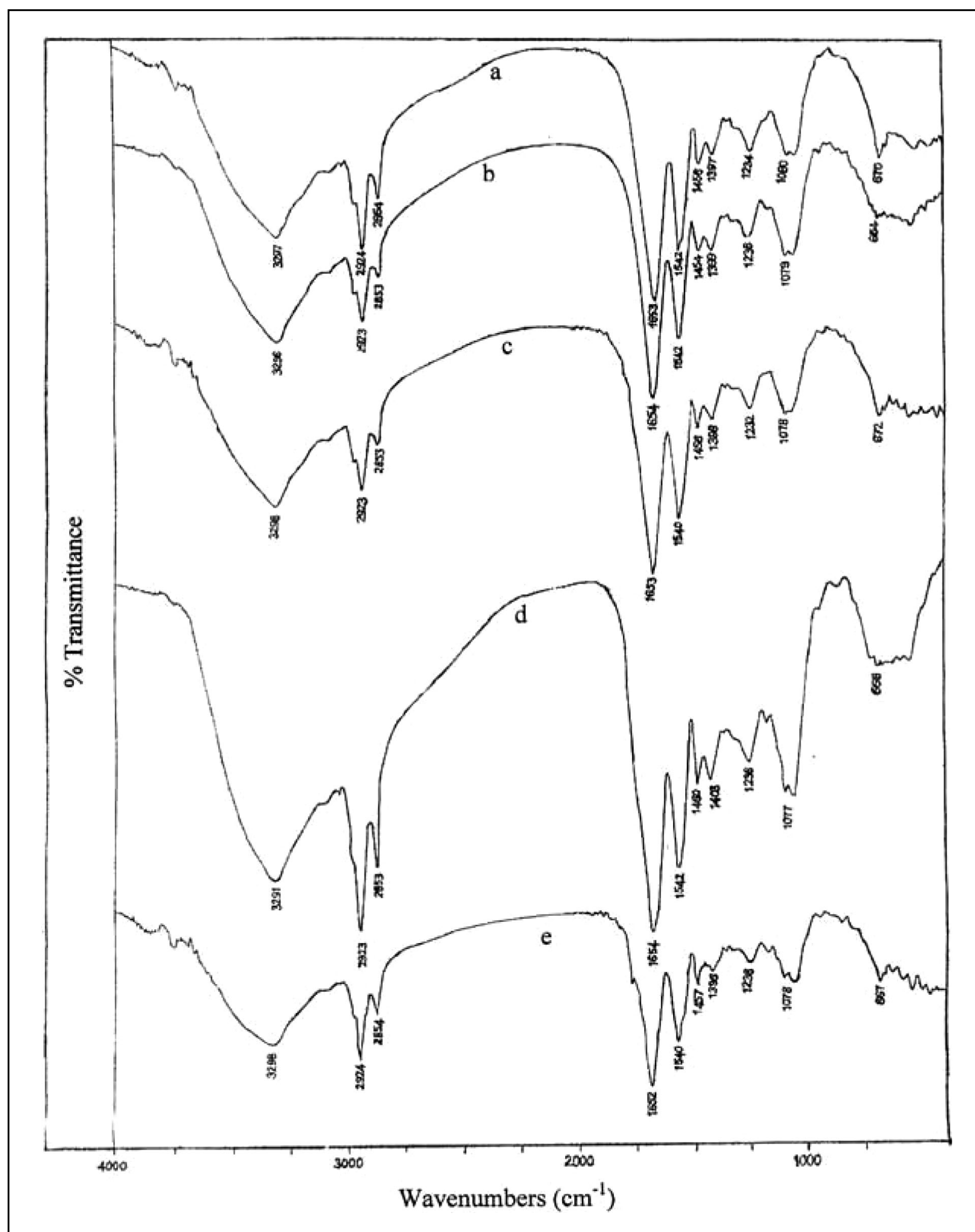

Figure 1. FT-IR Spectra of Liver tissues of mice, *Mus musculus.* (a) normal, (b) mercury treated, (c) *Tribulus terrestris* fruit extract alone, (d) *Tribulus terrestris* fruit extract followed by mercury treatment, (e) Mercury followed by *Tribulus terrestris* fruit extract.

Table 1. Infrared absorption frequencies (cm^{-1}), relative intensities ($\log I_o / I$) and tentative assignments of fundamental frequencies of Liver samples

Frequency cm^{-1}	Normal	Mercury treated	*T. terrestris* fruit extract alone	*T. terrestris* fruit extract followed by Hg treatment	Hg followed by *T. terrestris* fruit extract	Tentative Assignments
3290-3300	0.0670	0.0626	0.0742	0.1217	0.0352	O–H sretching vibrations / N–H stretching vibrations
2922-2924	0.0536	0.0878	0.0925	0.0248	0.0296	CH_2 asymmetric stretching; lipid, protein
2852-2854	0.1027	0.1287	0.1322	0.1406	0.0678	CH_2 symmetric stretching; mainly lipids, Proteins
1539-1543	0.0543	0.0691	0.0552	0.1313	0.0408	C–N stretching/ N–H bending; Amide II
1454-1462	0.1303	0.1504	0.1482	0.2394	0.0932	CH_3 asymmetric bending; Protein
1396-1400	0.1357	0.1504	0.1534	0.2467	0.1030	CH_3 symmetric bending; Protein
1234-1238	0.1409	–	0.1636	0.2747	0.0981	PO^-_2 asymmteric sretching Amide III
1077-1080	0.1357	–	0.1586	0.2394	–	PO^-_2 symmeteric sretching (glycogen)
667-670	0.1357	0.1811	0.1586	0.3661	0.0932	C-H bending vibrations

Note: I_o corresponding to ~1653 cm^{-1} (amide I) and I corresponding to the bands mentioned in the first column.

primarily from N-H bending and C-N stretching vibrations. The amide absorptions are considered sensitive to protein conformation; hence an increase or a decrease in the ratio of the intensities of the bands at ~1541 cm^{-1} (amide II) and ~1653 cm^{-1} (amide I) could be attributed to a change in the composition of the whole protein pattern. The ratio of the peak intensities of the bands observed ~1541 cm^{-1} and ~3297 cm^{-1} due to N-H bending and O-H stretching respectively could be used as indicators of the relative concentration of the protein to water of biological tissues.

The overall spectral profile is similar except for the variation in intensities of the bands. The most widely used modes in protein structural studies are amide I, II and III. The broad band at ~3297 cm^{-1} have been assigned in the present study to O-H stretching, the amide bands of proteins have made a small contribution to it. The band observed at ~2923 cm^{-1} and at ~2853 cm^{-1} are due to the asymmetric and symmetric stretching modes of the methylene chain in the membrane lipids. The sharp bands observed at ~1653 cm^{-1} and at ~1541 cm^{-1} are assigned to the in-plane C=O stretching vibration (amide I) and to the C-N stretching/N-H bending vibration (amide II) of the tissue proteins respectively (Parvez *et al.*, 1999). The amide I band are primarily associated with the stretching motion of the C=O group. This C=O band is sensitive to the environments of the peptide linkage and also depends on the protein's overall secondary structure (Max Diem *et al.*, 1999). The bands observed at ~1456 cm^{-1}

and ~1396 cm^{-1} are mainly due to asymmetric and symmetric CH_3 bending modes respectively of the methyl groups of protein. The medium intensity band observed at ~1235 $cm^{-1}$is that of the PO^-_2 asymmetric stretching modes of the phosphodiester indication of phospholipids and the amide III / CH_2 wagging vibration from the glycine backbone and protein side chain.

Protein plays a vital role in the physiology of living organisms. All the functions of an organism are regulated by enzyme and hormones, which are proteins. If any alteration takes place in the protein turnover, it may have an adverse effect on the important and complex groups of biological materials, comprising the nitrogenous constituents of the body and food intake and thus performing different biological events to maintain homeostasis of the cell. Therefore the protein content of a cell can be considered a diagnostic tool to determine the physiological phases of a cell (Manoj & Ragothaman, 1999).

The depletion of protein profile induces diversification of energy to meet the impending energy demands during the toxic stress (Mc Leay & Brown, 1975; Jagadeesan & Mathivanan, 1999). Similar types of results were observed in *Cyprinus carpio* and *Catla catla* when exposed to Lindane and cadmium respectively (Jana & Bandyopadhya, 1987; Vincent & Ambrose, 1994). Margarat *et al.* (1999) have also observed the decreased level of protein content in liver tissue of mice when treated with mercury.

The liver is the site for metabolic activity and it is also capable of biotransformation of foreign chemicals (Chris Kent, 1998). Liver is the vital organ for detoxification of unwanted and toxic substances (Hussain *et al.*, 1999; Kavitha & Jagadeesan, 2004). It acts as an intrinsic protein, which has a very high turnover rate. The liver synthesizes a great amount of protein, which is needed ostensibly for repair of damaged cell organelle and tissue regeneration.

The band observed at ~1151 cm^{-1} and ~1168 cm^{-1} are assigned to the C-O stretching mode of glycogen. The band at ~1080 cm^{-1} has been assigned to the symmetry phosphates; the stretching of glycogen also makes a contribution to the intensity of this band. The band at ~1065 cm^{-1} is assigned to the CO-O-C symmetric stretching of Lipids. (Annic Perromat *et al.*, 2001). The band at ~1045 cm^{-1} frequently found in glycogen rich tissues, can be assigned to C-O stretching coupled with C-O bending of C-O-H carbohydrates (Rigas & Wong, 1992). The weak bands at ~930 cm^{-1} may be due to the anti-symmetric stretching mode of the DNA or to a phosphate monoester band of phosphorylated protein and nucleic acids. The most characteristic absorption of Poly-nuclear aromatics results from C – H out of plane bending in the 900 – 675 cm^{-1} region.

The carbohydrate metabolism is also disturbed when animals were exposed to environmental stress condition. Glycogen as the principal store of chemical energy, exercises an extremely important function in furnishing the energy requisites of the cell. The ability to overcome the stress laid on tissues in animal, caused by environmental contamination, mainly depends on the carbohydrate contents. Glucose is one of the most important bio-chemical substance, which gives immediate energy in an organism (Jagadeesan & Mathivanan, 1999).

Generally, the liver tissue stores the energy rich molecules, glycogen which is a glucose polymer and glycogen exhibits absorption due to C-O and C-C stretching and C-O-H deformation motions with peak at ~1080 cm^{-1}. The band observed at ~1080 cm^{-1} in the liver of the control tissue has almost disappeared in the tunicate tissue indicating a marked fall of the glycogen content.

The stressful situations mainly disturb the rate of carbohydrate metabolism through the level of glycogen profile in toxicant exposed animal. Glycogen, a reserve energy source is decreased during mercury treatment. (Table 1) A fall in glycogen profile in the liver tissue indicates the possibility of glycogenolysis

and also an extensive utilization of energy stores. This stepped up utilization is to meet the extra demands of energy necessitated by the quick and brisk movement, which the animal shows in its behavioral response during the initial period of mercury treatment.

In the present investigations, *M. musculus* showed a remarkable recovery from the adverse effect of mercury poison. When the mice were exposed to mercury poison and *T. terrestris* extract treatment (both pre and post treatments), they showed a restoration in the level of biochemical constituent profiles in the liver tissue. The recovery could be attributed to the restoration of regulatory function of phosphorylases by elimination of toxicant (Holcombe *et al.*, 1976; Jagadeesan & Mathivanan, 1999; Margarat & Jagadeesan, 1999) from the endocrine glands like pancreas and adrenals. The disturbance in carbohydrate metabolism is caused by mercury and the recovery is not spontaneous but progressive.

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