Original Research Article

FTIR-ATR Studies on Phenylhydrazine Induced Hyperbilirubinemia in Wistar Rat

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ABSTRACT

Hyperbilirubinemia is a condition of increased bilirubin in serum and analysis of this high bilirubin and other related components achieved in the serum by routine methods in clinical biochemistry and due to certain limitations. Current modalities of diagnosis have limitations such as poor sensitivity, specificity, reproducibility but expensive. Diagnostic methods should have greater sensitivity and also facilitate early detection of diseases to provide better prognosis. Further this FTIR-ATR spectroscopic method aids in analyzing multiple parameters / component in single spectral analysis which could be the added advantages to determine the other specified components over other existing methods.

In the present work, FTIR- ATR spectroscopic techniques will be employed to evaluate biomarkers by studying the variations on bio molecule composition in blood serum of control and experimental animals. Experiments were conducted on Phenylhydrazine induced hyperbilirubinemia wistar rat and biochemical and FTIR-ATR spectral analysis were done to rule out the clinical condition. The FTIR-ATR spectral analysis for hyperbilirubinemia was compared with other routine biochemical components assay followed by statistical methods.

Findings on induction of hyperbilirubinemia viz. Biochemical studies as well as FTIR-ATR spectral approach clearly establishes the effectiveness of the development of hyperbilirubinemia compared with control group of experimental wistar rat. 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. Spectroscopy is the powerful tool for identifying types of chemical bonds /functional groups to identify the components of the sample qualitatively and quantitatively. The biochemical evaluations obtained with routine existing methods are comparable with the results of FTIR-ATR spectral analysis to diagnose the hyperbilirubinemia conditions in experimental wistar rat.

FTIR - ATR spectroscopic imaging has significant advantages composed to many other imaging methods for the characterization of biomolecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds such as carbohydrates , ester, albumin, proteins, nucleic acid well as inter atom chemical bonds. The ability to diagnose the early onset of disease, rapidly, non- invasively and unequivocally has multiple benefits. Quantitative estimation of Total Bilirubin in serum/ by FTIR –ATR spectrophotometric method is additional and useful in the diagnosis of hyperbilirubinemia due to any cause and is an indicator of liver functions.

Key Words: hyperbilirubinemia, FTIR-ATR spectral analysis phenylhydrazine, induction studies, wistar rat, biochemical analysis.

INTRODUCTION

Bilirubin (from the Latin ``bilis,'' meaning bile, and ``rubor,'' meaning red) is a bile pigment formed during the catabolism of heme-containing compounds, primarily hemoglobin. Bilirubin is the degradation product of heme, the bulk of which is derived from hemoglobin of senescent erythrocytes and hepatic hemoproteins. Bilirubin is potentially toxic, but is normally rendered harmless by binding to plasma albumin, and efficient hepatic clearance. The bilirubin is removed from the bloodstream by the liver and eliminated from the body in the bile, which passes from the liver into the intestines. Hyperbilirubinemia is the condition associated with abnormally high levels of bilirubin in the blood serum and that may interrupt the elimination of bilirubin from the blood and cause jaundice.

Laboratory tests are often used to evaluate patients' pathological conditions. Over the years, technological advances and automation have made tests easier and led to very accurate, more precise and timely results. Most of the studies conducted with animal model focused using conventional methods for clinical correlation. Further lot of research studies focusing on induction of different diseases with drugs and evaluation of drug efficacy with alternate method in addition to conventional methods. [1-7] But these existing methods involve specific reagents and equipment and the costs of which have increased tremendously over the years. Further to perform tests, skilled experts are required to run the calibration and quality control. Also these sophisticated equipments need special care and good maintenance. Moreover the multiple parameter analysis might require different analytical techniques. Hence researchers seek alternative methods to analyze those samples timely and economically with accuracy, precision and user friendly. Based on certain draw backs and advantages in recent years FTIR-ATR spectroscopic method is chosen among the other spectroscopic methods in diseases diagnosis. [8] Excessive accumulation of bilirubin, as a result of enhanced production or impaired elimination, results in yellow discoloration of the skin, sclera, and mucus membranes, which is termed jaundice / icterus [9] is a yellowish pigmentation of the skin, the conjunctival membranes over the sclerae (whites of the eyes), and other mucous membranes caused by hyperbilirubinemia. Quantitative estimation total bilirubin, Aspartate Amino Transferases (AST / SGOT and ALT (SGPT) in serum by attempted with routine existing methods.

The FTIR-ATR spectroscopy is based on the phenomenon known as Total Internal Reflection (TIR) [10,11] As water is a good absorbent of infrared radiation, it affects the actual spectral response of the test material and dominated in the FTIR spectrum of serum sample. This radiation strikes the interface between the IRE and the serum and tissue sample composed of a lower refractive index. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the serum sample held in contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns (0.5μ -5.0 μ) beyond the crystal surface and into the sample. The depth of penetration of infrared radiation from denser IRE into the test material depends on refractive indices of the materials to be investigated and the wave number of the infrared radiation. As the sample absorbs IR radiation at certain frequencies, the resultant totally reflected radiation (or) evanescent wave will be attenuated (altered) in regions of the infrared spectrum where the sample absorbs energy. This attenuated IR radiation of evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and it is detected by the detector in IR spectrometer.

Earlier literature studies shows FTIR- ATR spectroscopic analysis on different bio molecules (conformations of peptides, polypeptides and proteins [12,13]

and amino acids lipids, proteins and carbohydrates, [14] bilirubin [15] IgA and IgM, [16] plasma protein, urea, creatinine [17] etc., Due to ethical issues on human, current study focus using FTIR-ATR method to detect the disorders / disease in animal models. The experimental animals were induced phenylhydrazin to induce hyperbilirbie diseases in animal models. The chemical bonding stretches reveal biochemical composition in blood / serum, different tissue homogenate including liver, kidney, heart, skeletal muscle, etc., helps in determining the detection and severity of disease conditions. Literature studies are available for rat induced model to study the blood parameters jaundice and hyperlipidemia etc., [18,16,19,20,15] But studies on rats induced with using hyperbilirubinemia is limited with FTIR-ATR spectroscopic studies. In the present work, FTIR-ATR spectroscopic technique is employed to study the spectral differences in the serum of normal and pathological blood samples. Further it can be an alternate as well as additional tool in clinical analysis compared with other techniques for understanding the biological nature of the disease for prognosis and management.

MATERIALS AND METHODS
Procurement Experimental Animals:

Experimental wistar rat were obtained and maintained as per standard protocol to meet guidelines of animal ethical committee as described. This study was approved by animal house of Research and Development, Saveetha Medical College and Hospital, Thandalam Chennai, India. All experiments were carried out according to the guidelines for care and use of experimental animals, and are approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethical Committee. Six (both sexes weighing between 120 and 150 g) wistar rats per cage were housed in polypropylene cages (32.5 ×21×14) cm lined with raw husk which was renewed every 8 hours.

The animal house was maintained at an average temperature ($24.0\,^0C \pm 2\,^0C$) and 30-70 % RH, with 12 hr. light-dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). Animals received humane care and were fed with commercial pellet diet and the animals were acclimatized for one week before the start of the experiment.

Experimental studies on promotion of Hyperbilirubinemia (HB):

Promoting of hyperbilirubinemia was achieved by subcutaneous injection of Phenylhydrazine (75 mgs / kg body weight) daily for 14 days as per the standardized procedure along with control animals. [21] The phenylhydrazine used in this study is 97% pure (100gm) obtained from Sigma Aldrich, Mumbai to conduct induction study.

Collection and Processing of Sample:

At the end of experiment the wistar rats were fasted overnight. Blood samples of the 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 FTIR-ATR spectral studies.

Analysis of Bio components by using Conventional Methods:

Biochemical quantification of blood serum of both control and induced HB were done using standard procedures. The composition/components are the preferred indicator with respect to the patho physiological condition of the system. The blood serum were analyzed quantitatively for biochemical parameters include glucose, urea, creatinine, calcium, phosphorus, uric acid, total bilirubin, SGOT,SGPT, total protein, albumin, cholesterol, triglyceride, HDL etc., by enzymatic assay method using respective commercial diagnostic kits. [22-33]

Sample Preparation for FTIR-ATR spectral analysis:

50 µl Serum sample was placed on the IRE crystal and the water content on the serum samples were air dried for water evaporation to eliminate the stray absorption bands due to water and holder is mounted in sample window of the spectrophotometer. The sampling window is scanned as the background and 32 scans are co added with spectral resolution of $1cm^{-1}$ > All spectra were base line corrected and normalized to acquire identical areas under the curve. FTIR spectral measurements were carried at room temperature and each measurement was repeated to ensure the reproducibility of the spectra. These spectra were subtracted against the background of air spectrum. After every scan, the crystal is cleaned with isopropyl alcohol or methanol soaked tissue and a background of new reference air was taken to ensure the crystal cleanliness.

Biochemical evaluation by FTIR-ATR:

The FTIR-ATR spectral analysis for the blood serum in control as well as in experimental animals was carried out according to standard procedure. Spectroscopy is the measurement and interpretation of absorption and emission of electromagnetic radiation when atoms, molecules or ion move from one energy level to another. The absorption energy causes an electron or molecules to go from an initial energy state (ground state) to high energy state (excited state) which could take the form of the increased rotation, vibration or electronic excitation. By studying these changes in energy state scientists are able to learn more about the physical and chemical properties of the molecules. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule.

Statistical Analysis:

All statistical analysis were 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. One way analysis of variance (ANOVA) Independent sample student t tests were used to compare continuous variables between two groups. A two sided p value < 0.05 was considered statistically significant.

RESULTS

Biochemical Evaluation of Blood serum in Phenylhydrazine promoted HB Wistar rat

The induction studies in Wistar rat showed markedly increased absorbances and variations in FTIR-ATR spectral analysis concludes positive effect of inducing agents to develop the pathological status of the hyperbilirubinemia as a result the total bilirubin and direct bilirubin level and other parameters level are shown in Table 1
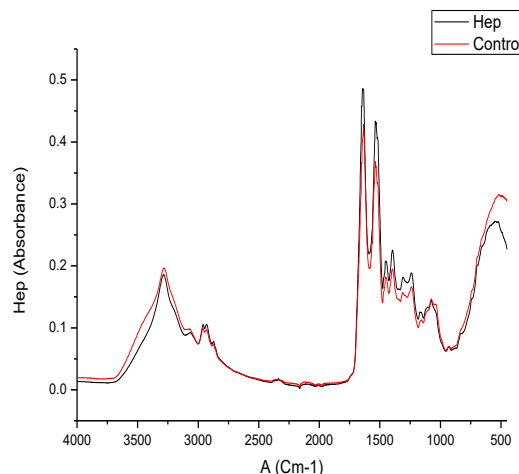
FTIR-ATR Spectral studies of Blood serum Phenylhydrazine promoted HB in Wistar rat

FTIR-ATR spectral analysis carried out to identify the various analytes in the experimental samples of blood serum for phenylhydrazine induced hyperbilirubinemia in male Wistar rat. To evaluate clinical status of the hyperbilirubinemia, overlaid spectral pattern of blood serum of control and induced hyperbilirubinemia in male Wistar are represented in Fig.1. FTIR-ATR spectroscopic imaging has significant advantages composed to many other imaging methods for the characterizations of biomolecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds as well as inter atom chemical bonds which in turn helps in disease diagnosis well as inter atom chemical bonds. The vibration band assignment is done with the idea of the group frequencies of the various analyses present in the sample given in Table 2

Table1. Changes in biochemical composition levels in Blood serum of control and in wistar rat induced with Phenyl Hydrazine

Biochemical Parameters	Control	Phenyl Hydrazine Injected
Total Bilirubin	0.67 $\pm$ 0.39	3.9 1$\pm$.81***
Direct Bilirubin	0.23 $\pm$ 0.11	1.9 1. $\pm$11***
SGOT (IU/L)	19.65 $\pm$ 8.7	51.7 $\pm$12.81**
SGPT	23.33$\pm$ 9.1	70 .5 $\pm$14.1**
ALK.P	76.7$\pm$ 10.8	159.8$\pm$ 15.8**
Total Protein (gm/dl)	7.16$\pm$2.89	7.31$\pm$1.81*
Albumin(gm/dl)	4.35$\pm$1.11	4.5$\pm$.96†
Globulin (gm/dl)	2.86$\pm$0.77	3.06$\pm$0.50†
Plasma Glucose mg /dl	112.7$\pm$5.42	129$\pm$2.10†
Uric acid (mg /dl)	4.8 $\pm$1.11	4.85$\pm$ 0.3†
Calcium(mg /dl)	7.9$\pm$2.43	8.19$\pm$1.72†
Total Cholesterol (mg /dl)	167$\pm$31.10	188$\pm$5.72†
Triglyceride (mg /dl)	120$\pm$30.89	144$\pm$3.11†
HDL Cholesterol (mg /dl)	47$\pm$9.80	39$\pm$3.09†
T3(ng/dl)	161$\pm$15.87	178$\pm$7.32†
T4(μ/dl)	5.9$\pm$1.20	5.99$\pm$.90†
TSH (mIU/dl)	4.8$\pm$1.33	4.97$\pm$ 4.05†
Urea (mgs/dl)	40$\pm$7.10	41$\pm$6.40†
Creatinine (mgs/dl)	0.88$\pm$0.4	0.91$\pm$0.5†

† NS * $P<0.05$ ** $P<0.01$ ***$P<0.001$

Fig.1 FTIR-ATR spectral overlaid pattern of blood serum of healthy control and phenylhydrazine induced hyperbilirubinemia in experimental male Wistar rat

Table2. FTIR Vibration Band assignment of blood serum of control and phenylhydrazine induced male wistar rat blood serum

S.No	Wave Number (cm-^{1})	Vibration 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 protein and 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=N and 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 CH3 groups.
10	1395	Free Amino Acid and Fatty Acids;
11	1313	Amide III erythrocyte
12	1240	Amide III and Asymmetric PO2 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 glucose-Muco Poly saccharide
17	934	Ribose , Phospholipids
18	532	Polysulfidic S-S stretch in cystic acid

Table3. The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the serum of control and induced HB experimental rats

Peak ratio	Wave Number (cm $^{-1}$)	Absorbance		P Value
		Control	HBI	
(Protein) $_{sym\&asym}$vib-(Lipids –FA-TGL) /HDL cholesterol ester	$I_{2961}/_{1742}$	4.2393$\pm$0.177	5.3080$\pm$0.179	0.0001
Amide I / Ribose –phospholipid	$I_{1634}/_{934}$	6.1594$\pm$0.174	6.9971$\pm$0.179	0.001
Amide II/ Mucopoly-Glu-str.	$I_{1538}/_{1040}$	2.6661$\pm$0.177	3.4345$\pm$0.175	0.0003
(Lipoprotein)$_{asym.}$vib./ Glucose-str	$I_{1453}/_{1076}$	1.2394$\pm$0.176	1.4406$\pm$0.176	0.0005

The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the serum of control and induced HB experimental rats represented in Table 3. The absorbance obtained in hyperbilirubinemia rat are significantly high for (Protein) $_{sym\&asym}$ vib-(Lipids –FA-TGL) / HDL choletsreol ester (I_{2961}/I_{1742}), amide I / Ribose –phospholipid (I_{1634}/I_{934}), amide II / Mucopoly-Glu-str. (I_{1538}/I_{1040}) as well as (Lipoprotein)$_{asym}$.vib. And Glucose-str. (I_{1453}/I_{1076}) compared with absorbance in control healthy rats and the p values calculated are 0.0001, 0.001, 0.0003 and 0.0005.

DISCUSSION

FTIR-ATR spectroscopy is a clinically satisfactory method suitable for clinical routine measurements. The great advantage of FTIR-ATR spectroscopy is high sensibility that permits the determination of many components even in very small amount. The spectrum is like a finger print of a head of the molecular species making up of the sample. FTIR-ATR spectrometry is a useful tool for determining the concentration of multiple bio molecules in micro samples of all biological samples. The singular advantage of FTIR-ATR over other techniques is convenience. Diagnostic tests provide objective information about a person's health. used for risk assessment purposes used to monitor the course of a disease or to assess a patient's response to treatments, or even to guide the selection of further tests and treatments.

Exposure to phenylhydrazine causes damage to red blood cells, potentially resulting in anemia and consequentially hyperbilirubinemia. [34-36] the induction study results implicated oxidation products of phenylhydrazine and not proteolytic degradation as major contributors to phenylhydrazine induced protein damage in red blood cells ghosts. Phenylhydrazine directly oxidizes hemoglobins and has a property to exacerbate G6PD deficiency and the precipitation of unstable hemoglobins. The high total bilirubin obtained in rats correspond to even higher total bilirubin in humans because bilirubin does not bind as well to rat albumin as it does to human albumin. [37] Serum levels of enzymes like SGOT, SGPT, alkaline phosphatase etc., are very sensitive markers employed in the diagnosis of liver diseases. (Table 1)

The phenylhydrazine induced wistar, the bilirubin levels were found to be higher (3.9 1$\pm$0.81 mg/dl and 1.91$\pm$ 0.65) and statistically highly significant (p<0.001) among control and hyperbilirubinemia experimental male Wistar rat. [38] Further this study also reports that phenylhydrazine induces hepatic damage

resulted moderately raised serum AST, ALT and ALP (51.7$\pm$12.81, 70.5$\pm$14.1 and 159.8$\pm$15.8) respectively and the values obtained are statistically significant (p<0.01). The increased serum ALT, AST and ALP indicating chemical induced hepatocellular toxicity is significant and showed as a specific marker of liver injury due to toxic drugs, alcohol and virus, [39] paracetamol and acetaminophen. [40] In contrast with this, the other author documented that phenylhydrazine did not increase AST and ALT levels in serum of rats. [21]

Further treated with phenylhydrazine developed significant hepatic damage and oxidative stress which was observed from substantial increase in serum cholesterol and triglycerides level also significantly elevated. This is indicative cellular leakage and loss of functions integrity of the cell membrane in liver. [41,42] Moreover, phenylhydrazine administration in rats significantly increased the level (p<0.05) of serum creatinine (1.6$\pm$0.5) and urea (58$\pm$6.4) as compared to control rat (serum creatinine 0.88$\pm$ 0.5 and urea - 40$\pm$7.10) which support the earlier studies. [43,37] The other parameters like total protein, albumin, globulin, glucose, uric acid, calcium, HDL Cholesterol,T3, T4, TSH, etc., studied were do not showed significant difference among control and phenylhydrazine injected rats (Table 1).

The experimental study shows that the amide I band is caused by N-H stretching vibrations and amide II band is caused by N-H stretching coupled with C-N stretching vibrations of protein are recorded at 1634 cm^{-1} and 1538 cm^{-1} respectively and these observations were contrast to the earlier study where the authors documented that the amide -A band is caused by the N-H stretching vibration and the amide-B was the first overtone of the amide II vibration. [44] The earlier research findings shows specific frequencies glucose component for identified in blood [45] were 1365, 1152, 1109, 1080 and 1035 cm^{-1} and current study shows specific frequencies identified from

1076 -1040 cm^{-1}. Below 1240 cm^{-1} a weak absorption of 934 cm^{-1} is considered due to P-O symmetric stretching of phospholipids and the authors observed that the absorption due to the presence of fatty acids, ceramides and ribose and phospholipids. [46] The study also document that elevated biomolecules like bilirubin, AST, ALT, ALP, cholesterol and triglycerides which are needed ostensibly for the repair of damaged cell organelles and tissue regeneration.

The ratio of internal parameter calculation for absorbance is predominantly higher in phenylhydrazine induced hyperbilirubinemia than the control indicates the changes on biochemical variations after induction with phenylhydrazine injection. In order to quantify spectral differences in these regions, four intensity ratio parameters show highly significant (P<0.001). The internal peak ratio parameters analysed findings were almost similar to that of study of earlier findings. [20,16] Based on these (Protein) $_{sym\&asym}$ vib-(Lipids –FA-TGL) / HDL choletsreol ester, amide II / Mucopoly-Glu-str. and (Lipoprotein)$_{asym.}$vib./ Glucose-str. are considered as biomarker to evaluate hyperbilirubinemia in male Wistar rat.

CONCLUSION

Induction of rats with phenylhydrazine showed the development of hyperbilirubinemia with significant increased total bilirubin and direct bilirubin level (p<0.001), AST, ALT and ALP (p<0.01) and urea and creatinine (p<0.05) compared with control male Wistar rat concludes that the phenylhydrazine induce significant hepatocellular toxicity. The absorbance peak ratio for hyperbilirubinemia studied were (protein) $_{sym\&asym}$ vib-(lipids –FA-TGL) / HDL choletsreol ester (I_{2961}/I_{1742}), amide I /ribose–phospholipid (I_{1634}/I_{934}), amide II / mucopoly-glu-str. ($I_{1538}/_{1040}$) as well as (lipoprotein)$_{asym.}$vib. and glucose-str. (I_{1435}/I_{1076}) compared with absorbance in control healthy rats. Based on these the absorbances of peak ratio of wave length

I_{1453}/I_{1076} was highly significant in screening followed by I_{2961}/I_{1742} and I_{1634}/I_{934} and the peak ratio calculation for (lipoprotein)$_{asym.}$vib./glucose-str. considered as biomarker to evaluate hyperbilirubinemia in male Wistar rat induced with phenyl hydrazine.

REFERENCES
1. Singer EA, Cooper DS, Levy EG, Ladenson PW, Braverman LE, Daniels G, Greenspan FS, Mc Dougall IR, Nikolai TF. Treatment guidelines for patients with hyperthyroidism and hypothyroidism. JAMA 1995; 273:808-12.
2. Roos A, Linn- Rasker SP, Van Domburg RT, Tijssen, JP Berghout. Archives of Internal Medicine. 2005; 165:1714-1720.
3. Dehghani F, Namavar MR, Noorafshan A, Karbalay- Doust S, Esmaeilpour T. Evaluation of the kidney extract on gentamicin-induced nephrotoxicity in rat. Kidney Res 2011; J.1 (1) 24-32.
4. Sastry AVS, GirijaSastry V, Appalanaidu B, Srinivas K, Annapuran A. Chemical and pharmacological evaluation of aqueous extract of seeds of *Cassia occidentalis.* J Chem Pharm Res 2011; 3(2): 566-575.
5. Saud Alarifi, Amin Al- Doais, Saad Alkahtani SA, Al- Farraj, Mohammed Saad Al-Eissa B, Al- Dahmash, Hamad Al-Yahya, Mohammed Mubarak. Blood chemical changes and renal histological alterations induced by gentamicin in rats, Saudi Journal of biological sciences 2012; 19,103-110.
6. Rajasekaran SR, Anandan Nishad KM. Anti Hyperlipidemic Activity of AcalyphaIndica Linn. On Atherogenic Diet Induced Hyperlipidemia. Int J Pharm Sci 2013; Vol. 5 Suppl. 4, 699-701.
7. Swati R, Dhande Malvika, S, Gursahani Vilasrao, Kadam J. Standardization of

cholesterol suspension induced Hyperlipidemia in rats. World Journal of Pharmacy and Pharmaceutical Sciences 2014; Volume 3, Issue 12, 1107-1123.

8. Zanyar Movasaghi, Shazza Rehman, Ihteshamur Rehman. Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues, Applied Spectroscopy Reviews 2008; Volume 43, Issue 2.

9. Weizheng W, Wang Stephen D Zucker. Encyclopedia of Gastroenterology 2005; Pages 219-222.

10. Katon JE, Micron. Infrared Microspectroscopy. A Review of Fundamentals and Applications 1996; .27(5): p. 303-314.

11. Baulsir C F, Simler R J. Advanced Drug Delivery. Reviews 1996 ; 21 : 191-203.949 137

12. Krimm S Bandekar J. Vibrationa spectroscopy and confirmation of peprtides, polypeptides and proteins. Protein chemistry 1986; 38: 181-364.

13. Venayaminov SY Kalnin NN. Quantitative IR spectro photoometry of peptide compounds in water (H_2O) solution I. Spectral parameters of amino acid residue absorption bonds. Biopolymers 19; 30: 1243-1257.

14. Kanagathara N, Thirunavukkarasu M, Jeyanthi CE, Shenabagarajan P. FTIR and UV-Visible Spectral Study on Normal Blood Samples. International Journal of Pharmacy and Biological Sciences 2011; (1) 74-80.

15. Gunasekaran S, Uthra D, Sailatha E, Anita B. FTIR Spectral Study on Jaundice blood samples before and after treatment. Asian Journal of Chemistry 2010; 22, 51-56.

16. Sankari G, Krishnamoorthy E, Jayakumaran S, Gunasekaran V, Vishnupriya V, Shyama Subramanian, Subramaniam S, Surapaneni Krishna Mohan. Analysis of serum Immunoglobulin's using FTIR spectral measurements, J Biology and Medicine 2010; 242-48.

17. Cyril Petibois, Cazoria GA, Cassagne A, Deleris G. Plasma protein contents determined by Fourier transform infrared spectrometry, Clinical chemistry 2001; 47(4) 730-738.

18. Renukadevi TS, Gunasekaran S, Wesley HJ, Sarone AJ." Analysis on renal failure patients blood samples: characterization and efficacy study". Indian Journal of Science and Technology 2009; 2, 0974-6846.

19. Haas SL, Möller R, Fernandes A, Dzeyk-Boycheva K, Hohmann J, Hemberger S, Elmas E, Brök – hmann M, Bugert P, Backhaus, J. Spectroscopic Diagnosis of Myocardial Infarction and Heart Failure by Fourier Transforms Infrared Spectroscopy in Serum Samples. Applied Spectroscopy, 2010; 64, 262-267.

20. Gunasekaran S, Renuga Devi TS Sakthivel PS. Efficacy of simvastatin on chronic Renal failure patients -A Spectroscopic Approach. Asian Jornal of Chemistry 2008; Vol.20.N0.1. pages 167-476.

21. Lan Zhang, Bo Yuan, Hui Ping, Wang, Ya Gao. Therapeutic Effect of *Agaricusasiliensis* on Phenylhydrazine-Induced Neonatal Jaundice in rats, Hindawi Publishing Corporation [Internet]. Bio Med Research International Volume 2015; Article ID 651218, 6, pages. Available from http: /dx.doi.org/10.1155/2015/651218.

22. Soos M, Siddle K. Characterizationof monoclonal antibodies directed against human thyroid stimulating hormone, Journal of Immunological Methods 1982; 51(1): 57-68.

23. Allain CC, Poon LS, Chan CS, Richmond WS, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem. 1974; 20: 470-475.

24. Mc Gowan MW, Artiss JD, Stranberg DR, Zak BA . A peroxidase coupled method for the colorimetric determination of serum triglycerides. Clin Chem 1983; 29.538-542.

25. Izzo C, Grillo F Murador E. Improved method for the determination of high density lipoprotein cholesterol. Clin Chem 1981; 27: 371-374.

26. Friedwald WT, Levy RI, Frederickson DS. Estimation of the concentration of low lipoprotein cholesterol in plasma without use of preparative ultra centrifuge. Clin Chem 1972; 18: 499-502.

27. Sasaki T, Matsy S, Sonae A. Effect of acetic acid concentration on the colou reaction in the ortho toluidine boric acid method for blood glucose estimation. Rhinsho Kagak. 1972; 1: 346-353.

28. Lowry OH, Roseborough NJ Farr AL. Protein measurement with Folin'sphenol reagent. J Biol Chem. 1951; 193: 265-275.

29. Owen JA, Iggo JP, Scangrett FJ Steward JP. Determination of creatinine in plasma and

serum, a critical examination. J Biochem 1954; 58:426-437.

30. Marsh WH, Fingerhut B Miller H. Automated and manual direct methods for the determination of blood urea. Clin Chem 1976; 11:624.

31. Caraway WT. Uric acid: Standard Methods in Clinical Chemistry. In: Seligson D, 4th (ed.), New York, Academic Press. 1963; 239-247.

32. Culling FA. Handbook of Histopathological and Histochemical techniques, 1979. 3rd (ed.), Butter worth and Co. Publisher Ltd., New York.115-117.

33. Reitman S Frankel S. Estimation of Aspartate aminotransferase and Alanine aminotransferase. Am J Clin Pathol 1957; 28:56-63.

34. Stern A. Drug-induced oxidative denaturation in red blood cells. Semin Hematol.1957 ; 26:301–306,.18

35. Chakrabarti S, Naik. AA, Reddy GR. Phenylhydrazine mediated degradation of bovine serum albumin and membrane proteins of human erythrocytes. Biochim Biophys Acta 1990 ; 1028:89–94.

36. Itano HA, Hosokawa K, Hirota K. Induction of haemolytic anaemia by substituted phenylhydrazines. Br J Haematol. 1990; 32:99–104.

37. Ahlfors CE Shapiro SM. Auditory brainstem response and unbound bilirubin in jaundice (jj) Gunn rat pups. Biol Neonate 2001; 80:158–162.

38. Pratt DS, Kaplan MM. "Evaluation of a normal liver- enzyme results in asymptomatic patients," The New England Journal of Medicine, 2000; vol. 342, no. 17, pp. 1266-1271.

39. Sherlock S, Dooley J. Drugs and the liver. In: Disease of liver and Biliary system. 11 ed. Oxford: Blackwell Scientific publications. 2002; p. 322-356.

40. Suresh Kumar SV, Sujatha C, Syamala J, Nagasudha B Mishra S H. Protective effect of root extract of Operculina turpethum Linn against paracetamol induced hepato toxicity in rats. Indian Journal of Pharmaceutical Sciences 2006; Vol: 68, 32-35.

41. Sapakal V, Chadge, R. Adnaik R, Naikwade N Magdum C. Comparative hepato protective activity of Liv-52 and Livomyn against carbon tetrachloride -Induced hepatic injury in rats. Int J Green Pharm 2008; 43:79–82.

42. Raju S, Uma Maheshwara Rao V, Sreeramulu Reddy K, Ramya G Vasanth Kumar G. Effect of benzoin resin on the serum bilirubin levels in temporary jaundice induced by phenylhydrazine: a preliminary study, Asian *J Pharmaceut Res Health Care.* 2011;Vol.3 Issue 3. P 68-71.

43. Brodersen R. Binding of bilirubin to albumin. CRC Crit Rev Clin Lab Sci. 1980; 11:305–399.

44. Gunasekaran S, Uthra D. FTIR and UV-Visible Spectral Study on Normal and Jaundice Blood Samples. Asian Journal of Chemistry 2008; 20, 5695-5703.

45. Zeller H, Novak P Landgraf R. Blood glucose measurements by infrared spectroscopy Int J Artificial Internal Organ 1989; 12, 129.

46. Vishnu Priya V, Sankari G, Malika Janu, Sarapaneni KM, Aishwarya TS, Saraswathi P, Chandra Saol Gopan VS. Auto fluorescence and Fourier Transform – Infra red (FTIR) spectral investigation on diethyl nitrosamine (DEN) induced hepato cellular carcinoma, treated with pericarp extract of garcinia mangostanelin in rats. J Clinical and diagnostic research 2010; 4:3289-3297.

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