

# Laser Spectroscopic Diagnosis of Photodynamic Therapy in Rats

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**ABSTRACT:** The purpose of this study is to diagnose the photodynamic therapy (PDT) by using different optical techniques for characterization of tissue. The feasibility of many of these tools has been demonstrated in animal experiment (*in vivo*). PDT was used as a treatment of breast cancer. The model of breast cancer was induced by Ehrlich ascites administered in the thigh of the mice to bear solid tumor. These tissues were injected by the photosensitizer (TMP) and irradiated with diode laser at wavelength 650nm (irradiance 398 mW/cm<sup>2</sup>). We evaluate the normal group, abnormal and treated tissues by using fluorescence spectroscopy, and the vibration transition changes of each group by using FTIR and Raman spectroscopy.

The fluorescence spectra of coenzymes flavins, amino acids tryptophan and lipid porphyrin were discussed by using suitable line excitation wavelength 450 nm, 405 nm and 280 nm respectively. The fluorescence emission spectra (FES) were recorded. The tumour control shows a prominent peak at 605.5nm, while in treated and normal at 606 nm and 601 nm, respectively, which due to porphyrin. The emission peaks of flavin of the three groups were identical; appear at 513nm, the normal control and the treated are much more intense than the tumor. In case of tryptophan spectra gives height intense in tumor was positioned in 330.5 nm. The normal peaks appear at 330.5 nm, it even lower than the treated which poisoned in 331nm.

The result of fluorescence studies confirmed that the PDT treatment is an effective method of breast cancer (falvin & porphyrin gives 100% and tryptophan 75%).

The results of Raman and FTIR are also analyst and discussed.

**KEYWORDS:** photodynamic therapy (PDT), fluorescence spectroscopy, Raman spectroscopy, FTIR, laser diode, TMP photosensitizer.

## I. INTRODUCTION

Optical spectroscopy recently has been a wide used as diagnostic techniques to provide information about molecular composition of the structure of tissue [1,2].

Photodynamic therapy (PDT) is an interesting treatment modality for cancerous and precancerous lesions in addition to a number of other diseases. Disease leads to change in the molecular composition of tissue and one may assume that such changes occur simultaneously or even preceded modulation in cell morphology and tissue structure. PDT is a local treatment modality based on a combination of a selectively localized light-sensitive drug, (known as a photosensitiser) and light of an appropriate wavelength. The light-activated form of the drug reacts with molecular oxygen to produce reactive oxygen species (ROS) and free radicals [3], that are cytotoxic to cells of target tissue [4].

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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Vol. 6, Issue 5, May 2017

*Fluorescence Spectroscopy* is an emerging technology with potential applications in experimental stages for clinical diagnosis and discrimination of cancer, normal and treated tissues. Many studies showed evidences of different fluorescence properties of normal and many types of cancer, which are detectable by means of fluorescence spectroscopy. When excited at suitable wavelength, several bimolecular species gives rise to a fluorescence emission over a wide range in the UV-visible region. Cancers increase the rate of metabolism and give rise changes in endogenous fluorophores. The difference arises due to the active nature of the endogenous fluorophores including tryptophan, flavin and porphyrin. The fluorescence of the endogenous molecules plays an important role in analyzing the tumor [5,6,7]. Wenchong et al[8] presented porphyrin is a tumor maker in the cancer blood. Sankari et al[5] observed the relationship between porphyrin, tryptophan and cancer cell proliferation in a liver tumor, and the abnormal cell proliferation reduction when treated by administered drug the porphyrin and tryptophan are controlled and reformation. Malsilamani et al[9] and Karthikeyan et al[10] discussed the relationship between porphyrin and proliferation of cancer cell in an animal tumor.

*Raman spectroscopy* is a unique molecular vibration technique that has found its remarkable way in routine clinical oncology application because of its excellent capability of non destructively probing, the changes of molecular composition in biochemical vibration markers associated with tumor processing[11] Proteins and lipids, and carbohydrate –amid I were found in the bands 1440-1460  $\text{cm}^{-1}$  and 1650 – 1680  $\text{cm}^{-1}$  respectively, of human breast cancer tissue and non-diseased tissue, present in literature<sup>1</sup>. Garcia et al,[2] has shown the bands of Raman in breast cancer and normal tissue at 2854 $\text{cm}^{-1}$  and 2895  $\text{cm}^{-1}$  ( $\text{CH}_2$  stretching of lipids), 2937  $\text{cm}^{-1}$  ( $\text{CH}_3$  stretching of proteins) and 3010  $\text{cm}^{-1}$  related to CH stretch lipids.

*Fourier transform infrared spectroscopy (FTIR)* is an optical technique that provides highly specific spectroscopic fingerprints of the molecular composition of tissue. FTIR can also provide unique information on molecular structure, mainly for determining the structure of proteins, mutation of nucleic acids and peroxidation of phospholipid [12].

## II. MATERIALS AND METHODS

a- *Ehrlich ascites carcinoma cell line*:- A line of Ehrlich Ascites carcinoma provided from National Institute (NCI) in Egypt. Present line was supplied through the courtesy of Prof. G. Kline, Amsterdam, Holand. The tumor was maintained in female Swiss albino Mice interperitoneal transplantation of cell. This cell line ( $5 \times 10^6$ ) was used to induct the solid tumor in the thigh. The tumors were taken 15 days to grow.

b- *Animals*:- 45 female Swiss albino mice (25-30 g body weight) were divided into three groups, normal control (group1), tumor control (group2) and treated group injected by TMP photosensitizer 0.5 mg/kg and radiated with diode laser 650 nm at irradiance 398  $\text{mW}/\text{cm}^2$  (group 3)

c- *Photodynamic therapy protocol*:-

1- *Drug Administration*:- the tumor bearing rats (group3) were injected intra-tumor with the drug dose of TMP 0.5mg/kg, which purchased from Frontier Scientific (Logan, USA) ( the needle was inserted laterally at the tumor midline and positioned approximately at the center of the tumor)[13].

*Light treatment and equipments* :- Two hours after the drug administration , the animals were anaesthetized and irradiated on the thigh, with the visible light GaAlAs diode laser (photon lase III, DMC, Sao Carlos, Brazil) at wavelength 650 nm, power 50 mW (irradiance 398  $\text{mW}/\text{cm}^2$ ), the laser exposure time was 10 minute.

The animals were maintained in a dark room after injected by (TMP), which are sacrificed after 24 hours,<sup>(11)</sup> samples were collected and analyzed, the samples have been put in plastic containers.

The fluorescence emission spectra (FES) have been analysed using FP-6300 spectrofluorometer, the samples that have been collected were equipped in flat quartz cell and excited with three different wavelength 450 nm and 405 nm 270 nm to evaluate the flavin, porphyrine and tyrosine respectively. The spectra were collected and analysed.

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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A compact dispersive Raman microscope (Bruker-Senterra II) was used in this study. The laser excitation line used was 785nm of AlGaAs laser.

FT/IR 4100 (Jasco) was used to measure the absorption spectra of samples. The samples were prepared, whereas sliced to 50  $\mu\text{m}$  by cryostat and put in glass slit and inserted in FTIR device.

During the period of spectral analysis samples have been stored in deep freezer at temperature  $-20^{\circ}\text{C}$  [14].

### III. RESULTS AND DISCUSSION

**3-1- Fluorescence spectroscopy:-** This work present, the auto fluorescence spectra of the normal, malignant and treated tissue, which measured at the emission wavelength from 300-700 nm, for lipid pophyrin, amino acids tryptophan and coenzyme flavin. According to the Beer Lambert's law, the optical absorption is proportional to the concentration of the sample.

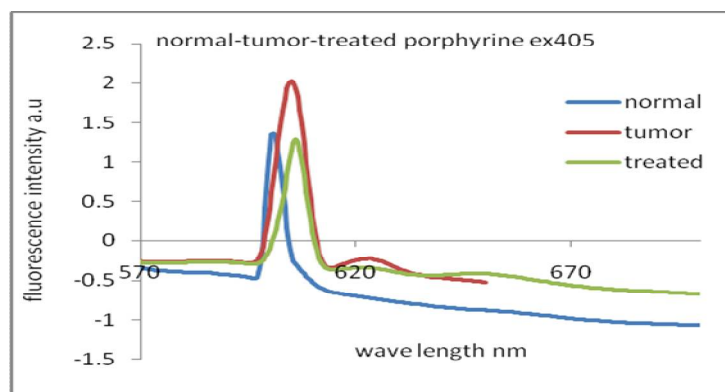


Figure (1) shows the different intensities Of fluorescence spectra of porphyrine for normal, malignant and treated tissues corresponding to the excitation wavelength 405 nm, differences were observed in emission spectra for three tissue samples, the positions 601 & 0, 605&623, 606&621 nm emissions are due to normal, tumor and treated tissues respectively. The intensity of the tumor tissue is higher than the normal and the treated tissues.

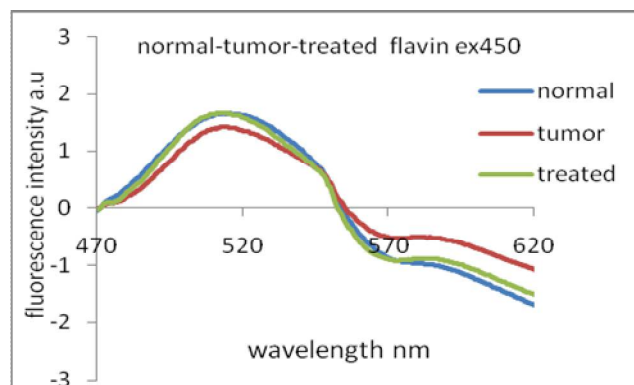


Figure (2) presents fluorescence spectra of three sampls, when we used excitation 450 nm/513nm emissions, corresponding to Flavin. The normal and the treated tissue gave identical spectra, however the tumor spectrum showed the lowest intensity.

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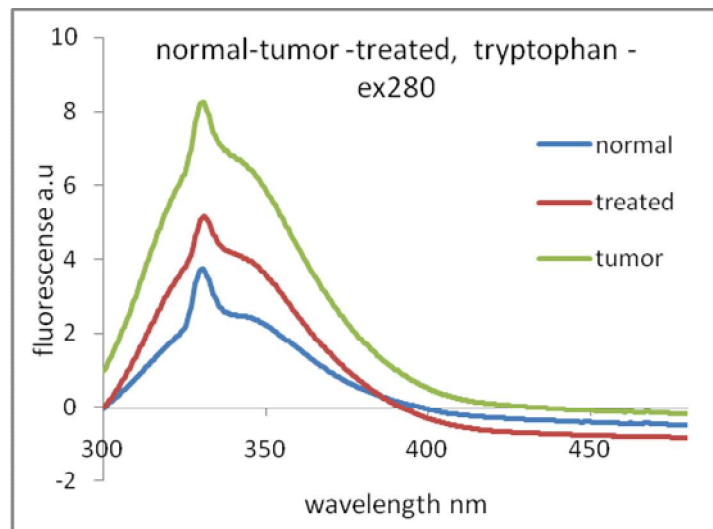


Figure (3) shows spectra of FES of the three groups at 280 nm excitation wavelength corresponding to tryptophan fluorescence. The emission of the three samples normal, malignant and treated tissues present the prominent intensity around 330.5 nm. The tumor peak was higher than the normal and treated. This matched with the results of (Sankari et al [5], Surapaneni et al[15]).

Table (1):- Presents fluorescence intensity at (arbitrary unit) of the three fluorophores in the normal, tumor and treated tissues.

Fluorophores	Normal	Intensity a.u	Tumor	Intensity a.u	Treated	Intensity a.u
Porphyrin	601	1.364	605.5	1.996	606	1.289
	-----	-----	623	-0.229	621	-0.340
Flavin	513.5	1.668	513.5	1.416	513.5	1.668
Tryptophan	330.5	3.7358	330.5	8.24467	331	5.14524

Ratios of the fluorophore between two intensities in each two samples. Which present the accumulation of each fluorophrs in the samples. Ratios of the fluorophore between two intensities in each two samples. Which present the accumulation of the fluorophrs in the normal, tumor, treated tissues.

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Fluorophors	I Tumor/I Normal	I Tumor/ I Treated	I Normal/I Treated
Porphyrin	146 %	155%	105%
Flavin	85%	85%	100%
Tryptophan	221%	160%	73%

We extract from the table (2) a comparison between the intensity of normal and the intensity of treated tissues for porphyrin, tryptophan and flavin, revealed reduction of porphyrin in treated tissue, which gives hundred percentages, in tryptophan reach 75%, while in flavin increased reaching hundred percentages.

In breast cancer, the ratios of the fluorescence intensities at the emission peaks, 340, 440 and 460 nm that attributed to tryptophan, had high ratios in a high histological grade, while in low grade tumor had low ratios [16].

Accumulation of porphyrin in tumor attributed to a porphyrin is an ingredient of heme derived from intermediate porphyrinogens in the biosynthesis pathway which are from the proto-heme. Porphyrine is a small amount in all living cells, the synthesis of protoporphyrin is under feedback control; that is, cell produce it at a rate just sufficient to match their heme levels, but with excessive cellular proliferation the feedback mechanism loses control, and the excess porphyrin thus produced appear in the tissue and blood [17]. Also Protoporphyrin (PpIX) is a porphyrin derivative that combines with ferrous iron to form the heme of hemoglobin and with ferric or ferrous iron to form the prosthetic groups of substances such as myoglobin, catalase, and the cytochromes. Abnormal metabolism was observed in blood, plasma, serum, and tissue cancerous, which indicated that cancer cells accumulate more PpIX than normal cells and tissues accumulate [18,19].

In flavin, the reasons for the decreased autofluorescence seem to be a decrease in the amount of oxidized forms of flavins in malignant tissue. Another reason for decrease in tumor autofluorescence might be a change in the relative nicotinamide adenine dinucleotide (NADH) and the weakly fluorescent oxidized form of NAD<sup>+</sup> in malignant tumor [13].

In conclusion the differentiation between three types of tissues was done by comparison of their intensity ratios between each two of them. The results showed that the PDT is much more effective.

**3-2-FTIR spectroscopy:** -The differences between normal, malignant and treated tissues were dependent on the numerical changes of several band intensities and frequencies. As seen in figures (4) and (5) the spectra are quite complex and contained several bands arising from the contribution of different functional groups belonging to proteins, lipids and other [20].

The most widely used modes in infrared spectra of the protein structure are amide region and the C-H region. The amide regions 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 contribution from C-N stretching vibration. The amide III absorption is normally weak and arises primarily from N-H bending and C-N stretching vibrations. [5] the region of amide I (1600-1700) cm<sup>-1</sup> we have two absorption bands 1671 cm<sup>-1</sup> and 1616 cm<sup>-1</sup> related to  $\beta$  turn and  $\beta$  sheet in normal tissue, bands 1679 cm<sup>-1</sup> and 1612 cm<sup>-1</sup> appear in tumor tissue, and in treated tissue appear at 1640 cm<sup>-1</sup> & 1669 cm<sup>-1</sup>. These bands of tumor are much higher intense than in normal and treated tissue [18].

The range 1300-2000 cm<sup>-1</sup> in figure (4), which consists the lipid band, the ester C=O stretching vibration at 1750-1720 cm<sup>-1</sup>, is sensitive for the structural changes in the biological membranes. The broad band consists two components around 1742 and 1727 cm<sup>-1</sup>. First, these components were thought to reflect the sn-1 and sn-2 ester bonds of the glycerol backbone [20]. The infrared band of fatty acyl ester at the sn-2 position in the microsomal membranes shifted to the red wave number position (1731, 1730 and 1732 cm<sup>-1</sup>) in the normal, malignant and treated tissue respectively, the normal and treated tissue have high intensity than that in malignant [18,19]. Strongly associated with lipid implying that any shift in the frequency of this band can be directly correlated with an alteration in the state of intermolecular hydrogen bonding of the interfacial region of the phospholipids structure with water molecules and/or some functional groups of other molecules. [20] The other reason that the fast growth of cancer needs more nutrition and energy, it might utilize lipid to supply nutrition and energy, the lipid substance not to be accumulated in cancer cell and tissue [21].

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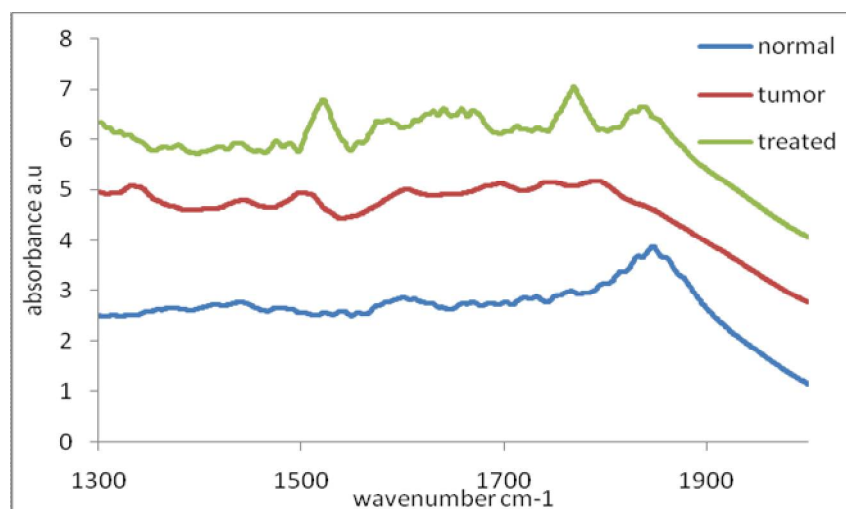
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In Figure (5) the lipid spectra contain a large proportion of methyl, methylene and carbonyl bands in the region 2600-3700  $\text{cm}^{-1}$  [22]. The absorbance band at 2922 & 2930  $\text{cm}^{-1}$ , and 2851 & 2852  $\text{cm}^{-1}$  which correspond respectively to the antisymmetric and symmetric stretching vibrations of methylene ( $-\text{CH}_2$ ) groups, the membrane lipid which is directly related to the lipid acyl, primarily saturated, chains. The band at 2956 & 2958 & 2958.5  $\text{cm}^{-1}$  is associated to asymmetric  $\text{CH}_3$  stretching mode of the methyl group, and less contribution of proteins [21,22,23]. In malignant tissue the intensity of methyl was decreased while increased in methylene [20]. The bands at 2965  $\text{cm}^{-1}$  and 2850  $\text{cm}^{-1}$  were related mainly to the stretch of proteins.

The changes in frequencies, intensities and band shapes of these bands may provide further information about the structural changes associated with malignancy. In our result the malignant tissue of the symmetric  $\text{CH}_2$ , 2927  $\text{cm}^{-1}$  band decreased when compared to the corresponding bands in normal tissue [2].

Daicovicu et al., [24] shows that an animal's treated by 5- ALA PDT the levels of lipid peroxides and protein carbonyls were increased both in plasma and tumor, and Saczko et al., [25] present in their literature the level of lipids peroxidation was higher for cells after PDT, comparing to control cell <sup>(25)</sup>. According to our results we observe the FTIR spectra, in treated tissue bands of the protein content have lower peaks in comparison to malignant tissues but still higher than the normal. And the lipid bands are the highest in treated tissue when compared with normal and malignant. Subsequently, the treatment of malignant tissue by PDT is successful.



Figurem (4) shows the FTIR spectra in the range 1300-2000  $\text{cm}^{-1}$  for normal, malignant and treated tissue. which consist the lipid band, the ester  $\text{C}=\text{O}$  stretching vibration at 1750-1720  $\text{cm}^{-1}$  [20]. The infrared band of fatty acyl ester at the sn-2 position in the microsomal membranes shifted to the red wave number position (1731, 1730 and 1732  $\text{cm}^{-1}$ ) in the normal, malignant and treated tissue respectively

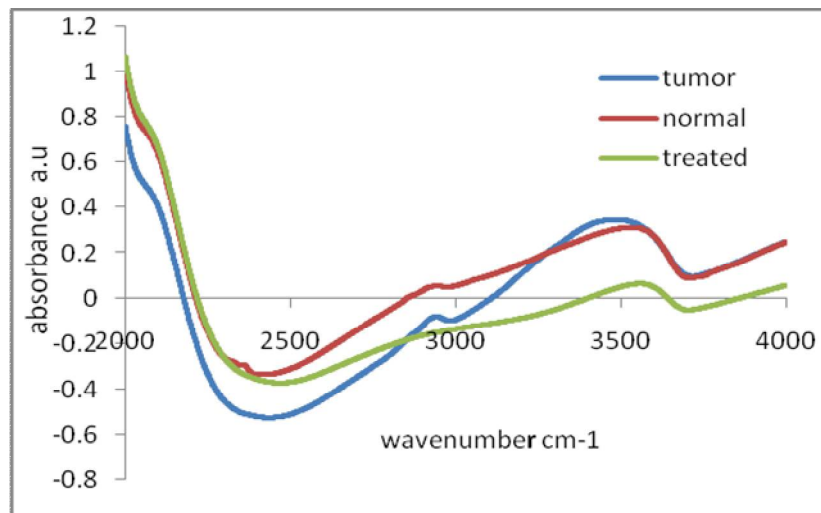


Figure [5]:-FTIR spectra ( $2000-4000\text{ cm}^{-1}$ ) for comparison between control normal tissue, malignant tissue and treated tissue in the thigh of mice, the bands of FTIR spectra in this region consist lipids and proteins

**3-3 Raman spectroscopy:-** Figure (6) shows the Raman spectra of normal, malignant and treated tissue. Table (3) presents all peaks and their assignments. The main spectral differences of typical Raman microscope at the interval  $500$  to  $2000\text{ cm}^{-1}$  which may differentiate diagnostically the sample.

The prominent vibrational bands spectra in treated tissues  $512, 592, 669, 802, 859, 969, 1005, 1306, 1444, 1546, 1608, 1665$  and  $1738\text{ cm}^{-1}$ . The spectra of neoplastic tissue showed band at  $517, 617.5, 694, 801, 858.7, 970, 1003, 1058, 1038, 1307, 1454, 1559, 1615, 1655,$  and  $1737\text{ cm}^{-1}$  and in the non neoplastic tissue displayed the Raman spectra at  $530, 604, 696, 806, 864, 1006, 1047, 1071, 1301, 1443, 1554, 1603.5, 1657$  and  $1746\text{ cm}^{-1}$ .

The intensity was found to be larger in treated tissue than in normal, and was found to be even lower in neoplastic tissue in the bands  $512, 697, 859,$  and  $1608\text{ cm}^{-1}$ , almost these bands attributed to proteins. However, the lipids bands  $1071, 1301, 1443$  and  $1657\text{ cm}^{-1}$  in the normal is greater than the tumor and was found to be even lower in treated tissue. Even though the bands of tumor tissue  $806, 1554, 1006\text{ cm}^{-1}$  are at high intensity when compared to the treated and normal tissue [2,26,27,28,29,30].

In figure (7) the region  $2000-3700\text{ cm}^{-1}$  included normal, tumor and treated tissue, and Presented four bands spectra  $2101.5, 2103, 2107\& 2225, 2220, 2226\& 2851.5, 2847, 2844$  and  $2937, 2969, 2927\text{ cm}^{-1}$  respectively. In normal tissue the two bands of lipids  $2225\text{ cm}^{-1}$  and  $2851.5\text{ cm}^{-1}$  are higher than the tumor and treated tissue. Prominent Raman bands in the region  $2500-3700\text{ cm}^{-1}$  at  $\sim 2851.5\text{ cm}^{-1}$  ( $\text{CH}_2$  stretching of lipids) and  $2937\text{ cm}^{-1}$  ( $\text{CH}_3$  stretching of proteins), and abroad band of water in the region of  $3010-3550\text{ cm}^{-1}$  were found to be with small variation in intensities. The variation of intensity in the two bands of  $2851.5\text{ cm}^{-1}$  and  $2937\text{ cm}^{-1}$  can be lead to a comparison between normal and abnormal tissue spectra based on their vibrational spectra of lipids and proteins [27].

In Raman spectroscopy: (i) After treatment of malignant tissues lipids contents are increased while proteins decreased. (ii) Proteins peaks decreased after treatment of malignant tissues, but it was still higher than in normal tissues.

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Table (3):- General band assignments of the Raman spectra of normal , malignant and treated tissues based on literatures.

Normal cm <sup>-1</sup>	Raman Intensity	Tumor cm <sup>-1</sup>	Raman Intensity	Treated cm <sup>-1</sup>	Raman Intensity	Assignment &References [26], [27], [30], [28] ,[29], [2] ,[31]
530	12.7565	517	10.3182	512	34.8743	<b>510-525</b> <sup>[23]</sup> Pyrimidine ring deformation
604	9.9565	617	12.4168	592	30.8463	<del>617-600</del> <sup>[23]</sup> C=O bending
696	17.8482	694	8.9585	697	37.00	<b>700</b> <sup>[20]</sup> Cholesterol
806	11.1260	801	55.2257	802	53.6201	<b>805</b> <sup>[23]</sup> P-O stretching
864	37.5726	858.7	35.1782	859	38.4550	<b>854</b> <sup>[18]</sup> , C-C stretching protein
969	10.3353	970	27.8096	969	27.9370	<b>972</b> <sup>[30]</sup> PO <sub>3</sub> <sup>2-</sup> stretching
1006	10.5311	1003	38.4616	1005	31.0047	<b>1005</b> <sup>[28]</sup> C-C ring breathing of phenylalanine
1047	8.7493	1038	10.0745	-----	-----	<b>1047</b> <sup>[30]</sup> C-O-P stretching
1071	42.4788	1058	24.4812	-----	-----	<b>1055-1070</b> <sup>[30]</sup> CO-O-C symmetric stretching
1301	36.8430	1307	14.4761	1306	10.1718	<b>1302</b> <sup>[28]</sup> - <b>1299</b> <sup>[27]</sup> CH <sub>2</sub> twisting and bending
1443	67.4281	1454	8.3125	1444	14.3733	<b>1445</b> <sup>[26]</sup> CH <sub>2</sub> bending mode of protein and lipids
1554	3.9999	1559	20.6665	1546	14.9258	<b>1550</b> <sup>[30]</sup> Amide II
1603.5	2.2093	1615	9.2741	1608	10.4162	<b>1600</b> <sup>[30]</sup> Tyrosine ring vibration
1657	58.1148	1655	27.8857	1665	18.40281	<b>1660-1655</b> <sup>[29]</sup> C=O amide I,



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1746	15.8450	1737	14.6609	1738	8.1579	<b>1745<sup>[21]</sup>-1730<sup>[30]</sup></b> C=O stretching
2851.5	17.9544	2847	3.1724	2844	2.3829	<b>2840-2875<sup>[27]</sup></b> CH <sub>3</sub> symmetric stretch of lipids
2937	3.2552	2969	2.8011	2927	3.8631	<b>2937<sup>[27]</sup>-2968<sup>[31]</sup></b> CH <sub>3</sub> asymmetric stretching protein

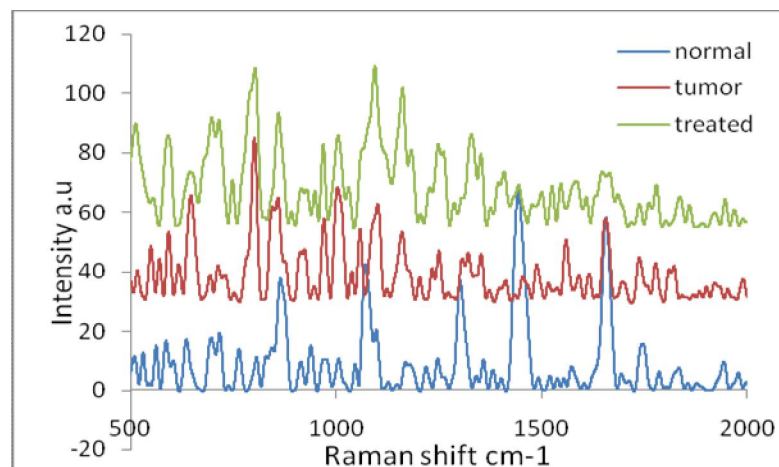


Figure [6]:- shows the Raman spectra of normal tissue at the thigh of mice, malignant tissue (breast cancer model) and treated tissue which treated by PDT method in the region (500-2000 cm<sup>-1</sup>).

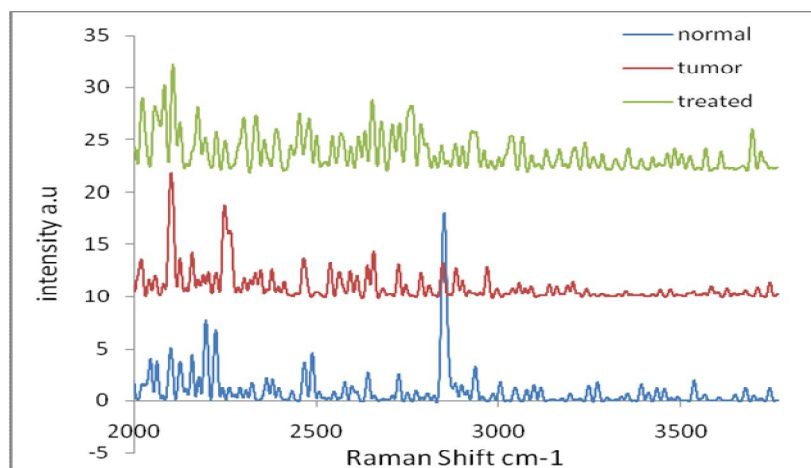


Figure [7]:-shows the Raman spectra of normal tissue of mice, malignant tissue (breast cancer model) and treated tissue, which treated by PDT in the region (2000-3750 cm<sup>-1</sup>)

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## IV. CONCLUSION

All techniques used in the study have rapid results in diagnosis of malignant and treated tissues. In fluorescence spectroscopy each fluorophore needs specific wave length to be excited and it give accurate result after treatment. The FTIR samples must be prepared first before analysis, however in Raman spectroscopy it can be used directly without preparation the samples and it gives the sharp peaks in the analysis spectrum.

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