



COMPARATIVE STUDIES ON BIOCHEMICAL, HISTOPATHOLOGICAL AND FTIR-ATR SPECTRAL VARIATIONS OF GENTAMYCIN INDUCED RENAL DISEASE IN WISTAR RAT

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ABSTRACT

Establishment of FTIR-ATR spectroscopic method in disease diagnosis was attempted with induction of renal disease status in wistar rat with gentamycin. The routine methods were used to analyse the biochemical variations to determine the renal disease status via colorimetric, ELISA and Histopathologically in addition to FTIR-ATR spectral analysis. To achieve this, experiments were conducted with wistar rat injected with gentamycin for 30 days. At the experiment termination blood serum and organs were subjected to rule out the bio markers towards development of renal disease. The spectral bands (4000 – 450 cm⁻¹) obtained from serum as well as to different organs was characterized for biomarkers to assess the diseases status which aid in complete screening to control and management of the disease. The chemical bonding stretches reveals the nature of bio molecule levels in serum and different organs. The results shows that the peak ratio for amide I / glucose-str. (I1634/I1076) as well as amide II / ribose-phospholipid (I1538/I934) were significantly increased in gentamycin induced renal than control male Wistar rat with renal disease. The absorbance for internal peak ratio of blood serum for amide III erythrocyte / (cystic acid) s-s-str. (I1313 /I532) is highly significant among gentamycin induced. These results also support fatty change in liver due to hypoxia and persisting fatty change in liver, inflammation in kidney, interstitial inflammation and granulomatous reaction as well as increased urea and creatinine levels in blood. FTIR-ATR spectral analysis in may be suggested addition to other routine methods. FTIR-ATR infra-red spectrum provides information about biochemical components in blood as well as in different tissues are discussed.

KEYWORDS: Wistar rat, Renal disease, induction, gentamycin, FTIR-ATR spectral analysis.

INTRODUCTION

Kidneys are pair of organs which filter blood and remove toxins from the body and send toxins to bladder followed by removal of toxins during urination. Kidney failure occurs when kidneys lose the ability to filter waste from blood sufficiently. Many factors can interfere with kidney health and function such as toxic exposure to environmental pollutants, certain acute and chronic diseases, severe dehydration, kidney trauma etc., the glomerular filtration rate estimation for diagnosing renal function typically measures the variance of the many molecules involved with the system disorder. Several molecular studies also contributed to the finding of potential biomarkers related to kidney disease. Loss of blood flow to the kidneys due to blood pressure and anti-inflammatory medications, kidney stones, an enlarged prostate, blood clots within urinary tract damage

glomerulo nephritis, vasculitis, thrombotic thrombocytopenic purpura etc., causes renal failure. The treatment includes diagnosis of the elevated levels of renal biomarkers like urea and creatinine. Many of the symptoms of renal failures include high blood pressure, high cholesterol levels, anemia, high calcium deposits in blood vessels. Recent years have witnessed the unprecedented development and integration of genomics, epigenetics, transcriptomics, proteomics and metabolomics, as well as a growing interest in biomarkers and process-specific biomarker panels in renal diseases.

The screening of renal diseases could be possible with analyzing single or multiple parameters as biomarkers. Urea and creatinine levels in blood were considered to be the most important bio molecule to rule out the renal

dysfunction. Impaired renal function or increased tissue protein breakdown are associated with increased urea levels, whereas liver damage associated with decreased levels. Measurement of serum creatinine and blood urea are useful in the diagnosis, treatment and follow up of renal diseases / renal failure.

The clinical diagnosis is the process that identifies a possible disease or disorder that can be diagnosed through various diagnostic tests involving components of multiple techniques and procedures that identifies the internal physiological changes occurring due to the disease which is followed by treatment and prognosis. Laboratory tests are often used to evaluate patient's pathological conditions. Body fluids are considered as attractive source for clinical markers. Many of the literature studies explained the analysis of different body fluids using routine methods like ELISA (Enzyme Linked Immuno Sorbant Assay), RIA (Radio Immuno Assay), IF (Immuno Fluorescence Assay), CLIA (Chemical Luminance Immuno Assay) and Spectrophotometer (Singer *et al.*, 1995; Roos A *et al.*, 2005; Dehghani F *et al.*, 2011; Sastry AVS *et al.*, 2011; Saud Alarifi *et al.*, 2012; Rajasekaran S R and Anandan Nishad K M 2013 and Swati *et al.*, 2014) with animal model for clinical correlation. These methods has made exponential progress for the last many years but suffer from few limitations occurring as result of low specificity and lack of efficacy and involve specific reagents, equipment and skilled labour with escalating costs have made these methods obsolete and replaced with new generation automation which are timely, accurate, precise, user friendly etc.,.

Spectroscopic are based on the wave length of light absorbed and the interaction of different frequency of light either emitted or absorbed by the electromagnetic spectrum with matter. The study on biochemical composition evaluation by spectroscopic techniques can be used not only for understanding the biological nature of the disease, but also for the diagnosis of the disease. In recent years the spectroscopic tools which has shown more consideration in biological sciences include Immunosensors (Peter R D *et al.*, 2001), UV-Visible (Gunasekaran S *et al.*, 2010 and 2008), Raman (Kees Maquelin, 2000), Nuclear Magnetic Resonance (NMR) (Bales *et al.*, 1984 and Bruce L, 1991), Vibrational spectroscopy (Catherine Kendal *et al.*, 2009), Infra-red (IR) (Herbert *et al.*, 1998; Heise H M *et al.*, 2000; and Gamze Hosafic *et al.*, 2007); FTIR (Zanyar Movasaghi *et al.*, 2008) etc.,.

The concept of using FTIR -ATR in the analysis of biological samples like different organs in addition to blood serum dates back over half a century (Blout ER and Mellors R 1949 and Kraft C and Sergo V, 2006). FTIR-ATR spectroscopic technique applied to understand the variations of bio molecule composition in blood serum / organs and spectral differences to identify the clinical status of the diseases using biomarkers.

Further, FTIR-ATR spectroscopic method where the spectrum obtained due to different bond stretching and functional groups exist in serum / tissue sample might be the additional information to the routine methods FTIR-ATR spectroscopy is a useful tool for determining the concentration of multiple bio molecules in micro samples of all biological samples and has significant advantages compared to many other imaging methods for the characterization of bio molecules. The singular advantage of FTIR-ATR over other techniques is convenience and can be effectively employed as a diagnostic tool in clinical chemistry and can be an additional method in clinical analysis.

Several reports have showed the successful application of FTIR-ATR imaging in studies on identification of various pathological changes in tissues such as brain (Nadia Amharref *et al.*, 2006 and Abdelilah Beljebba *et al.*, 2010), lung (Christoph Kraff *et al.*, 2008), myocardium (Toyran N *et al.*, 2006), liver (Le Naour F *et al.*, 2009 and Gautam R *et al.*, 2012). Nowadays this technique has become an independent modality, especially in its imaging system for searching spectral biomarker of different diseases include thyroid (Kamatchi S *et al.*, 2016); atherosclerosis (Sankari G *et al.*, 2010); myocardial infarction (Haas S L *et al.*, 2010); renal failure patients (Renugadevi *et al.*, 2009); breast cancer tumors (Dimitrova M 2009); prostate cancer (Mackanos M A *et al.*, 2009); jaundice (Gunasekaran S *et al.*, 2008 and 2010) and protein plasma in blood (Cyril Petibois *et al.*, 2001).

The analysis of blood parameter and nature of organs affected by different diseases due to induction has been limited with FTIR-ATR spectroscopic analysis. Yet, these studies have helped for the detection of these diseases in human population and FTIR-ATR spectroscopic method was found to be most efficient. Induction experiments, the animal model is chosen to achieve the study on renal diseases diagnosis using FTIR-ATR spectroscopy in addition to routine methods available. The blood serum and different tissue homogenate including liver, kidney, lung, heart and skeletal muscle were considered as specimen for the detection and severity of disease conditions. The results can be best employed in the qualitative and quantitative analysis of bio molecules in biological fluid and tissues or organs. This study focusing early detection of renal diseases with the existing biochemical, histological methods available and compared with FTIR-ATR spectroscopic method.

MATERIALS AND METHODS

Procurement of animals

For the experimental purpose male Wistar rats weighing about 100-150 gm were purchased from Saphthagiri Livestock and Organic Research Farm, Tamilnadu, India, were housed in Animal House of the Saveetha Medical College, Saveetha University, Thandalam, Chennai, India the animal house was maintained at an average

temperature ($24.0^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and 30-70 % room humidity, with 12hrs Light- dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). The experimental rats received human care and were fed with commercial pellet diet and the animals were acclimatized for one week before the start of the experiments.

Experimental Design of Developing Renal Disease (RD) in male Wistar rat

The experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study proposal was also approved by the Institutional Animal Ethical Committee Experimental animals. (Six rats per cage) were housed in polypropylene cages ($32.5 \times 21 \times 14$) cm lined with raw husk which was renewed every 48 hours.

Gentamicin (GM) is an effective aminoglycoside antibiotic that is still widely used against serious and life threatening infections by gram-positive and gram-negative aerobic bacteria, but nephro toxicity and oxidative damage limits its long term clinical use (Whelton A and Solez K 1983; Abdel-Naim A B *et al.*, 1999; Al-Majed A A *et al.*, 2002; Abou El-Sooud K 2003; Karahan I *et al.*, 2005; Kuhad A *et al.*, 2006; Priyamvada S *et al.*, 2008; Khan S A *et al.*, 2009; Chaware V J *et al.*, 2011; Babu S V 2011; Kore K J *et al.*, 2011 and Sharma R *et al.*, 2011). To develop renal disease in male Wistar rat, Gentamycin (80 mg / 2 ml vial) obtained from Ranbaxy Laboratory Pvt. Ltd. India and injected sub cutaneously with dosage of 100 mg/kg body weight was fixed. The development of renal disease in male Wistar was conducted for three weeks with daily dosage following the standard procedure of Saud Alarifi *et al.*, 2012 and the experiment was terminated on 21st day.

Collection and Processing of experimental Samples

At the end of each experiments the male Wistar rats were fasted overnight. Blood samples of the male Wistar rats were withdrawn on from the heart under mild anesthesia before killing and collected in plain tubes. Blood serum was separated by centrifugation at 3000 rpm for 15 minutes and preserved for further biochemical analysis. For FTIR-ATR spectral analysis, the serum samples were properly preserved in ice bags and immediately transported to the wet lab for spectral studies.

The fresh homogenization of organs leads to loss of cell structure. A high-quality preparation of a tissue homogenate represents an optimum compromise in diagnosis instead of homogenization of whole tissue. At postmortem, the heart, liver, lungs, kidney and muscle were immediately excised from adult rats and washed with saline and refrigerated (-20°C).

Lyophilization of organs tissue were done by Scanvac cool safe, 55-9 Denmark vacuum concentrator at Central Institute of Brackish water Aquaculture, Indian Council of Agricultural Research, Govt. of India, Chennai. Further, the freeze dried samples grounded to powder using mortar and pestle and preserved in desiccators containing silica gel till FTIR-ATR spectral analysis. For histological studies, excised organs after saline wash, were fixed in 10% buffered neutral formalin for further processing and analysis.

Quantification of Bio molecules by Routine Methods

The quantitative analysis of blood composition as well as tissue components of different organs is a major field in the clinical chemistry. The composition/ components are the preferred indicator with respect to the patho physiological condition of the system. The blood serum of experimental male Wistar rat were analyzed in a reputed clinical laboratory in Chennai. Quantitative analysis of bio molecules were carried out include glucose, urea, creatinine, calcium, phosphorus, uric acid, total bilirubin, SGOT, SGPT, total protein, albumin, cholesterol, triglyceride, HDL (High Density Lipoprotein) *etc.*, by enzymatic assay method using respective commercial diagnostic kits (Soos M and Siddle K 1982; Allain *et al.*, 1974; Mc Gowan M W *et al.*, 1983; Izzo C *et al.*, 1981; Friedwald W T *et al.*, 1972; Sasaki *et al.*, 1972; Lowry O H *et al.*, 1951; Owen J A *et al.*, 1954; Marsh W H *et al.*, 1976; Culling FA 1979 and Reitman S and Frankel S 1957). The serum total T_4 , T_3 and TSH concentrations were determined by ELISA method (detection kits provided by Transasia, Zemun, and SCG) in a reputed clinical laboratory in Chennai (Berger A J and Itykan M S F 1997).

FTIR-ATR Analysis of blood serum and organs

FTIR-ATR spectral measurements of serum samples and lyophilized tissues of different organs of experimental male Wistar rats were carried out at Sophisticated Analytical Instrumentation facility (SAIF-SPU), St. Peter's University, Avadi, and Chennai-600054, using Perkin Elmer Spectrum-Two FTIR Spectrophotometer with Attenuated Total Reflectance accessory having highly reliable and single bounce diamond as its Internal Reflectance Element (IRE).

Sampling Analysis in FTIR-ATR Spectroscopic Technique

FTIR-ATR sampling technique that provides excellent quality data in conjunction with the best possible reproducibility of any IR sampling technique. For the successful generation of infrared spectrum, the sample must be in direct contact with the ATR crystal, because the evanescent wave or bubble only extends beyond the crystal $0.5 \mu - 5.0 \mu$. Diamond is the best ATR crystals material because of its robustness and durability. The most frequently used small crystal ATR material is diamond because it has best durability and chemical inertness. The lyophilized tissues sample (0.5-1.0 mg) is finely ground using mortar and pestle were used for

spectral analysis using Perkin Elmer spectrum Two FTIR-ATR Spectrometer To minimize band distortion due to scattering of radiation, the sample should be ground to particle of 2m (the low end radiation wave length) or less in size. The finely powdered sample is then pressed in to a transparent disc in an evaluable die at sufficiently high pressure. As a result spectra can be obtained from a wide variety of solid materials. Once the solid has been placed on the crystal area, the pressure arm should be positioned over the crystal/ sample area, the pressure arm locks into a precise position above the diamond crystal. Force is applied to the sample, pushing it to the diamond surface.

In the case of blood serum, 50l serum sample could be prepared by spreading a small volume of serum on ATR crystal, allowing to dry followed by measuring the absorption spectrum of the film (Heise H M *et al.*, 2000 and 1995). It is known that the strong absorption band of water in the mid IR region is hindered and to eliminate the same, the serum samples are air dried to form a thin uniform film on the ATR crystal. The infrared transparent ATR crystal without sample was scanned as background for each spectrum and 16 scans were coadded at a spectra resolution of $\pm \text{cm}^{-1}$. The spectra were baseline corrected and they were normalized to acquire identical area under the curves and the maximum absorbance values corresponding characteristics band were noted.

Histo pathological Study

The formalin fixed tissues were dehydrated in ascending grades of ethyl alcohol and cleared in xylene (Krasilnikova O A *et al.*, 2002). Tissue samples were then impregnated with three changes of molten paraffin wax, then embedded and blocked out. Tissue Sections of 5 μm thickness (Palmero S 1989) were cut by rotator microtome. At least 25 tissue sections for each organs were assessed. The sections were processed and passed through graded alcohol series, stained according to Bancroft J D and Stevens A (1999) using the conventional histological stains with haematoxylin and eosin (Luna L G 1968), cleaned in xylene and cover slipped in DPX. Histological examination was done under 10X magnification using Trinocular Reseachziess Microscope (Gottingen, Germany) and further obtained from 10 random and on microscopic fields per animal at X 45 and X100 objective.

Statistical Analysis

All statistical analysis was performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows. The data were not normally distributed. And therefore Non - parametric tests were performed. Descriptive statistics were presented as numbers and percentages. The data were expressed as Mean and SD. A one way analysis of variance (ANOVA) Independent sample student t test / was used to compare continuous variables between two groups. A two sided p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Kidney Disease is one of the worst public disease in developing countries. The stages of CKD (Chronic Kidney Disease) are mainly based on measured or estimated GFR (Glomerular Filtration Rate). However, this method is not sensitive enough on early stages of the pathology and thus do not offer accurate diagnostic value. The death of 50% experimental rats of the 150 mg group after one week of treatment might indicate that a dose of 150 mg / kg body weight for seven consecutive days causes dramatic nephro toxicity in rats. A previous study showed that a dose of 100 mg / kg body weight for six days resulted in significant nephrotoxicity in rats (Cuzzocrea S *et al.*, 2002 and Dehghani *et al.*, 2011). Early detection and treatment can often limit or avoid the chronicity effects of the disease. Scenario currently dominated by kidney biopsy and established biomarkers such as serum creatinine, albuminuria and proteinuria novel biomarkers could potentially provide vital diagnostic and prognostic information. The results of quantification of biomarkers aid to evaluate the healthy and pathological conditions of control and experimental animals.

Biochemical Evaluation of Blood serum in Gentamycin Induced RD in Wistar rat

The renal markers assayed include like creatinine, urea, uric acid, calcium, phosphorous, total protein, albumin etc., are indicative of the functional efficiency of kidney. The other biochemical parameters such as cholesterol, triglycerides, HDL cholesterol, T3, T4, TSH, etc., were also analysed for clinical correlations. Table 1 shows various bio chemical parameters and enzymes in blood involved in evaluation of the organ functions concern with renal disease.

Table 1: Changes in biochemical composition levels in Blood serum of control and experimental groups in wistar rat.

Biochemical Composition in blood serum	Control	Gentamycin injected
Urea (mgs/dl)	40±7.10	63±7.20**
Creatinine (mgs/dl)	0.88±0.4	2.56±0.5***
Total Protein (gm/dl)	7.1±2.89	8.6±2.96*
Albumin(gm/dl)	4.3±1.11	5.9±1.56*
Globulin (gm/dl)	2.8±0.77	4.1±0.98*
Plasma Glucose mg /dl	112±5.42	139±4.48*
Uric acid (mg /dl)	4.8 ±1.12	6.9±1.33†
Calcium(mg /dl)	7.9±2.43	9.0±1.72†
Total Cholesterol(mg /dl)	167±31.10	170±3.82†
Triglyceride (mg /dl)	120±30.89	134±4.07†
HDL Cholesterol (mg /dl)	47±9.80	41±4.09†
T3(ng/dl)	161±15.87	178±8.19†
T4(µ/dl)	5.9±1.20	5.7±1.09†
TSH (mIU/dl)	4.8±1.33	5.2±3.11†
SGOT	55 ±5.1	63 + 5.6†
SGPT	61± 7.5	70 ±6.9†

† NS * P<0.05 ** P<0.01 ***P<0.001

The result indicates increased level of creatinine (2.56±0.5) in gentamycin induced in Wistar rat is highly significant (p<0.001) and is a useful indicator of renal dysfunction (Perrone R D 1992). Elevated creatinine level in serum is usually associated with various renal and earlier stage of renal disease. The study also shows that the level urea (63±7.20) obtained is more significant (p<0.01) among healthy control animals. The other parameters like total protein (8.6±2.96), albumin (5.9±1.56), globulin (4.1±0.98) and glucose (139±4.48) are statistically significant (<0.05) are considered to be biomarker to assess clinical status of renal disease. The result of this study is in accordance with the findings of earlier research studies (Ekor M et al., 2010; Hughes A et al., 1996; Abdel-Naim et al.1999; Lovari J et al.,2008; Francescato H S D C et al. 2001; Saad A et al.,2009; Sueishi K et al.,2002; Travlos T S et al., 1996; Kore K J et al., 2011;Babu S V et al., 2011; Soliman K M et al., 2007 and Chaware V J et al., 2011). Further, nephrotoxicity demonstrated in the present study is also associated with increased creatinine and urea in blood are of special significance to evaluate the renal function (Frank C M D 1993; Karahan I et al., 2005; El-Ashmawy H M et al., 2006 and Zeeni, N et al., 2007) The present study recorded increased blood level of AST (70±6.9) also evident that gentamycin induced renal damage is directly related to such increment. Increased blood level of AST is usually coincided with renal damage and further indicated that nephrotoxicity is closely associated with an increase in lipid peroxidation (Iseri S et al., 2007 and Stryer L 1988) and the drug causes generation of reactive oxygen species and inhibits the activity of antioxidant enzymes like SGOT, SGPT etc., (Table 1).

FTIR-ATR Spectral studies of Blood serum and different organs of Gentamycin induced RD in male Wistar rat

Since the constitution of body fluids based on their molecular structure, have highly specific bands in the spectral region because of their molecular structure which aids in diagnosing the mechanism of human diseases.

Table 2: FTIR-ATR Vibrational band assignment of bio molecules of healthy control serum of male Wistar rat.

S. No.	-1 Wave Number (cm)	Vibrational Band assignment
1	3283	N-H stretch due to protein and urea
2	3071	Amide B band due to overtone of Amide I band and olefinic group C-H stretch Lipids of Unsaturated fatty acid
3	2961	C-O-C Asymmetric / Symmetric stretch vibrations of methyl group of Protein and C- H Lipids (Fatty acids and TGL)
4	2931	Asymmetric stretching vibrations of methylene group of protein and lipids
5	2879	Symmetric stretching vibrations of methylene group of proteinand lipids
6	1742	C=O group of cholesterol ester (HDL)
7	1634	Aryl substituted C=C Amide I band mainly due to C=O, C=Nand N-H stretching
8	1538	Amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein.
9	1453	Asymmetric bending vibrations of lipids, proteins of CH ₃ groups.
10	1395	Free Amino Acid and Fatty acids;
11	1313	Amide III erythrocyte
12	1240	Amide III and Asymmetric PO ₄ stretching vibration mode of Nucleic acid
13	1165	Ring vibrational mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric cholesterol ester, Phosphoric acid
14	1115	Stretching vibration of glycogen
15	1076	C-O chacterization stretching of glucose
16	1040	Primary alcohol C-O stretch glucosemuco polysaccharide
17	934	Ribose, phospholipids
18	532	Polysulfidic S-S stretch in cystic acid

The spectrum analysis gives information about atomic and molecular energy levels, molecular geometries, chemical bonds in the functional groups, interactions of molecules as well as the qualitative and quantitative analysis of the sample. Fourier transform Infrared spectroscopy (FTIR) is a technique which characterize the biomolecules because it relies on the characteristic absorbance of corresponding molecular vibration of functional groups in the chemical compounds bio molecules such as carbohydrates, lipids, proteins etc.,. Attenuated total reflection spectroscopy is based on the phenomenon known as Total Internal Reflection (TIR) (Katon J E and Micron 1996 and Baulsir CF and Simler RJ , 1996). A new diagnostic approach based on FTIR-ATR spectrometry has been introduced which provides a rapid, reliable, and easy way to perform blood test for the diagnosis of renal failure. A vibration band assignment is done with the idea of the group frequencies of the various analytes present in the sample are represented in Table 2. To evaluate clinical status of the renal disease, overlaid spectral pattern of blood serum of control and induced renal disease male Wistar are represented in Fig.1.

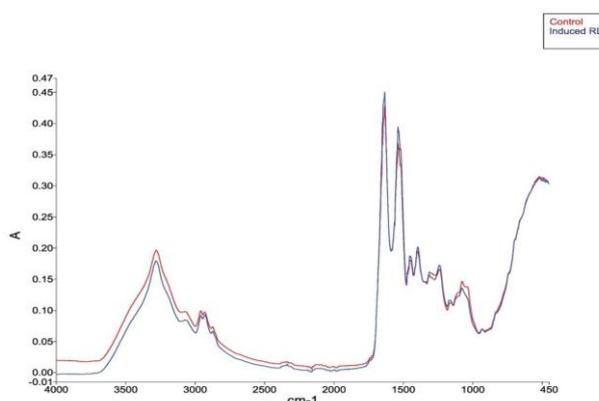


Fig. 1: FTIR-ATR spectral overlaid pattern of blood serum of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

The intensities of infra-red spectra provide quantitative information and the absorption positions reveal qualitative characteristics about the nature of chemical bonds, their structure and their molecular environment. The important absorption bands arise from NH, C=O, C-H and X-O bonds found in urea, carbohydrates, proteins, lipids and nucleic acids (Benjamin, et al., 2008). The study shows that the most intense bands for urea is 3400 cm⁻¹ and spectral results obtained are well supported by the clinical values and agreed with the earlier studies (Renuga Devi T S R et al., 2009; Gunasekaran S et al., 2010). The peaks between 1640-1620 cm⁻¹ is intense absorption band in proteins is the amide peak, which is

observed at 1652 cm^{-1} . Amide-I is mainly associated with both the C=O and C-N stretching vibration and is also related to the backbone conformation (Grismer J T et al., 1996; Shaw R A et al., 1996; Do-Hyun Kim Ilev, 2007). The vibrations recorded in this study on lipids are the CH_2 stretching (2850 - 2920 cm^{-1}) bending or scissoring (1450-1480 cm^{-1}) were contrast to previous study of Moharram M A et al., 1996 and they documented that the lipid finger print region containing phosphate and di ester stretch modes (1000-1450 cm^{-1}), and the C=O stretching vibration (1700-1750 cm^{-1}). The spectral region (3600-3000) cm^{-1} comprises of C-H, O-Hand N-H stretching vibrations of the protein. The prominent absorption peak 3300 cm^{-1} is due to the N-H stretching mode (amide A) of proteins. (Sankari G et al., 2010) and similar observations are recorded in this study. Further, both present and the study of Sankari G et al., 2010 shows that the asymmetric and symmetric stretching C-H vibrations of methyl and methylene group are found to be present around 2930–2875 cm^{-1} . The strong absorption and at 1650 cm^{-1} correspond to C=O stretching vibrations (amide I) whereas the vibration and at 1542 cm^{-1} is attributed as amide II arising of N-H bending vibrations strongly coupled with C-N stretching of proteins. The absorption peaks in the region (1400-1200 cm^{-1}) arise due to the C-H deformation of methyl and methylene group of the proteins on the basis of their most characteristic IR absorption peaks which support the study of Cyril Petebois et al., 2001. For albumin, the best correlation with results obtained by a comparison method was found using the N-H absorption region (1600-1480 cm^{-1}) common to all plasma proteins and concluded that the FTIR-ATR spectrometry is a useful tool for determining concentrations of multiple bio molecules in micro samples of plasma.

The asymmetric and symmetric P-O stretching vibrations are found to be around 1245 cm^{-1} and 956 cm^{-1} respectively. The spectral region 1250-925 cm^{-1} is predominantly occupied by C-O-C asymmetric and symmetric vibrations of phospholipids of proteins and the same was reported by Sankari G et al., 2010 and Renuga Devi T S R et al., 2009 and concluded that a systematic approach has been made using FTIR-ATR spectroscopic technique to study the spectral difference between the healthy and renal failure patients blood samples.

The spectral region 1234-934 cm^{-1} is predominantly occupied by C-O-C asymmetric and symmetric vibrations of phospholipids of proteins and carbohydrates (glycogen and glucose) and similar findings was noticed in earlier study where the author was described about the C-O-C asymmetric and symmetric vibrations of phospholipids of proteins rather than carbohydrates and documented that the spectral region 1081 cm^{-1} corresponds to phosphate and glycogen. Randhawa H S 2003 also reported that the spectral region 1250– 925 cm^{-1} is predominantly occupied by C-O-C asymmetric and symmetric vibrations of phospholipids of proteins.

The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the serum of control and induced renal disease in Wistar male rat given in Table 3. The internal peak ratio are calculated for the amide I/Glucose-str. (I1634/I1076), amide II/ribose – phospholipid (I1538/I934), amide III Erythrocyte/(cystic acid) s-s-str. (I1313/I532) and (amide III) asym PO₄ of NA/glycogen-vib. str (I1240/I1115).

Table 3: The changes in the FTIR- ATR band internal peak ratio calculation for various molecules in the serum of control and induced RD experimental rats.

Peak ratio	Wave Number (cm^{-1})	Absorbance		P value
		Control	RDI	
Amide I / Glucose-str	I1634/1076	2.9242± 0.183	3.3190± 0.169	0.0029
Amide II / Ribose -phospholipid	I1538/934	5.2902± 0.177	5.7314± 0.157	0.0326
Amide III Erythrocyte/(Cystic acid) s-s-str.	I1313/532	0.4974± 0.015	0.5102± 0.019	0.1522
(Amide III) asym. PO ₄ of NA / Glycogen-vib. str	I1240/1115	1.3227+ 0.183	1.4275+ 0.180	0.0873

The results shows that peak ratio for amide I / glucose-str. as well as amide II/ribose phosphor lipid are significantly increased gentamycin induced renal than control (p value 0.0029 and 0.0326). The small changes in the absorption is also appreciable in the FTIR-ATR spectra as it depends on the short existing, effective evanescent wave with 0.5 μ -5 μ depth of penetrations. The absorption ratio of symmetric amide III Erythrocyte/(cystic acid) s-s-str. and (amide III) asym. PO₄ of NA /

glycogen-vib.str of induced renal and control rats are not significant and p values obtained are 0.1522 and 0.0873 respectively The trends observed on absorptions of internal peak ratio of renal rat was more than control which support earlier studies (Renuga Devi T S R et al., 2009 and Gunasekaran S et al., 2010). The results of FTIR-ATR spectral analysis of variations in bio molecules is resembles to that of quantification of bio molecules in this study.

The overlaid FTIR-ATR spectral bands in different organs of male Wistar rat control and induced renal disease are represented in Fig.2a-e

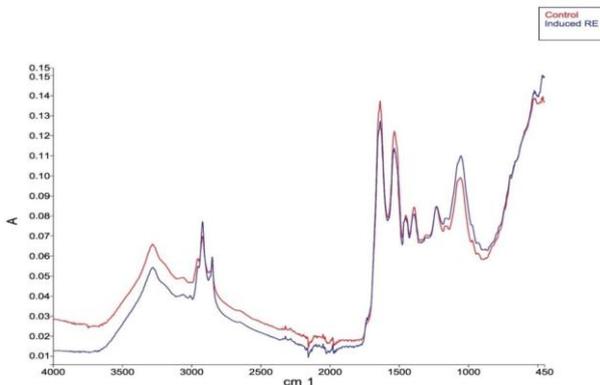


Fig. 2a: FTIR-ATR spectral overlaid pattern of kidney of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

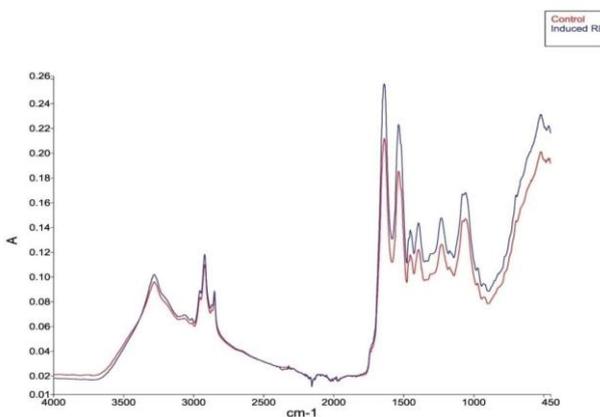


Fig. 2b: FTIR-ATR spectral overlaid pattern of liver of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

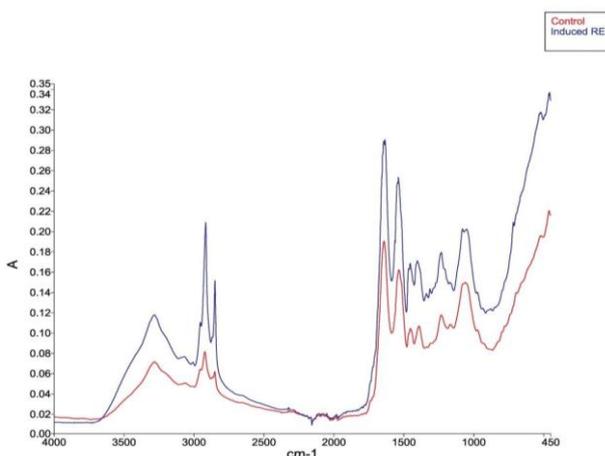


Fig. 2c: FTIR-ATR spectral overlaid pattern of lung of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

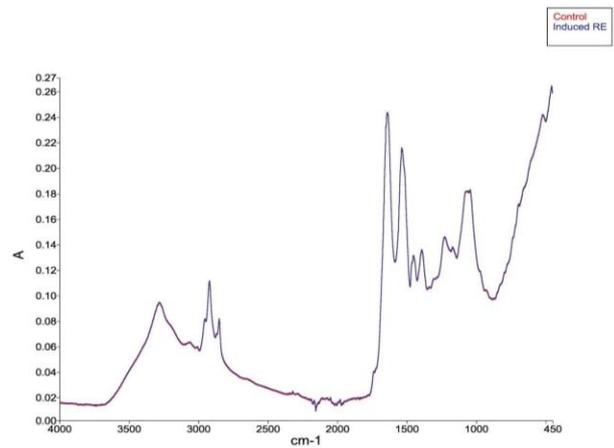


Fig. 2d: FTIR-ATR spectral overlaid pattern of heart of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

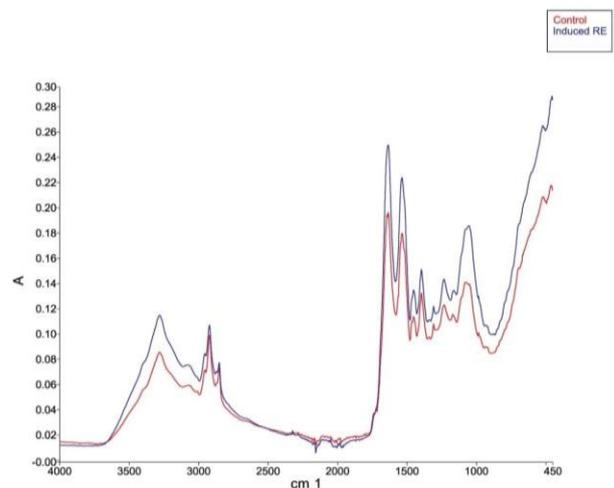


Fig. 2e: FTIR-ATR spectral overlaid pattern of muscle of healthy control and gentamycin induced renal disease in experimental male Wistar rat.

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For internal peak ratio studies on different organs, the wave length chosen for change in sensitive peaks and no change in sensitive peaks are include (Lipoprotein)_{asym} and Amide I (I2931/ I1634), (Lipoprotein)_{sym.vib} and Amide II (I2879/I1538), HDL choletsreol ester and (Nucleoprotein-Amide III) _{asym}.PO4 (I1742/I1240) as well as Mucopoly-Glu and (Cystic acid)_{s-s} -str. (I1040/ I1532). The absorbance ratio shows that increased values obtained for liver, kidney, lung and muscle and not for heart indicates induction with gentamycin does not show any molecular changes on heart (Table 4). The statistical calculation for all four different peak ratio (I2931/I1634, I2879/I1538, I1742/I1240 and I1040/I532), also showed highly significant ($p < 0.01$) for kidney, liver, lung and muscle for and not significant for heart whole support the FITA-ATR spectral analysis of the study.

Table 14: The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the lyophilized organs of control and induced RD experimental rats.

Peaks ratio	Liver	P value	Lung	P value	Kidney	P value	HEART	P value	Muscle	P value					
	Cont.	RDI		Cont.	RDI		Cont.	RDI		Cont.	RDI		Cont.	RDI	
I 2931 (Lipoprotein) asym. str.	0.3998 ± 0.012	0.4613 ± 0.020	0.0116	0.4271± 0.022	0.7218± 0.022	0.0001	0.5091± 0.015	0.6051± 0.012	0.0001	3.2831± 0.118	3.2516 ± 0.209	0.19	0.4320± 0.023	0.5062± 0.020	0.0001
I1634 Amide I															
I2879 (Lioprotein) sym. vib	0.3824 ± 0.016	0.5928 ± 0.013	0.0001	0.3803± 0.015	0.5990± 0.011	0.0001	0.4599± 0.021	0.5210± 0.023	0.0171	0.3774± 0.008	0.3754 ± 0.007	0.245	0.3449± 0.013	0.4299± 0.016	0.0002
I1538 Amide II															
I1742HDL choletsreolester	0.2834 ±0.013	0.3114 ±0.013	0.0001	0.2720± 0.013	0.3782± 0.014	0.0001	0.2975± 0.012	0.3274± 0.020	0.0015	0.2664± 0.014	0.2614± 0.009	0.3186	0.2919± 0.019	0.3322± 0.020	0.0001
I1240 (AmideIII) asym. PO4 of NA															
I 1040Mucopoly-Glu-str.	0.7311 ±0.013	0.8331 ±0.013	0.0001	0.6371± 0.014	0.7671± 0.009	0.0001	0.7141± 0.016	0.8720± 0.020	0.0001	0.7554± 0.007	0.7574 ±0.020	0.1025	0.5397± 0.027	0.70040. ±0.19	0.0002

Histological studies on Gentamycin Developed RD in male Wistar rat

Studies on histological alterations in renal tissues due to gentamycin are limited and have not been fully identified. Severe and extensive histological alterations in the renal tissue induced by gentamycin as seen in the present work suggest the potential of gentamycin to cause oxidative damage to macromolecules and cellular organelles. The findings of the present study showed that exposure to gentamycin resulted in granulomatous inflammation in kidney progressive tubular, glomerular and interstitial histological alterations (Fig. 3 a, b, e & f). Further, epithelium with interstitial inflammation and granulomatous reaction observed in the present work is different from the findings of previous investigations of the tubular necrosis and degenerative changes (Al-Majed *et al.*, 2002; Ekore *et al.*, 2010; Nitha and Janardhanan, 2008; Ali *et al.*, 2011; and Dehghani *et al.*, 2011). The earlier literature studies shows that tubular damage in proximal convoluted and distal tubules, necrosis, interstitial oedema, perivascular oedema and multiple focal collections of mononuclear cells in the interstitium, and calcification of the necrotic renal tubules (Chinnapareddy *et al.*, 2011 and Saud Allarifi *et al.*, 2012) but the present study do not observe any tubular changes, oedema, necrosis etc.,. Effect of gentamycin on the liver resulted in fatty change

(Fig. 3 c- e) due to hypoxia which lead to liver injury causes hepatic inflammation and prolonged hypoxia may lead to right-side heart failure, which results in ischemia and congestive hepatopathy (Crenesse D *et al.*, 2001; Gasbarrini A *et al.*, 1997; Gadsboll N *et al.*, 1989; Hoffman B J *et al.*, 1990 and Holley H C *et al.*, 1989). Further, gentamycin significantly increased the serum enzyme levels, namely ALT, AST and ALP indicating chemical induced hepatocellular toxicity and similar observations was observed. The renal injury was based on focal chronic pyelonephritis, severe congestion, focal coagulation necrosis etc., Sections from control group showed normal histological structure of kidney tissue. In renal sections from the rats receiving gentamycin group showed epithelium with interstitial inflammation and granulomatous reaction. "Acute tubular necrosis" is associated with interstitial inflammation, usually late in the course of the disease. Gentamycin therapy was implicated as a possible cause of the interstitial inflammation is noticed. The similar reports were documented in earlier reports and in their study interstitial inflammation was significantly greater in the female rats even though the renal failure was significantly more severe in the male rats (Olivier Kourilsky *et al.*, 1982). Other organs like muscle and heart are normal (Fig.3 hand i).

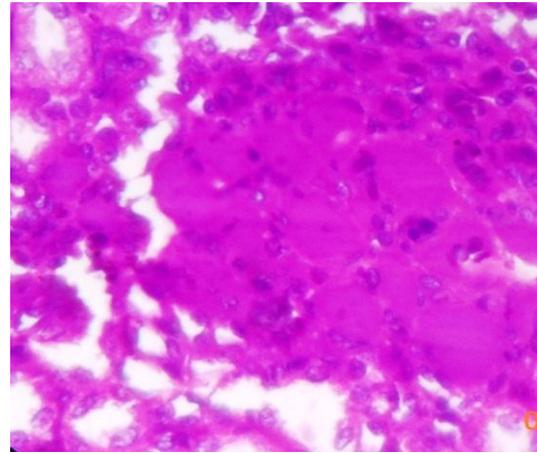
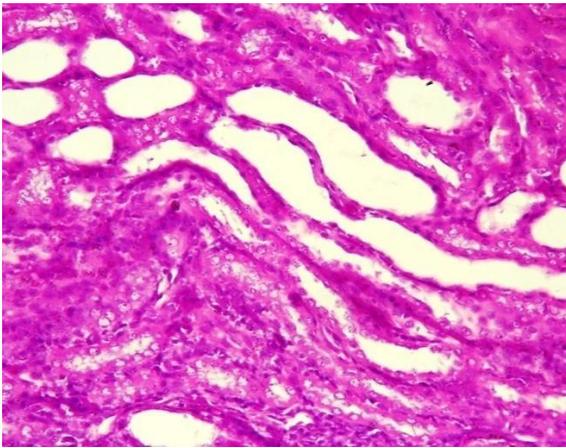


Fig. 3a: Normal Kidney: Glomeruli and tubules. Fig. 3b: Gentamycin treated Kidney H&E x40 Granulomatous inflammation.

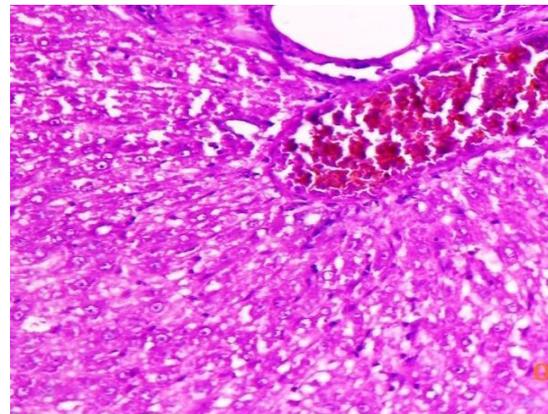
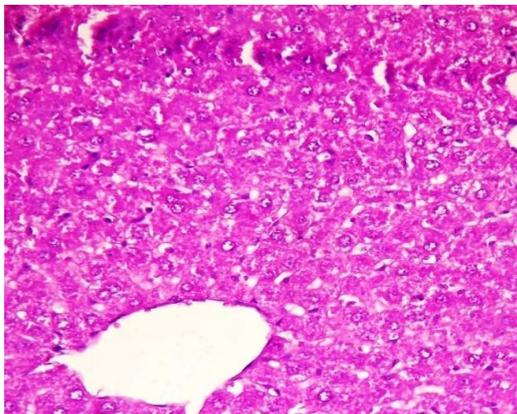


Fig. 3c: Control group: Liver. Fig. 3d: Gentamycin treated liver fatty H&E x40 change due to hypoxia.

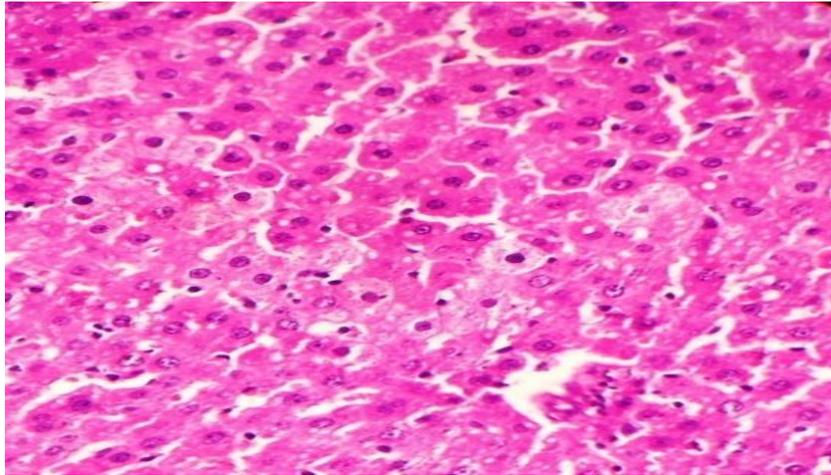


Fig. 3(e): Induction: Liver: Microvesicularsteatosis (Fatty change) H&Ex40.

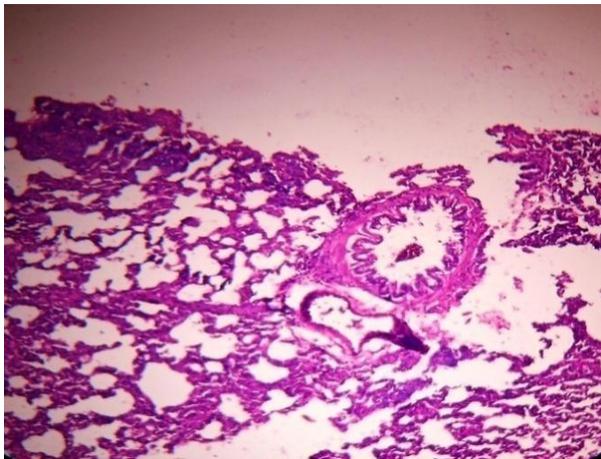


Fig. 3(f): Normal lung parenchyma H&Ex40.

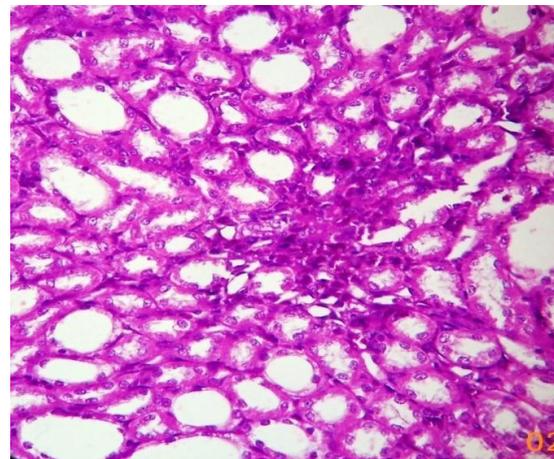


Fig. 3(g): Gentamycin treated Lungs Interstitial inflammation.

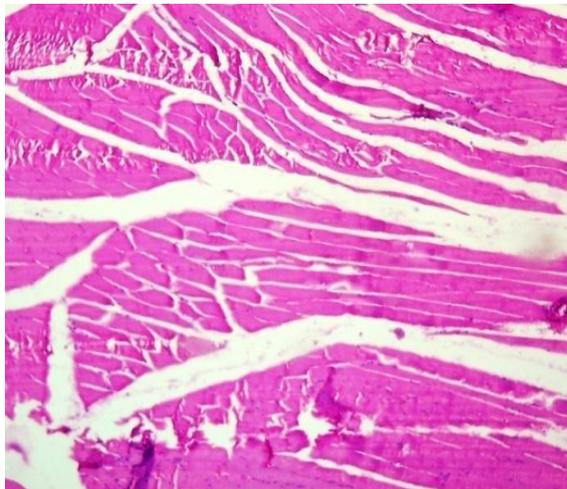


Fig. 3(h): Control group: Muscle.

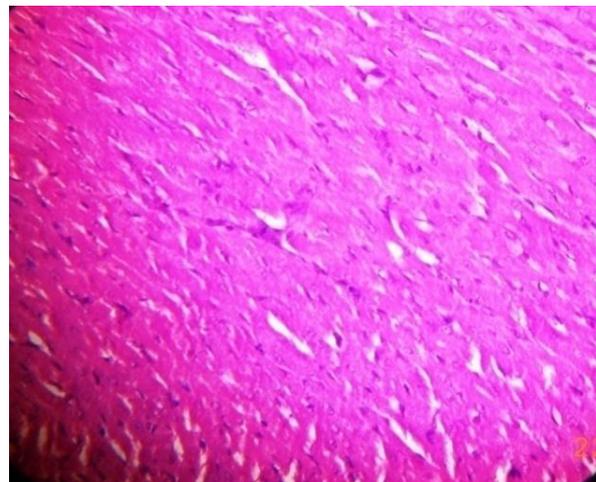


Fig. 3(i): Control group: Heart.

CONCLUSION

The increased level of creatinine and urea in gentamycin induced male wistar rat were highly significant ($p < 0.001$) and is a useful indicator of renal dysfunction and particularly associated with earlier stage of renal disease. The other parameters like total protein, albumin, globulin and glucose were also statistically significant and considered to be biomarker to assess the clinical status

of renal disease. The results shows that the peak ratio for amide I / glucose-str. (I1634/I1076) as well as amide II / ribose-phospholipid (I1538/I934) were significantly increased in gentamycin induced renal than control male Wistar rat with RD. The absorbance for internal peak ratio of blood serum for amide III erythrocyte / (cystic acid) s-s-str. (I1313 /I532) is highly significant among gnetamycin induced was support other methodologies.

The absorbance of internal peak calculation ratio of the lyophilized organs for the (lipoprotein)_{sym} str.a amide I (I2931/I1634), (lipoprotein)_{sym}.vib. and amide II (I2879/I1538), HDL cholesterol ester and (amide III)_{asym}.PO4of NA (I1742/I1240) as well as mucopolysaccharide and (cystic acid)s-s -str (I1040/I532) were indicates that the organs showed fatty change in liver due to hypoxia and persisting fatty change in liver . Further epithelium with interstitial inflammation and granulomatous reaction was also observed. The absorbance peak ratio obtained for the wave lengths I 2931/I1634, I2879/I1538, I 1742/I1240 and I1040/I532 were significantly high for liver, kidney, lung and muscle but not for the heart. FTIR-ATR spectral analysis gives additional information with in addition to other routine methods.

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