Use of dispersive Raman spectroscopy to detect the cytotoxic action of *viscum album* in adenocarcinoma of colon

Daniela Franco Lopes
Institute of Research and Development (IP&D), Universidade do Vale do Paraíba (UNIVAP), Av. Shishima Hifumi, 2911 Urbanova, 12244-000 São José dos Campos, SP, Brazil

Landulfo Silveira, Jr.[a)]
Group of Biomedical Engineering, Universidade Camilo Castelo Branco (UNICASTELO), Rod. Pres. Dutra, Km 138, Eugênio de Melo, 12247-004 São José dos Campos, SP, Brazil

Marcelo Nobuo Sibata, Aloísio Cunha de Carvalho, Cristina Pacheco-Soares, and Jamil Saade
Institute of Research and Development (IP&D), Universidade do Vale do Paraíba (UNIVAP), Av. Shishima Hifumi, 2911 Urbanova, 12244-000 São José dos Campos, SP, Brazil

Marcos Tadeu T. Pacheco
Group of Biomedical Engineering, Universidade Camilo Castelo Branco (UNICASTELO), Rod. Pres. Dutra, Km 138, Eugênio de Melo, 12247-004 São José dos Campos, SP, Brazil

(Received 21 January 2008; accepted for publication 4 November 2008; published 5 March 2010)

In the past decades it was noticed that there was a gradual increase in neoplasia diagnostics in animals, which are attributed to the longevity achieved by efficient vaccines, antibiotics, diagnostics, and food quality. The aliphatic oncology uses chemotherapics that are citostatic association, citostatic antibiotics, antimetabolic citostatics, and antimetabolic hormones in tumor treatment. Later on, the oncology introduced the imunomodulated antineoplasics from vegetals which belongs the *viscum album*. This study verified the efficiency of the extract of the injectable *viscum album* with concentrations D3 (0.1 μg/mL), D6 (0.1 ng/mL), D9 (0.1×10^{-3} ng/mL), D12 (0.1×10^{-6} ng/mL), and D30 (0.1×10^{-24} ng/mL) in culture of Caco-2 cells *in vitro* evaluated by correlating the spectral information obtained by dispersive Raman spectroscopy at 830 nm excitation with the cell viability determined by the Mitochondrial Activity (MTT) assay test. Results of MTT indicated that *viscum album* acted in the mitochondrial activity by reducing cellular viability in the Caco-2 cells in the concentrations of D3, D6, and D9. It was found that there was a good correlation of the Raman spectrum analyzed by principal components analysis, with the values of the MTT, and such result indicated that Raman could constitute in a sensitive tool for the detection of changes in the mitochondrial activity of Caco-2 cells. © *2010 Laser Institute of America.*

Key words: Injectable homeopatic medicine, neoplasm, colon, cell culture, MTT assay, dispersive Raman spectroscopy, principal components analysis (PCA)

I. Introduction

In the biomedical field, spectroscopic techniques using light as an excitation source such as fluorescence and Raman spectroscopy have been applied in the identification and characterization of biological samples by means of laser excitation. Such studies started in end of 1970s and beginning of 1980s with the use of fluorescence spectroscopy to detect and identify benign and malignant tissue[1,2] and the first Raman spectrum obtained from a native protein using an He–Ne laser as the excitation source.[3] Of both techniques, Raman spectroscopy has advantages because Raman spectrum can provide the most detailed information about the chemical composition of the sample under study.[4]

The Raman effect is a fundamental process in which energy is exchanged between light and matter. A monochromatic light can interact with molecules mainly by scattering or absorption of photons. Most of the scattered light will have the same frequency as that of the incident light. However, a small fraction of the incident light can go into setting molecules in the material into vibration. The energy for this must come from the incident light. Since light energy is proportional to frequency, the frequency change of this scattered light must equal the vibrational frequency of the scattering molecules. This process of energy exchange between scattering molecules and incident light is known as the Raman effect.[4–6]

Raman spectroscopy has been applied for characterization of human diseases *in vitro* and *in vivo* such as colon,[7]

[a)]Author to whom correspondence should be addressed; Landulfo Silveira, Jr., Group of Biomedical Engineering, Universidade Camilo Castelo Branco—UNICASTELO, Núcleo do Parque Tecnológico de São José dos Campos, Rod. Pres. Dutra, km 138, São José dos Campos, SP, Brazil, 12247-004; FAX: (55) (12) 3905-4648 ext. 209; electronic mail addresses: landulfo.silveira@unicastelo.br, landulfo.silveira@gmail.com

skin,[8] and breast cancer,[9] vascular diseases, mainly atherosclerosis,[10,11] blood, and urine.[12,13] Results show the possibility to identify the disease status by analysis of biochemical changes in the cell/tissue structure and in the analytes. Advantages of Raman spectroscopy for biomedical applications include no need for sample preparation, results are obtained in almost real time, and the biochemistry of important tissue alterations could be assessed.

Veterinary medicine is one area which could benefit from advances in disease identification through optical methods. Cancer diagnosis in small pets has been increasing over last ten years, which can be attributed to the advances of technology that produces vaccines, antibiotics, and foods that provide increase in the longevity of domestic animals and more efficient and early diagnostics.[14]

The physics of dynamized systems continues being the great challenge in the academic community, despite the 200 years of the homeopathy, where clinical experience and expressive results are still doubtful due to insufficient information that characterize the phenomenon, in which the experiments are limited to mere hypothesis where the absence of evidence does not mean evidences of absence.[15]

The aliphatic oncology uses chemotherapics that are citostatic association, citostatic antibiotics, antimetabolic citostatics, and antimetabolic hormones in tumor treatment. Later on, the oncology introduced the imunomodulated antineoplasics, from the vegetal class of which belongs the *viscum album*. In 19th century, Steiner, the father of the antroposophy, and Wegman introduced the *viscum album* in the treatment of human carcinoma. It is estimated that more than 80 000 human beings have been treated with injectable homeopathic therapy in the last 30 years, complementing conventional treatment[16] that are managed normally by subcutaneous, intravenous, or peritumoral injection.[17] Current clinical studies had demonstrated that *viscum album* was efficient in the evolution control of radium osteocondrosarcoma and hemangioma of splen, conferring analgesia without use of other allopathic pharmacologies in dogs without provoking detectable clinical side effects.[18] Another clinical study, using the injectable *viscum album* concomitant to vincristina sulfate in dogs carrying venereal tumor demonstrated that chemotherapy time can be reduced, as well as side effects such as the leucopenia promoted by the use of this chemotherapy.[19]

The potential for the Raman spectroscopy to address qualitative and quantitative problems in cellular and molecular biology in real time, costless, and high accurate is being investigated. Shaw *et al.*[20] used dispersive Raman spectroscopy to the online determination of the biotransformation by yeast of glucose to ethanol, aiming the noninvasive online determination of the progress of microbial fermentation. Raman spectral characterization of biochemical changes related to cell cycle dynamics within single living human osteosarcoma cells *in vitro* have been proposed by Swain *et al.*,[21] indicating changes in relative cellular lipid, nucleic acids, and proteins contribution to Raman spectral signatures depending on the phase of cell cycle. Advantages include no need for cell staining, labeling or marking. Chan *et al.*[22] demonstrated the use of Raman spectroscopy to monitor changes in a population of *E. coli* cells that occur during overexpression of a protein, the extracellular domain of myelin oligodendrocyte glycoprotein. Duarte *et al.*[23] proposed the detection of IgG and IgM antibodies against toxoplasmosis in cats blood serum by dispersive Raman spectroscopy, rapid, and without need for reagent.

The objective of this study is to verify if dispersive raman spectroscopy (DRS) using infrared laser excitation (830 nm) could be used to evaluate cell viability in cultures of Caco-2 cells after inoculation of *viscum album* injectable extract in concentrations of D3 (0.1 μg/mL), D6 (0.1 ng/mL), D9 (0.1×10^{-3} ng/mL), D12 (0.1×10^{-6} ng/mL), and D30 (0.1×10^{-24} ng/mL), diluted by using the Hering method,[24] in order to determine its antineoplastic efficiency in Caco-2 cells. It is proposed the correlation of the Raman spectral information obtained from cell suspension, after inoculation of vegetable extract in cell culture, with the percentage of cellular viability (measured through mitochondrial activity), furnished by the mitochondrial activity (MTT) assay test, through principal components analysis (PCA) applied to the scores. The main goal is to obtain a rapid, accurate, cheap, and feasible evaluation of cell viability without need for cost and time consuming cell preparation of MTT tests.

II. Material and methods

Cell culture. The Caco-2 cells (CR069, adenocarcinoma of colon) were donated from the Bank of Cells of Rio de Janeiro state and had been stored in the Laboratory of Dynamic Cellular Compartments, IP&D, UNIVAP. Cell line was routinely cultured using Minimum Essential Medium (MEM, Gibco) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mM/mL), and fungizone (0.25 μg/mL). Cells were cultured at 37 °C in 5% CO_2 atmosphere.

Incubation with viscum album. (a) The first plate was destined to the MTT test, it was composed by 24 wells that received a cellular volume from 2×10^5 cells/mL. Wells were divided in six groups of four samples: one control and five for each dilution incubated with the *viscum album* in concentration of D3 (0.1 μg/mL), D6 (0.1 ng/mL), D9 (0.1×10^{-3} ng/mL), D12 (0.1×10^{-6} ng/mL), and D30 (0.1×10^{-24} ng/mL). After incubation cells had been analyzed by optical microscopy, and the aspect of the cells were registered through photomicrographs. In order to analyze the cytotoxity, the MTT test was used (4,5 dimetiltiazol-2yl)-2–5-difenil-2H bromine tetrazolate, 1 mg/mL, MTT, Sigma, St. Louis), which measures the mitochondrial activity that is related to the cell viability. The culture medium was removed and 200 μL of MTT was added to the remaining cells and incubated at 37 °C for 1 h, after which 200 μL of DMSO (sulfoxide dimetil) was added with agitation of 30 min for dilution of formazana crystals. The reading was carried out at spectrophotometer Spectra Count TM (Packard) with 570 nm filter. (b) The group analyzed by Raman was cultivated in plates of 12 wells, two for each dilution and two for control, having followed the same standard for the MTT assay. After 24 h the culture cells were scraped and the cellular suspension transferred to a quartz cuvette and submitted to Raman

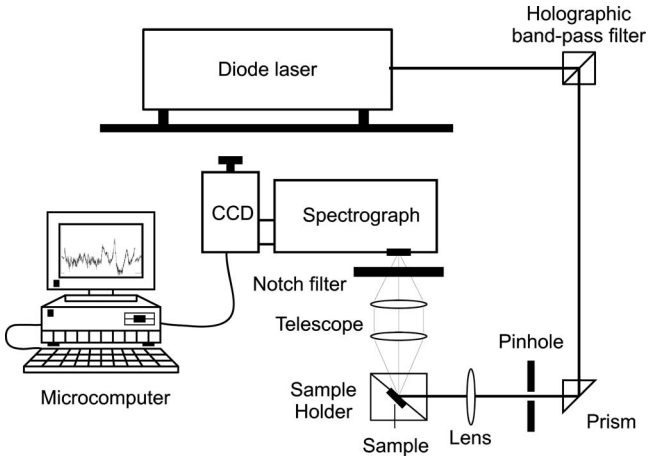

FIG. 1. Schematic diagram of the dispersive Raman spectrometer with excitation wavelength of 830 nm, laser power of 80 mW, and spectral range of 800–1800 cm^{-1}.

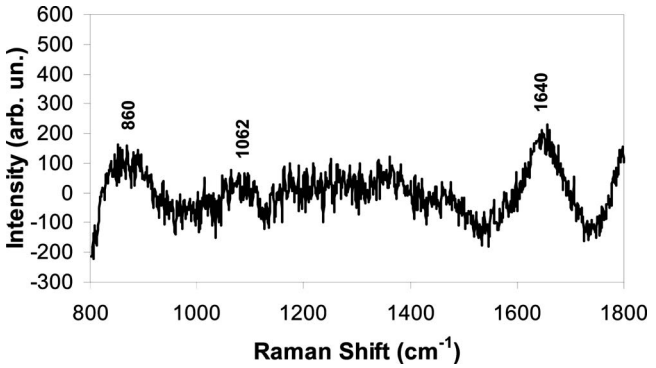

FIG. 2. Mean Raman spectrum of *viscum album* in the dilution of D3 (0.1 $\mu g/mL$). Excitation wavelength of 830 nm, laser power of 80 mW, and spectral resolution of 8 cm^{-1}.

spectroscopy and were collected two spectra per sample.

Cells used in the MTT assay cannot be used for Raman scanning because the MTT test evaluates the capacity of the mitochondrial enzyme succinato desidrogenase to reduce the MTT to formazan crystals for the toxicity analysis, rupturing the cell membrane and Raman uses intact cells to measure *viscum* absorption.

Raman system. The dispersive Raman system used to collect Raman signal is presented in Fig. 1 and is composed by a diode laser as an excitation source at 830 nm with output power of about 80 mW (Micro Laser Systems Inc., model L4830S). The monochromatic laser light is directed to the sample by a set of lens and prism, and after scattering the light is collected by a set of lenses and directed to the spectrograph before passing through a notch laser band-rejection filter. The signal is then dispersed by an imaging spectrograph (Chromex, model 250 IS, with a 600 lines/mm grating) in the wavenumber range 800–1800 cm^{-1} and detected by a liquid-nitrogen cooled, deep depletion, charge coupled device (CCD) with controller (Princeton Instruments, model LN/CCD-1024-EHR1). The CCD exposure (integrating) time was set to 30 s for all samples. This integrating time was chosen based on the saturation limit of the CCD camera with one scan to reduce the electronic readout noise to its minimum. One spectrum of each sample was collected.

Wavelength and intensity calibration was done by a routine developed in the laboratory by using a tungsten spectral calibration lamp for the intensity and the naphtalene ($C_{10}H_8$) with known Raman bands for the wave number. The cuvette background was not removed from gross spectrum because it is known that quartz has nonlinear elastic and inelastic scattering properties,[25] resulting in residues when simple subtraction is applied. The fluorescence background from cells that are collected among the scattered photons was removed by the same routine, through a 5th order polynomial that fits and subtracts the low order contribution from fluorescence. This fluorescence can reduce the signal-to-noise ratio by adding shot noise and overlapping the weak Raman signal, and its removal do not increase signal quality, just the final spectrum appearance. After calibration, spectra were saved in ASCII format for data processing using software MATLAB 6 (The Mathworks, Inc.). The mean error of the dispersive Raman spectrum with a stabilized laser calculated by taking five spectra and measuring the change in the intensity of a particular band is on the order of ±5%.

Statistical analysis. Processed Raman spectra were submitted to multivariate statistical analysis, in order to correlate the cell viability measured through MTT test and the spectral information from Raman signals. Since some spectral changes are very small and undetectable by visual inspection or simple intensity analysis of Raman bands, the PCA was used.[26] First, it was determined which principal component is responsible for the spectral variance. After, the cell viability was considered the independent variable and the intensity scores furnished by the PCA was the dependent variable, in a linear model with those variables, providing a quantification model based on the PCA. So, any change that occur in the spectrum depending on the amount of viable cell can be correlated with the PCA variables.

III. Results and discussion

All spectra were averaged using four measurements. Figure 2 shows the mean Raman spectrum of *viscum album* in the dilution of D3. It can be seen Raman bands in the position of 860, 1062, and 1640 cm^{-1}, which can be attributed to the quartz cuvette, by comparing the spectrum of *viscum album* with the spectrum of saline. Figure 3 shows the average Raman spectra from suspension of Caco-2 cells after incubation of *viscum album* with several doses. Spectra were separated with an offset intensity to remove overlapping, but the original band intensity was kept for PCA calculations. The noise of the spectra is attributed to the shot noise (statistical fluctuations of the detected light) that arrives with the unwanted fluorescence from cells and quartz cuvette.

It can be observed a change in the relative intensity of the Raman bands, mainly in the spectral region of 1200–1600 cm^{-1}. This spectral region is characterized by bands mainly from proteins and lipids from cells and tissues. Bands below 1200 cm^{-1} and above 1600 cm^{-1} could be attributed to the quartz cuvette.

The PCA was calculated using the Raman spectra of all dilutions. The PCA scores (PCs) were then correlated with

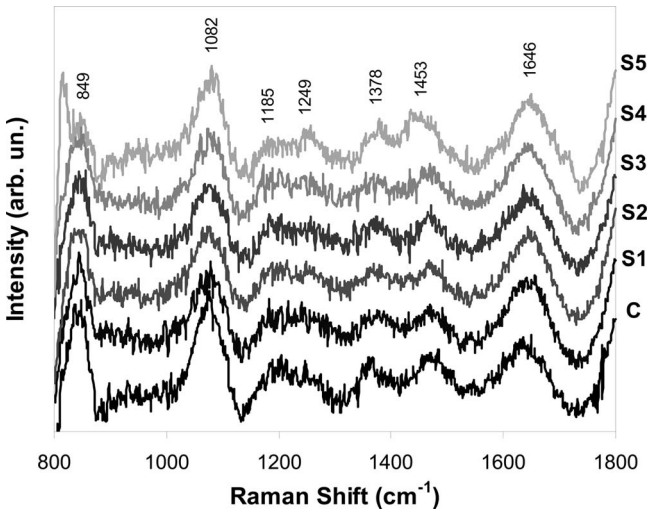

FIG. 3. Raman spectra of five samples of Caco-2 cells with the following dilutions of *viscum album* (from top to bottom): D3 (0.1 μg/mL), D6 (0.1 ng/mL), D9 (0.1×10^{-3} ng/mL), D12 (0.1×10^{-6} ng/mL), and D30 (0.1×10^{-24} ng/mL). Excitation wavelength of 830 nm, laser power of 80 mW, and spectral resolution of 8 cm^{-1}.

the cell viability obtained from the MTT assay. Based on these two inputs (spectra and cell viability), it is possible to find a correlation between both variables. Due to the fact that only a few variables can be responsible for the changes in the spectral data in a typical Raman experiment using only one sample type, the PCA model considered only the first two principal components. Figure 4 shows the loading vector spectra of the first two PCs. It is noted that the first vector represents the mean, while the second one represents the spectral variations.

The cell viability was compared to the PCA scores. It was found that the first two PCs are responsible for more than 90% of all spectral variation. Since only one variable can be responsible for most changes that occur in the spectra of Caco-2 cells after *viscum album* incubation, the intensity of the PC2 was used to correlate the MTT assay with the

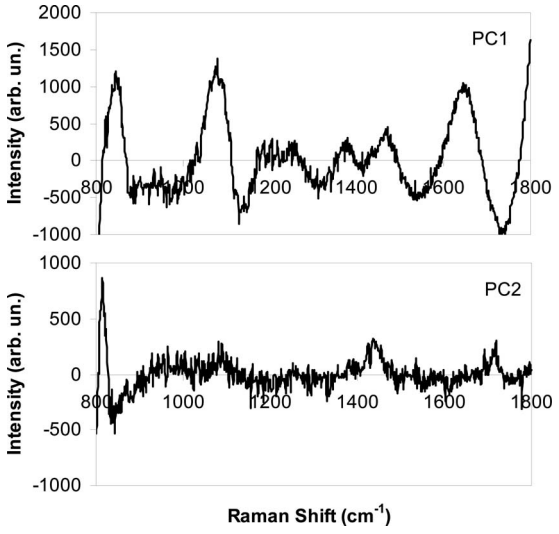

FIG. 4. Plot of the first two loading vectors (principal components spectra) calculated from the Raman data set for the PCA analysis.

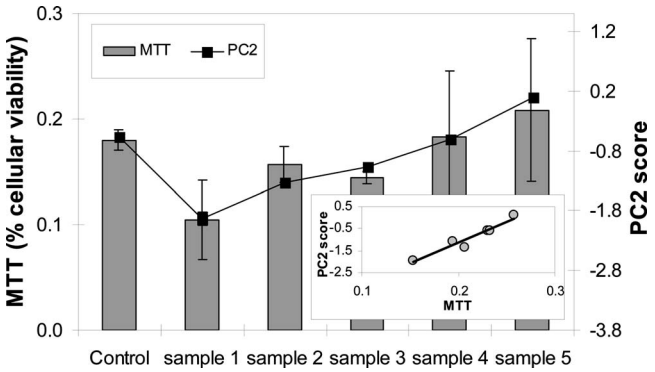

FIG. 5. Plot of the MTT assay versus the second PCA scores (PC2) calculated from the Raman spectra. Correlation coefficient $r^2=0.93$.

Raman information (Fig. 5). It was obtained a good correlation ($r^2=0.93$) with the measured MTT and the PC2 score (most of them inside the MTT error bars), showing the possibility of measuring this parameter by Raman spectroscopy.

The results of MTT assay shown in Fig. 5 indicates that *viscum album* have acted in the mitochondrial activity in the concentrations of D3, D6, and D9, reducing the cellular viability when inoculated in the Caco-2 cells. Also, the MTT could be predicted by the equation: $y=18.7x-3.94$, where y is the new MTT assay and x is the PC2 score from a new Caco-2 spectrum taken in the same experimental conditions.

The laser irradiation time for collecting Raman signal should be set to a minimum to guarantee a good signal to noise ratio without damaging cells by overexposure. Since the infrared excitation laser was set to 80 mW and all Raman data collection were done in about 30 s exposure time, it is believed that no measurable cell damage was induced by the laser since near-infrared is poorly absorbed by proteins and fat in cell cultures *in vitro*[27] or its effect is the same for all samples.

Raman signal from biological samples are extremely weak (10^{-9} scattered photons compared to the incident ones) and the fluorescence emission is the major problem to collect Raman signals.[28] By using infrared laser excitation, the fluorescence can be reduced to a minimum.[9–11,27]

The Raman signal from the Caco-2 cells were significantly intense despite the low cell concentration (2×10^5 cells/mL). It was found that small, but important differences were verified in the spectra of treated cells mainly in the region of 1200–1600 cm^{-1}. This region is being characterized to provide spectral information from proteins and lipids from cells, and the differences found in this work for this region can be used to quantify cell viability. This can be confirmed by the analysis of the loading vector PC2 (Fig. 4), with main spectral band in the 1440 cm^{-1} region, in which such information provided the best correlation to viability. Bands between 1000 and 1600 cm^{-1} carry information of the structural proteins (tyrosine, tryptophan, histidine, and phenylalanine).[29] The nucleotides present bands between 1100 and 1700 cm^{-1}, which are rich in nitrogenated bases.

The diagnostic methods currently used in oncology for disease confirmation, i.e., the so-called "gold standard," are characterized by disadvantages such as time consuming pro-

cedures, uncertainty by the effectiveness and therapeutic compatibility among cell/patient and antitumor drug, and uncertainties and emotional suffering after diagnostic confirmation. Spectroscopic diagnostic methods would address some questions due to the fact that procedures could be done less invasive, results could be obtained in real time, and costs could be reduced in analyzing the best drug for each tumor type allowing strategically changes during the therapies.[30]

The MTT assay is a current technique to evaluate cell viability, specifically the mitochondial activity. MTT can be related to the information of Raman spectrum in terms of protein since the cancerous cell mitochondrial activity can be correlated with the amount of protein degradation[31] and such degradation would alters the Raman scattering and consequent its intensity. Other study shows that the presence of *viscum album* induced the colon cell apoptosis due to activation of caspasis and inhibition of antiapoptotic proteins, accelerating cell death.[32]

The cell cultures of colon adenocarcinoma had been evaluated by Raman spectroscopy[33] and the high concentration of protein and nucleic acids could be evidenced in neoplastics cells when compared to normal cells, demonstrating that Raman could be a useful method of differentiation, therefore the amount of these compounds in neoplastic cells can be evaluated when analyzing anticancer chemoterapeutic drugs.

IV. Conclusion

It can be concluded that the dispersive Raman spectroscopy technique combined with the multivariate statistics PCA were highly correlated with the results of the chemosensitivity MTT assay test, mainly identifying changes in the spectral information of cellular proteins related to the cellular viability. Raman technique revealed to be a valuable and sensitive method for detection of change in the mitocondrial activity of Caco-2 cells, when submitted to the antineoplastic medicine *viscum album*.

[1] A. E. Profio and D. R. Doiron, "A feasbility study of the use of fluorescence bronchoscopy for the localization of samll lung tumors," Phys. Med. Biol. **22**, 949–957 (1977).

[2] R. Alfano, D. Tata, J. Cordero, P. Tomashefsky, F. Longo, and M. Alfano, "Laser induced fluorescence spectroscopy from native cancerous and normal tissue," IEEE J. Quantum Electron. **20**, 1507–1511 (1984).

[3] R. C. Lord and N. T. Yu, "Laser-excited Raman spectroscopy of biomolecules I. Native lysozyme and its constituent amino acids," J. Mol. Biol. **50**, 509–524 (1970).

[4] E. B. Hanlon, R. Manoharan, T. W. Koo, K. E. Shafer, J. T. Motz, M. Fitzmaurice, J. R. Kramer, I. Itzkan, R. R. Dasari, and M. S. Feld, "Prospects for *in vivo* Raman spectroscopy," Phys. Med. Biol. **45**, R1–R59 (2000).

[5] J. Twardoswki and P. Anzenbacker, *Raman and IR Spectroscopy in Biology and Biochemistry* (Ellis Horwood, New York, 1994).

[6] O. Sala, *Fundamentos da Espectroscopia Raman no Infravermelho* (Unesp, São Paulo, 1996), pp. 13–15.

[7] M. V. P. Chowdary, K. Kalyan Kumar, K. Thakur, A. Anand, J. Kurien, C. Murali Krishna, and S. Mathew, "Discrimination of normal and malignant mucosal tissues of the colon by Raman spectroscopy," Photomed. Laser Surg. **25**, 269–274 (2007).

[8] L. O. Nunes, A. A. Martin, L. Silveira, and M. Zampieri, "FT-Raman Spectroscopy study for skin cancer diagnosis," Spectroscopy Int. J. **17**, 597–602 (2003).

[9] A. S. Haka, Z. Volynskaya, J. A. Gardecki, J. Nazemi, J. Lyons, D. Hicks, M. Fitzmaurice, R. R. Dasari, J. P. Crowe, and M. S. Feld, "In vivo margin assessment during partial mastectomy breast surgery using Raman spectroscopy," Cancer Res. **66**, 3317–3322 (2006).

[10] J. T. Motz, M. Fitzmaurice, A. Miller, S. J. Gandhi, A. S. Haka, L. H. Galindo, R. R. Dasari, J. R. Kramer, and M. S. Feld, "*In vivo* Raman spectral pathology of human atherosclerosis and vulnerable plaque," J. Biomed. Opt. **11**, 021003–021011 (2006).

[11] L. Silveira, S. Sathaiah, R. A. Zângaro, M. T. T. Pacheco, M. C. Chavantes, and C. A. G. Pasqualucci, "Correlation between near-infrared Raman spectroscopy and the histopathological analysis of atherosclerosis in human coronary arteries," Lasers Surg. Med. **30**, 290–297 (2002).

[12] S. Pilotto, M. T. T. Pacheco, L. Silveira, A. B. Villaverde, and R. A. Zângaro, "Analysis of near-infrared Raman spectroscopy as a new technique for a transcutaneous non-invasive diagnosis of blood components," Lasers Med. Sci. **16**, 2–9 (2001).

[13] J. N. Y. Qu, B. B. Wilson, and D. Suria, "Concentration measurements of multiple analytes in human sera by near-infrared laser Raman spectroscopy," Appl. Opt. **38**, 5491–5498 (1999).

[14] R. C. Rosenthal, *Segredos em Oncologia Veterinária*, edited by Artmed (Artmed, Porto Alegre, 2004).

[15] M. C. M. Fonseca and V. W. D. Casali, "Revisão sobre as visões química, física e biocibernética da Homeopatia," Cultura Homeopática **14**, 6–10 (2006).

[16] E. Kaegi, "Unconvencional therapies for cancer: 3. Iscador," Can. Med. Assoc. J. **158**, 1161–1165 (1998).

[17] R. Ghelman, "Ação do Viscum album sobre a prenhez da rata albina (Rattus norvegicus albinus, Rodentia, Mammalia): Estudo morfológico e radiológico," Ph.D thesis, Universidade Federal de São Paulo, 2003.

[18] D. F. Lopes, A. C. Carvalho, and M. N. Sibata, "Estudo de casos clínicos oncológicos em caninos e felinos tratados com Homeopatia injetável (2005–2007)," *PUBVET* (Publicações em Medicina Veterinária e Zootecnia, 2007), p. 1 (www.pubvet.com.br/texto.php?id=68>).

[19] G. N. F. Lefebvre *et al.*, "Tratamento do Tumor venereo transmisivel(TVT) utillizando Viscum album em associação a quimioterapia," *Revista Clinica Veterinaria*, (2007), n-70, p. 78–86.

[20] A. D. Shaw, N. Kaderbhai, A. Jones, A. M. Woodward, R. Goodacre, J. J. Rowland, and D. B. Kell, "Noninvasive, on-line monitoring of the biotransformation by yeast of glucose to ethanol using dispersive Raman spectroscopy and chemometrics," Appl. Spectrosc. **53**, 1419–1428 (1999).

[21] R. J. Swain, G. Jell, and M. A. Stevens, "Non-invasive analysis of cell cycle dynamics in single living cells with Raman micro-spectroscopy," J. Cell. Biochem. **104**, 1427–1438 (2008).

[22] J. W. Chan, H. Winhold, M. H. Corzett, J. M. Ulloa, M. Cosman, R. Balhorn, and T. Huser, "Monitoring dynamic protein expression in living E-coli. Bacterial Celts by laser tweezers raman spectroscopy," Cytometry, Part A **71A**, 468–474 (2007).

[23] J. Duarte, M. T. T. Pacheco, R. Z. Machado, L. Silveira, R. A. Zangaro, and A. B. Villaverde, "Use of near-infrared Raman spectroscopy to detect IgG and IgM antibodies against Toxoplasma gondii in serum samples of domestic cats," Cell. Mol. Biol. **48**, 585–589 (2002).

[24] J. Satti, "Homeopathic Drug Standardization," Semin. Integr. Med. **3**, 113–122 (2005).

[25] R. W. Terhune, P. D. Maker, and C. M. Savage, "Measurements of Nonlinear Light Scattering," Phys. Rev. Lett. **14**, 681–684 (1965).

[26] Y. Z. Liang and O. M. Kvalheim, "Robust Methods for Multivariate Analysis: A Tutorial Review," Chemom. Intell. Lab. Syst. **32**, 1–10 (1996).

[27] F. F. Jöbsis-Vandervliet, "Discovery of the near-infrared window into the body and the early development of near-infrared spectroscopy," J. Biomed. Opt. **4**, 392–396 (1999).

[28] I. R. Lewis and H. G. M. Edwards, "*Handbook of Raman spectroscopy: from the research laboratory to the process line*" (Marcel Dekker, New York, 2001), p. 1054.

[29] A. Mahadevan-Jansen and R. Richards-Kortum, "Raman Spectroscopy for the detection of cancer and precancer," J. Biomed. Opt. **1**, 31–70 (1996).

[30] N. Stone, C. Kendall, J. Smith, P. Crow, and H. Barr, "Raman spectroscopy for identification of epithelial cancers," Faraday Discuss. **126**, 141–157 (2004).

[31] S. G. Velleman, D. C. McFarland, Z. Li, N. H. Ferrin, R. Whitmoyer, and J. E. Dennis, "Alterations in sarcomere structure, collagen organization,

mitochondrial activity, and protein metabolism in the avian low score normal muscle weakness," Dev., Growth Differ. **39**, 563–570 (1997).
[32]L. Y. Khil, W. Kim, S. Lyu, W. B. Park, J. W. Yoon, and H. S. Jun, :Mechanisms involved in Korean mistletoe lectin-induced apoptosis of cancer cells," World J. Gastroenterol. **13**, 2811–2818 (2007).
[33]K. Chen, Y. Qin, F. Zheng, M. Sun, and D. Shi, "Diagnoses of colorectal cancer using Raman Spectroscopy of laser-trapped single living epithelial cells," Opt. Lett. **31**, 2015–2017 (2006).