JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH

How to cite this article:
VISHNU PRIYA V, SANKARI G, MALLIKA JAINU, SURAPANENI K M, AISHWARYA T S, SARASWATHI P, CHANDRA SADA GOPAN V S. AUTO FLUORESCENCE AND FOURIER TRANSFORM – INFRA RED (FTIR) SPECTRAL INVESTIGATION ON DI ETHYL NITROSAMINE (DEN) INDUCED HEPATOCELLULAR CARCINOMA, TREATED WITH PERICARP EXTRACT OF *GARCINIA MANGOSTANA LINN IN* RATS.Journal of Clinical and Diagnostic Research [serial online] 2010 October [cited: 2010 October 15]; 4:3289-3297.

Available from
http://www.jcdr.in/article_fulltext.asp?issn=0973-709x&year=2010&volume=&issue=&page=&issn=0973-709x&id=988

ORIGINAL ARTICLE

Auto Fluorescence And Fourier Transform – Infra Red (FTIR) Spectral Investigation On Di Ethyl Nitrosamine (DEN) Induced Hepatocellular Carcinoma, Treated With Pericarp Extract Of *Garcinia Mangostana Linn In* Rats.

VISHNU PRIYA V*, SANKARI G**, MALLIKA JAINU***, SURAPANENI K M****, AISHWARYA T S*****, SARASWATHI P******, CHANDRA SADA GOPAN V S*******

ABSTRACT

The aim of the present study was to analyze the tumour activity of diethyl nitrosamine (DEN); a chemical carcinogen which induced hepatocellular carcinoma in the liver tissue samples of male albino rats. The analysis has been carried out in the following conditions; normal, tumour induced and tumour treated liver tissue samples. In all these cases, the action of the diethyl nitrosamine drug in inducing tumours in the liver has been investigated. *Garcinia mangostana* pericarp extract was the Ayurvedic drug which was chosen for the treatment of hepatocellular carcinoma. The control liver tissues, those in which tumour formation was induced by using diethyl nitrosamine and those in which the tumour was treated with the Garcinia mangostana pericarp extract after inducing tumor formation were analyzed by using auto fluorescence spectroscopy and fourier transform infra red (FTIR) spectroscopy. The qualitative spectral analysis was done by using auto fluorescence spectroscopy and the quantitative study was carried out by using FTIR spectroscopy. The tissue samples were experimented with auto fluorescence spectroscopy at the excitation wavelengths of 280nm, 325nm and 405nm, which exhibits emission due to tryptophan, collagen and porphyrin, respectively. It was observed that in all the tissue samples, the effect of diethylnitrosamine in inducing tumours was predominant. However, on treating the tumours with the pericarp extract of *Garcinia mangostana*, they responded positively. FTIR spectra have been recorded in the mid frequency region of 4000 – 450 cm^{-1} for all the liver tissue samples. The samples were analyzed quantitatively by using intensity ratio calculation among the selected absorption peaks to study the biochemical changes in the tissue samples. From this study, it was found that the total protein content was decreased in the liver tissues after inducing them with diethyl nitrosamine, thus causing the hepato cellular carcinoma. During the recovery phase, the decreased levels of the bio-chemical constituents were restored to near normal levels.

KEY WORDS: Auto fluorescence spectroscopy, Fourier Transform Infra Red (FTIR) spectroscopy, diethyl nitrosamine, hepatocellular carcinoma, pericarp extract, *Garcinia mangostana Linn.*

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INTRODUCTION

Optical techniques such as FTIR and fluorescence spectroscopy, Raman and light scattering can be used to distinguish between cancerous and non-cancerous tissues [1 – 4]. The underlying physical basis is that they are strongly influenced by the cellular structure and the chemical composition of the tissues. Due to its sensitivity to minute variations, fluorescence spectroscopy can provide quantitative biochemical information about the state of the tissues, which may not be obtained by using standard pathology. A number of fluorophores ranging from structural proteins to various enzymes and co-enzymes, are present in the human tissue and can be excited by ultra- violet and visible light. The fluorescence emission can differ significantly in normal and cancerous tissues due to the differences in the concentrations of the absorbers and the scatterers and also due to the size of the scatterers [5] – [7]. Infrared spectroscopy is a powerful method for the study of the molecular structure and the intermolecular interaction in biological tissues and cells. Feride et al [8] studied the effect of sreptozotocin (STZ) induced diabetes on rat liver and heart tissues by using FTIR spectroscopy. Chiriboga et al [9] studied the infrared spectra of normal and cancer liver tissues such as glycogen, DNA and RNA. Patrick et al [10] studied human colon tissues at the molecular level from the normal epithelium to the malignant tumour by pressure tuning FTIR spectroscopy.

Medicinal plants which are commonly included in the ayurvedic recipes for liver ailments have drawn much attention, as no reliable hepato - protective drug is available in modern medicine. Research investigations conducted on several natural plant products which are used for liver protection are well documented. *Garcinia mangostana* Linn. which is commonly known as "mangosteen", is a tropical evergreen tree and is an emerging category of novel which are sometimes called as "super fruits" and are presumed to have a combination of appealing subjective characteristics such as taste, fragrance and visual qualities, nutrient richness antioxidant strength [11] and a potential impact for lowering the risk of human diseases [12]. The pericarps of *G. mangostana* have been widely used as a traditional medicine for the treatment of diarrhoea, skin infections and chronic wounds in South East Asia for many years [13]. These are nature's most abundant sources of xanthones, which are natural chemical substances possessing numerous bio-active properties that help to maintain intestinal health, which neutralize free - radicals, which help and support joints and cartilage functions and promote immunomodulation systems [14]. These are extracted from the rind of mangosteen and contain 95% xanthones and also isoflavones, tannin and flavonoids [15]. In the present communication, the safe use of the *G. mangostana* pericarp extract has been shown by conducting an oral toxicity study in rats.

With this point of view, the present experimental work has been designed to study the effect of diethylnitrosoamine on the bio-chemical profile of the liver tissue of rat and also the protective effect of the pericarp extract of *Garcinia mangostana* against DEN induced hepatocellular carcinoma by using Fourier Transform Infra Red (FTIR) and auto fluorescence spectroscopy [16] – [19].

MATERIALS AND METHODS:

The study was conducted in the Department of Biochemistry, Saveetha Dental College, Saveetha Medical College and Hospital, Saveetha University and Department of

Biomedical Engineering, SSN Engineering College, Chennai, Tamil Nadu, India.

1. Animals

Adult Male Wistar Rats weighing 180 – 220 grams, who were maintained under asceptic conditions in the Department of Biomedical Engineering, SSN Engineering College, were used throughout the experiments and were fed with standard laboratory chow and water *ad libitum.* All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. Due clearance was obtained from the Institutional Animal Ethics Committee (IAEC) before the start of the study.

2. Drugs and chemicals

Diethyl nitrosamine (DEN) was purchased from Sigma Chemicals, St. Louis, MO and the *G. mangostana* pericarp extract powderobtained from Avasthagen Company, California, USA, as a compliment, was used in the present study. All other chemicals which were used were of analytical grade.

3. Animal treatment

The adult male Wistar rats were randomly divided into four groups: the Normal control group (Group A), the Diethylnitrosoamine (DEN) induced organ injury control group (Group B), the DEN induction with pericarp extract of the *Garcinia Mangostana Linn* treatment group (Group C) and six rats who were treated with the pericarp extract of the *Garcinia Mangostana Linn* alone to find out the effect of the drug on the liver if any (Group D). Each group consisted of 6 male Wistar rats (n = 6). The rats of groups B and C were administered orally with 0.01% diethyl nitrosamine which was dissolved in drinking water upto 16 weeks, which causes hepatocellular carcinoma. Simultaneously, the animals of group C were co - treated orally with *G. Mangostana* pericarp extract (400 mg / kg body weight) which was dissolved in drinking water as an intervention. The rats of control (group A) were on the standard laboratory diet alone and tap water *ad libitum.* The group D rats were administrated the *Garcinia mangostana* pericarp extract alone and tap water *ad libitum.* This was done to ensure whether the *Garcinia mangostana* pericarp extract by itself produced any side - effects in the liver. All the studies were conducted according to the guidelines which were described in the NIH Guide for the care and use of laboratory animals. The total weight of the diet was kept constant throughout the experimental period. After the scheduled treatment, the animals were sacrificed after obtaining complete ethical clearance from IAEC. The whole liver tissue was isolated immediately and was used for auto fluorescence and FTIR spectral studies. The development of hepatocellular carcinoma (HCC) in group B and the reversal of hepatocellular carcinoma to normal upon treatment with the pericarp extract of the G. mangostana in group C animals were confirmed by performing the histological studies of the liver tissues.

4. Sample Preparations for the Spectroscopic Study:

The whole liver tissue samples of each group of rats were isolated. The isolated whole liver tissue samples were then used for spectral analysis by the auto fluorescence and the FTIR spectroscopic methods. The tissue samples were put in separate plastic containers which were filled with clinical saline completely. Both the spectroscopic studies were conducted at the Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Madras (IITM). The samples were stored in a deep freezer at a temperature of -17°C till the spectral measurements were carried out.

5. Auto Fluorescence Spectral Measurement:

The fluorescence emission spectrum (FES) was recorded by using an ISA-Spex FluoroMax-2 spectro fluorometer. The main source that supplies UV radiation to this instrument is a xenon arc source. The main advantage of the FluoroMax-2 instrument is that it measures high sensitivity regardless of sample volume [20], [21]. The unique wavelength drive scans the

grating at speeds as high as 200nm/s. The grating grooves are blazed to provide maximum light in the UV and visible regions. The fluorescence emission spectra (FES) were recorded in the region of 400-700nm. The tissue samples to be recorded were cut into thin slices and were placed directly in the sample holder of the instrument.

6. FTIR spectral Measurement:

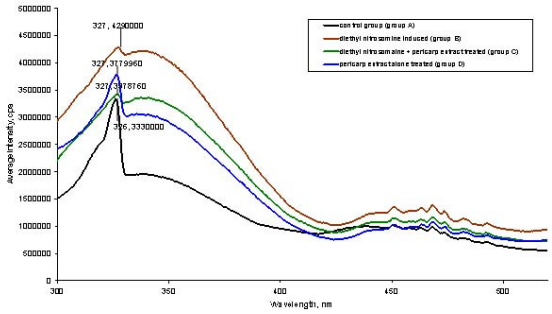
The whole liver tissue samples of each group of mice were isolated. The isolated whole liver tissue samples were lyophilized and were made into fine powder. The tissue powder samples and KBr (all in the solid dry state) were again lyophilized in order to remove most bound water, which could interfere with the prominent group frequencies. Five milligram of liver tissue sample was mixed with 100 mg of dried KBr and this was subjected to a pressure of 5x106 Pa and was made into a clear pellet of 13 mm diameter and 1mm thickness. Mid Infrared spectra in the region of 400 – 4000 cm^{-1} were recorded on a PERKIN – ELMER Spectrum One FTIR spectrophotometer which was equipped with a KBr beam splitter and an air - cooled DTGS (Deuterated Triglycine Sulfate) detector at SAIF, IIT Madras. The sampling window was scanned as the background and 32 scans were co -added with a spectral resolution of $1cm^{-1}$. The spectrometer was continuously purged with dry nitrogen. The absorption intensity of the peak was calculated by using the base line method.

RESULTS AND DISCUSSION:

1. Auto Fluorescence Spectral Analysis:

The liver tissue samples of each of the groups were first analyzed by auto fluorescence spectroscopic study. The fluorescence emission spectrum of the liver tissue samples were recorded at excitation wavelengths of 280nm, 325nm and 405nm, respectively. According to Beer - Lambert's law, the optical absorption is directly proportional to the concentration of the sample. From each spectra which was obtained, it was distinctly seen that there was a marked difference between the normal, the tumour induced and the tumour treated sample groups. The liver tissue samples were recorded individually for the four groups. Since the spectra obtained for the samples under a single group were alike, an average was calculated for their intensities and thus, an average emission spectrum was drawn for all the three groups separately.

[Table/Fig.1] shows the superimposition of the averaged fluorescence emission spectra (FES) of the liver tissue samples of groups A – D at 280nm excitation wavelength, which shows a prominent fluorescence emission peak at a wavelength of 327nm.



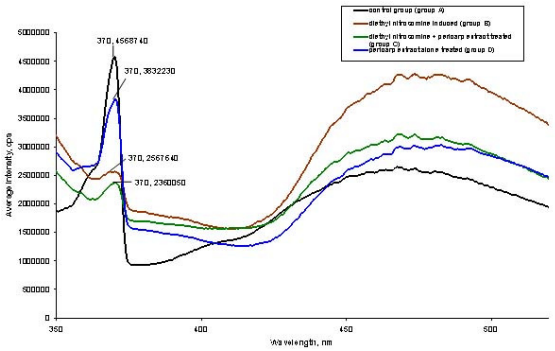
[Table/Fig.1]: Average Fluorescence spectra of four different liver sample groups - at Excitation wave length 280nm.

From the spectra, it was observed that the spectral characteristics of the control group (group A) tissue samples differed significantly from those of the groups B,C and D. Upon excitation at 280nm, the observed emission peak at 327nm was due to the fluorescence of the amino acid, tryptophan, as shown in [Table/Fig.1]. It is clearly inferred that the concentration and absorption of the amino acid goes very high, than that observed in normal healthy tissue, when diethyl nitrosamine drug is induced. This is indicated by the proliferation in the intensity corresponding to the tryptophan emission peak at 327nm. This confirms the denaturation of tryptophan due to the influence of the carcinogenic nature of diethyl nitrosamine, thus causing tumours in the tissue. When the *Garcinia mangostana* pericarp extract was administered after inducing diethyl nitrosamine to the tissue (group C), it was seen that there was a controlled revival of tryptophan, which

was indicated by the intensity value which was obtained, corresponding to 327nm, nearing the intensity which was obtained for the control group. Similarly, the spectrum corresponding to the *Garcinia mangostana pericarp extract* alone treated liver tissue samples (group D) indicated that there were no side - effects due to the extract. This was indicated from the intensity at 327nm for group D samples, which were almost closer to the intensity at 327nm for the control group samples.

In order to find more about the behaviour of other proteins, the fluorescence emission spectra of the samples were studied at 325nm excitation, which occurs due to the fluorescence of the fibrous protein, collagen. [Table/Fig.2] shows the superimposition of the averaged fluorescence emission spectra (FES) of all the four groups at 325nm excitation wavelength, which shows a prominent fluorescence peak at 370nm wavelength.

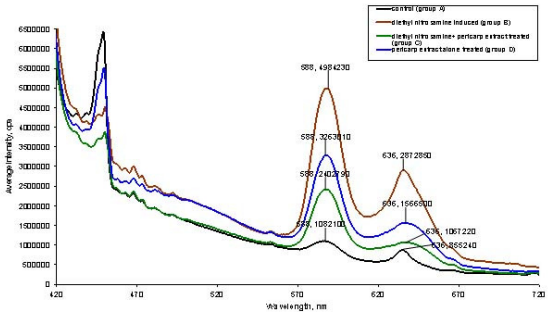
The well defined emission peak at 370nm indicates the concentration and absorption of the fibrous protein, collagen in the tissue. It was observed that there was deterioration of the collagen content, as diethyl nitrosamine was induced to the tissue, which was marked by a decrease in the fluorescence intensity when it was compared with the intensity which was obtained for the control group tissue samples. This confirms the toxic activity of diethyl nitrosamine more effectively.



[Table/Fig. 2]: Average Fluorescence spectra of four different liver sample groups – at Excitation wave length 325nm.

However, it was seen that when the *Garcinia mangostana* pericarp extract was administered to the diethyl nitrosamine induced tissue (group C), there was no regeneration of the collagen protein in the tissue. This was observed from the intensity values corresponding to the emission peak at 370 nm for group B and group C liver tissue samples, as obtained from the spectrum. However, it was also observed that the spectrum corresponding to the *Garcinia mangostana pericarp extract* alone treated liver tissue samples (group D) indicated that there were no side-effects due to the extract. This was indicated from the intensity at 370 nm for group D samples, which was almost closer to the intensity at 370 nm for the control group samples. Thus, the *Garcinia mangostana* pericarp extract was observed to be not much significant in the regeneration of the fibrous protein in the liver tissue.

In order to find about the behaviour of the heme content in the tissue, fluorescence emission spectra of the liver tissue samples were studied at 405nm excitation, which occurs due to the fluorescence of the porphyrin content of the samples. [Table/Fig.3] shows the superimposition of the averaged fluorescence emission spectra (FES) of the tissues of groups A to D at the 405nm excitation wavelength, which shows two prominent fluorescence peaks at 588nm and 636nm wavelength respectively.



[Table/Fig. 3]: Average Fluorescence spectra of four different liver sample groups-405nm.

From [Table/Fig.3], the well defined emission peaks at 588nm and 636nm indicate the presence of the heme group of the porphyrin in the tissue samples. It was observed that when diethyl nitrosamine carcinogen is induced to the liver tissue samples (group B), there was a sudden increase in the concentration of porphyrin,

which was seen from increase in the intensity corresponding to the band peaks at 588nm and 636nm respectively, when compared with that of the control group (group A). It was observed that when the *Garcinia mangostana* pericarp extract was administered as a treatment drug to the diethyl nitrosamine induced tissue samples (group C), the abnormal rise in the intensity which occurred at both the emission peaks disappeared and it approached the actual value of the concentration of the amino acid to a great precision, which is shown in [Table/Fig.3]. The spectrum peak corresponding to the *Garcinia mangostana* pericarp extract alone treated liver tissue samples (group D) indicates that there were no side - effects due to the extract. The sudden rise in the intensity peak may be attributed to the carcinogenic activity of diethyl nitrosamine, which leads to the excessive cell proliferation of the tumour cells. When the *Garcinia mangostana* pericarp extract was administered, the abnormal cell proliferation was curtailed and there was a controlled reformation of the porphyrin compound.

2. FTIR Spectroscopic Analysis:

The FTIR spectra of the normal liver tissues (group A), the diethyl nitrosamine induced liver tissues (group B), the diethyl nitrosamine followed by *Garcinia mangostana* pericarp extract treated liver tissues (group C) and *the Garcinia mangostana* pericarp extract alone treated tissue samples (group D) are shown in Fig.4. The relative intensities (log Io / I) and tentative assignments of the fundamental Infrared absorption frequencies are shown in [Table/Fig 4].

Frequency cm^{-1}	Control group	DEN induced	DEN induced treated by *G. mangostana*	*G. mangostana* treated alone	Vibrational Band Assignments
2923	0.15412	0.37530	0.18100	0.32127	CH_2 asymmetric stretching due to lipid and protein
2853	0.31856	0.56593	0.33033	0.48957	CH_2 symmetric stretching mainly due to lipids and proteins
1548	0.29612	0.42271	0.30157	0.37870	C–N stretching/ N–H bending; Amide II band
1461	0.55120	0.69656	0.63496	0.72217	CH_2 asymmetric bending due to protein
1402	0.64486	0.79386	0.66109	0.77430	CH_2 symmetric bending due to protein
1240	0.73280	0.88284	0.75316	0.89480	PO_2^- asymmetric stretching Amide III band
1063	0.81731	-	0.90315	0.84501	PO_2^- symmetric stretching (glycogen)

[Table/Fig 4]: Infrared absorption frequencies (cm^{-1}), relative intensities (log I_o / I) and tentative assignments of fundamental frequencies of liver tissue samples.

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

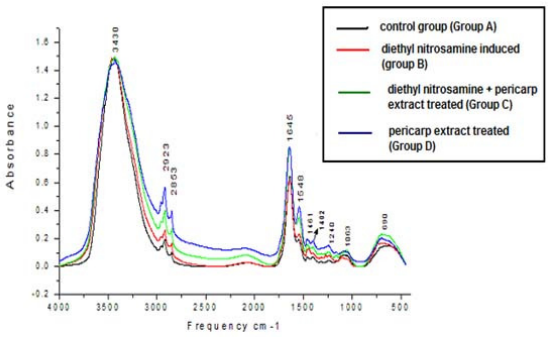
The FTIR spectrum of a sample exhibits characteristic absorption frequencies due to the specific functional groups which are present in the sample. FTIR spectroscopy offers the capability of identifying biochemical substances because of the highly distinctive features of the characteristic molecular vibrations which are rendered in the spectra. A vast amount of substances exist within the cells and most of those substances contribute specifically to the vibrational spectra. Changes in the composition of the cells' biochemistry should therefore be detectable by FTIR spectroscopic investigation. Although it is most likely that the variety of all those changes appear ambiguously in the spectra, multivariate data evaluation tools should provide access to the diagnostic features which are hidden among the abundance of spectroscopic information [22] – [27].

It is known that tissue proteins, carbohydrates and lipids play a major role as energy providers for animals who are exposed to stress conditions. Shaw et al [23] 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 the enzymes and specificity or by
iii altering the permeability properties of body membranes.

The infrared spectra of the proteins were characterized by a set of absorption regions which are known as the amide and the C-H regions. 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 primarily from the N-H bending and C-N stretching vibrations.

The amide absorptions are considered to be sensitive to protein conformation; hence, an increase or a decrease in the ratio of the intensities of the bands at ~1548 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 bands observed at ~1461 cm^{-1} and ~1396 cm^{-1} are mainly due to asymmetric and symmetric CH_3 bending modes respectively of the methyl groups of the proteins. The medium intensity band observed at ~1235 cm^{-1} is due to the $PO2^-$ asymmetric stretching modes of the phospho di ester indication of phospholipids and the amide III / CH_2 wagging vibration from the glycine backbone and protein side chain. The band at 1065cm^{-1} has been assigned to the PO^{2-} symmetric stretching symmetry phosphates; the stretching of glycogen also makes a contribution to the intensity of this band.



[Table/Fig 5]: FTIR Spectra of liver tissues of rats, (a) control, (b) diethyl nitrosamine induced, (c) diethyl nitrosamine followed by *Garcinia mangostana* pericarp extract, (d) *Garcinia mangostana* pericarp extract alone

The relative intensities (log Io / I) and tentative assignments of the fundamental Infrared absorption frequencies are shown in [Table/Fig 4]. The liver synthesizes a great amount of protein and glycogen, which is needed ostensibly for the repair of damaged cell organelles and tissue regeneration. Stressful situations mainly disturb the rate of carbohydrate metabolism through the levels of glycogen and protein profiles in toxicant exposed animals. Glycogen, a reserve energy source is decreased during the induction of diethyl nitrosamine, which is seen in [Table/Fig 4]. A fall in the glycogen profile in the liver tissue indicates the possibility of glycogenolysis. The depletion of the protein profile has also been observed in the liver tissue of rats, when treated with diethyl nitrosamine.

It was observed in this study, that the liver tissues of the rats showed a remarkable recovery from the tumour effect of diethyl nitrosamine. When the rats were exposed to diethyl nitrosamine and *Garcinia mangostana* pericarp extract treatment, they showed a restoration in the levels of biochemical constituent profiles in the liver tissues. The recovery could be attributed to the restoration of the regulatory function of the proteins and glycogen by elimination of the tumour causing toxicants.

CONCLUSION:

In the present study consisting of the spectral analyses, the following facts have been deduced; when diethyl nitrosamine was induced into the liver tissue, the carcinogenic activity of diethyl nitrosamine led to the denaturation of tryptophan, collagen and fluorescence completely, which signifies cellular, metabolic and pathological disorders. When *Garcinia mangostana* pericarp extract was administered to the tumour tissues, there was a regeneration of the normal cells in the tissues, thus enabling proper fluorescence, as shown in the fluorescence emission spectral graphs.

The decreased band areas of symmetric and asymmetric CH_2 stretching modes which are observed in the diethyl nitrosamine induced tissues, suggests the decreased composition of the lipid chains in the liver tissues of the rats. The decrease in the intensities of the amide bands in the tissues indicate a decrease in the protein quantity of the system and the destructive effect of the diethyl nitrosamine in the liver tissues. The decrease in the intensities of the symmetric and asymmetric stretching modes of the phosphodiester groups suggest a decrease in the relative content of the nucleic acids in the liver tissues, thus indicating the carcinogenic nature of diethyl nitrosamine. It was noted that when the *Garcinia mangostana* pericarp extract was administered to the tumour tissues, there was a revival in the amide and glycogen contents prominently, thus reversing the tumour activity in the liver tissues.

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