CHAPTER 7

Evaluation of the potential of Raman spectroscopy for prediction of chemopreventive response to silibinin and its nanoparticulates in DMBA-induced hamster buccal pouch carcinomas

7.1. Introduction

Oral cancer is a serious worldwide public health problem, with high incidence and mortality rates. It is estimated that approximately 275,000 new cases and 128,000 deaths will be attributed to oral cancer in the world in 2008 (Jemal et al., 2011). Oral cancer is the most common type of malignancy in certain countries, such as India, Sri Lanka, Pakistan and Bangladesh. In India, 320,000 new cancer cases are diagnosed annually in males, and 19% of this is oral cancer; in females out of 350,000 new cancer cases, 7% is oral cancer (Joshi et al., 2014). The most common oral cancer is oral squamous cell carcinoma (OSCC), which makes up 90% of all oral cancers. Tobacco smoking, smokeless tobacco and alcohol consumption are known to synergistically contribute to OSCC risk. For instance, betel-quid chewing is an important risk factor in Southeast Asia, where OSCC can account for up to quarter of all malignancies (Lee et al., 2011). Despite significant advances in surgical and treatment modalities, the five-year survival rate of oral cancer patients has not improved over the past couple of decades and remains at about 50% (Marsh et al., 2011). Therefore, early diagnosis and localization of malignant lesions together with appropriate curative treatment is critical to reducing the mortality rates of oral cancer patients. Therefore a fast, accurate and objective method for diagnosis of oral cancer is needed. Optical spectroscopic methods are being pursued as alternatives or adjunct to existing diagnostic methods. Optical diagnostic methods are being investigated to provide a non-invasive, rapid and real time diagnosis for the
early detection of cancer. A variety of optical based techniques such as fluorescence spectroscopy, elastic scattering spectroscopy, diffuse reflectance spectroscopy, infrared spectroscopy and Raman spectroscopy have been explored for development of newer diagnostic tools in oral cancer (Krishnakumar et al., 2009; Liu, 2011; Huang et al., 2013; Gurushankar et al., 2014). In recent years, Raman spectroscopy has attracted considerable attention due to its great potential for cancer diagnosis. Raman spectroscopy is a vibrational spectroscopic technique that can provide specific spectroscopic fingerprint information about the molecular composition, structure and biochemical constituents (Teh et al., 2008; Singh et al., 2012; Krishnakumar et al., 2013). Raman spectroscopy is based on the measurement of the vibrational energy levels of chemical bonds by measuring the inelastically scattered light following excitation. Raman spectroscopy using near-infrared (NIR) laser light excitation (e.g., 785 nm) is especially attractive as it holds significant advantages over previous spectroscopic techniques in tissue diagnosis, such as deeper penetration into the tissues and far less interference from tissue autofluorescence as compared to ultraviolet/shorter visible light excitation. The sensitivity of this technique is so high that a Raman spectrum is effectively a precise fingerprint of the biochemical makeup of the tissue. The Raman “fingerprint region”, between 1800 and 500 cm\(^{-1}\), is the region of the Raman spectrum, which correlates to the molecular vibrations of biochemical importance (Teh et al., 2008; Lui et al., 2012). The Raman spectra of the tissue reflect specific biochemical/biomolecular structures and conformations (i.e. biochemical signatures) including proteins, lipids and nucleic acid contents, providing the opportunity to distinguish between different tissue types at the molecular level (Luo et al., 2013). The specific and characteristic Raman bands can be used for qualitative and quantitative analyses. The qualitative
analysis provides important clues in the search for a specific diagnosis and the quantitative analysis of biochemical abnormalities is important in measuring the extent of the disease process, designing therapy and evaluating the efficacy of treatment. Recently, Raman spectroscopy has been successfully applied to clinical and preclinical models to monitor and predict therapeutic response under anti-cancer drug treatments (Krishnakumar et al., 2013; Rubina et al., 2013; Gonzalez-Solis et al., 2014).

In this study, 7,12-dimethylbenz[a]anthracene (DMBA) induced carcinogenesis model was developed in golden Syrian hamster buccal pouch (HBP) and to detect biochemical composition and structural changes at the molecular level in tumor tissues by Raman spectroscopy. It is an accepted and well recognized experimental model for studying biochemical, histopathological and molecular alterations occurring in oral carcinogenesis. DMBA induced molecular changes in the buccal mucosa of golden Syrian hamsters closely mimics or resembles to that of human oral tumor (Rajasekaran et al., 2013). Therefore, DMBA-induced oral carcinogenesis model is highly suitable for the study of the relationship between the biochemical alteration of the Raman spectra and also to assess the cancer chemopreventive potential of natural products and other anticancer agents.

Cancer chemoprevention is a promising anticancer approach aimed at delaying in onset of cancer, progression from precancerous lesion or recurrence after treatment by the administration of one or more naturally occurring and or synthetic agents. Silibinin (SIL) (*Silybum marianum*), a polyphenolic flavonolignan isolated from milk thistle, is one of the chemopreventive and anticancer agents that has attracted attention for the prevention of or treatment for cancer (Ramasamy and Agarwal, 2008; Sangeetha et al., 2010; Kiruthiga et al., 2010). However, the commercial oral
preparations are poorly absorbed with low bioavailability owing to its low solubility and do not provide effective treatment. To improve the pharmacokinetics properties and the bioavailability of the chemopreventive agents, tremendous advancement has been made by making nanoparticle-based therapeutic products. Recently, polymeric nanoparticles (NPs) have shown great potential as delivery systems for an increasing number of active molecules. The enhanced permeability and retention (EPR) effect of polymeric NPs near the tumor vasculature helps in passive targeting of anticancer drugs (Park et al., 2008; Parhi et al., 2012; Bertrand et al., 2014). Therefore, polymeric NPs could act as an effective delivery system for improving silibinin bioavailability and its anti-tumor effect. To enhance its anti-tumor efficacy, silibinin-loaded nanoparticles (SILNPs) were synthesized by the nanoprecipitation method with their average particle size of below 120 nm and it has been described in detail previous chapter 3. The present study is designed to investigate the biochemical composition and structural changes at the molecular level in response to antitumor effect of prepared SILNPs in comparison with free SIL against DMBA-induced oral carcinogenesis by Raman spectroscopic technique. Further, the multivariate statistical techniques, including principal components analysis (PCA) followed by linear discriminant analysis (LDA), were employed to develop effective diagnostic algorithms for differentiations between the control and the experimental groups. The majority of these algorithms undergo cross-validation analysis using the leave-one-out method to assess their validity. In addition, the receiver operating characteristic (ROC) curve was also employed to assess and compare the accuracy of both diagnostic algorithms.
7.2. Review of Literature

Huang, et al. (2003) have used near-infrared Raman spectroscopy to distinguish the normal tissues from malignant bronchial tissues. The observed spectra shows significant differences between the normal and malignant tissues particularly in the region 1000-1100, 1200-1400 and 1500-1700 cm\(^{-1}\), which contain signals related to protein and lipid conformations and nucleic acids. Further, tumor tissues showed higher percentage of nucleic acids, tryptophan and phenylalanine and lower percentage signals of phospholipids, proline and valine, as compared to normal tissues. In addition, the ratio of Raman intensities at 1445 to 1655 cm\(^{-1}\) provides good differentiation between the normal and malignant bronchial tissue. The results suggest that the near-infrared Raman spectroscopy provides significant potential for non-invasive diagnosis of lung cancer.

Kendall et al., (2003) have used Raman spectroscopy for the identification and classification of Barrett’s neoplasia in vitro. Raman spectral prediction with a consensus pathology classification model obtained sensitivities between 73% and 100% and specificities of 90-100%. The results suggest that Raman spectroscopy appears to provide a highly sensitive and specific technique for the identification and classification of neoplasia in Barrett’s oesophagus.

Stone et al., (2004) have used Raman spectroscopy to evaluate the possibilities of targeting pre-cancerous lesions in a number of epithelial tissues, and also discussed the use of misclassification costs to enhance the favoured outcome of very few false negatives for malignancy. Diagnostic predictive models have been constructed and optimised by multivariate analysis techniques. They have been tested using cross-validation or leave-one-out and demonstrated high levels
of discrimination between pathology groups (greater than 90% sensitivity and specificity for all tissues).

Murali Krishna et al., (2004) studied formalin-fixed oral squamous normal and carcinoma tissues using micro-Raman spectroscopy. They observed no noticeable spectral contamination due to formalin. The results showed that major differences between the normal and malignant spectra seem to arise from protein composition, conformation/structural changes and possible increase in the protein content in the malignant tissues. Further, the study validates the suitability of formalin fixed tissues for optical pathology in oral malignancy.

Crow et al., (2005) have used Raman spectroscopy (RS) to differentiate between prostatic adenocarcinoma (CaP) cell lines of varying degrees of biological aggressiveness. Raman spectra were measured from two well-differentiated, androgen-sensitive cell lines (LNCaP and PCa 2b) and two poorly differentiated, androgen-insensitive cell lines (DU145 and PC 3). The algorithm was able to identify the cell line of each individual cell with an overall sensitivity of 98% and a specificity of 99%. The results demonstrated the ability of RS to differentiate between CaP samples of varying biological aggressiveness. The results further suggest that RS shows promise for application in the diagnosis and grading of CaP in clinical practise as well as providing molecular information on CaP samples in a research setting.

Shetty et al., (2006) have utilised near-infrared Raman spectroscopy, a highly specific optical analysis technique, to build robust spectral, biochemical diagnostic models of oesophageal pathologies. The mean spectra obtained from selected regions reveals
that increased levels of glycogen in the squamous area compared with increased DNA levels in the abnormal region. The results suggest that Raman spectroscopy is a highly sensitive and specific technique for demonstration of biochemical changes in the carcinogenesis of Barrett’s oesophagus and further it is potential for in vivo application for real-time endoscopic optical diagnosis.

Kast et al., (2008) have used Raman spectroscopy with near-infrared light excitation to study the difference between the cancer and normal breast tissues in a mouse model. The results suggest that Raman spectroscopy can detect molecular preneoplastic changes prior to histologic alterations. They concluded that the rapid development and improvement of optic and computer technology, fiber-optic Raman probes may be used by clinicians to detect malignancy and by surgeons to guide surgical excisions in the not very distant future.

Aydin et al., (2009) have used surface-enhanced Raman scattering (SERS) to study the spectral differences between the healthy brain tissues and brain tumor tissues. They observed significant changes on SERS spectra from healthy brain tissues to tumor are the increase of the ratio of the peaks at around 723 to 655 cm\(^{-1}\). In addition, the spectral changes indicate increased protein content in tumors compared to healthy brain tissues. The result suggests that SERS spectra can be used for a quick diagnosis due to the simplicity of the sample preparation and speed of the spectral acquisition.

Teh et al., (2009) have used the near-infrared Raman spectroscopy to identify the normal and cancer tissues from larynx. They used Random forests method to develop effective diagnostic algorithm for classification of Raman spectra. They found significant spectral differences between the normal and malignant laryngeal
tissues in the region 1800-800 cm\(^{-1}\). They yielded diagnostic sensitivity of 88.0\% and a specificity of 91.4\% for laryngeal malignancy identification. The results suggest that near-infrared Raman spectroscopy in conjunction with random forests algorithm has a great potential for the laryngeal tumor diagnosis.

Bergholt et al., (2010) used image-guided Raman endoscopy for diagnosis of benign and malignant ulcers in the stomach. They found significant differences in Raman spectra among normal mucosa, benign ulcers and malignant ulcers, particularly in the spectral range of 800-900, 1000-1100, 1245-1335, 1440-1445 and 1500-1800 cm\(^{-1}\), which primarily contain signals related to proteins, DNA and lipids. The malignant ulcerous lesions are mainly associated with abnormal nuclear activity and decrease in the lipids when compared to benign ulcers. They obtained diagnostic sensitivities of 90.8\%, 84.7\% and 82.1\% and specificities of 93.8\%, 94.5\%, 95.3\% respectively for classification of normal mucosa, benign and malignant ulcers lesions in the stomach. The results demonstrated that image-guided Raman endoscopy technique associated with multivariate diagnostic algorithm has a great clinical potential for rapid, \textit{in vivo} diagnosis and detection of malignant ulcers gastric lesions at the molecular level.

Lieber et al., (2010) have assessed the ability of Raman micro spectroscopy to detect cancer field effects (CFE) by \textit{in vitro} study of organotypic tissue rafts approximating human skin. Raman spectra were measured from both epidermis and dermis after transfer of the rafts to dishes containing adherent cultures of either normal human fibroblasts or fibrosarcoma (HT1080) cells. Principal component analysis allowed discrimination between the groups with 86\% classification accuracy in the epidermis and 94\% in the dermis. These
results further suggest that Raman spectroscopy could be used for detecting CFE as a possible tool for non-invasive screening for tumor presence.

Kawabata et al., (2011) have applied near-infrared multichannel Raman spectroscopic (RAS) system to resected stomach with whole layers *ex vivo* at 1064 nm. The results revealed that RAS is useful for gastric cancer detection and when comparing cancer lesions, it discriminates between differentiated and undifferentiated types as well as early and advanced cancers, with even higher accuracy. The results suggest that RAS could help establish indications for endoscopic treatment by eliminating cancer lesions with an undifferentiated component or submucosal invasion.

Guze et al., (2011) have used micro-Raman spectroscopy to study the spectral differences between the normal and abnormal squamous cell carcinoma (SCC) in oral mucosa (*in vitro*). They observed significant differences between the Raman images of normal and malignant squamous cells. Their results revealed that cell surface protein greatly upregulated in SCC cells has been noticed at 1583 cm$^{-1}$. These results clearly show the potential of Raman spectroscopy in oral cancer pathology.

Raniero et al., (2011) carried out FT-Raman spectroscopy in the *in vivo* and *ex vivo* breast tissues of both healthy mice and mice with DMBA-induced mammary gland tumors in the 1800-900 cm$^{-1}$ wave number region. The results revealed that tumor tissues could have a higher protein concentration than the normal tissues and normal tissues could have a higher lipid concentration than the tumor tissues. The results further demonstrated that *ex vivo* measurements showed
the highest specificity (96%) and sensitivity (97%) as well as a largest percentage for correct discrimination (94%).

Su et al., (2012) have used confocal Raman spectroscopy to discriminate between malignant tissues/cells from normal. Specific bimolecular differences observed include an increase in protein and DNA concentration. The results revealed bimolecular difference between the normal and malignant conditions at the cellular level. The results suggest that Raman spectral diagnosis has provide a good technique for oral mucosal diseases.

Salman et al., (2013) have used Raman spectroscopy for the identification and characterization of murine fibroblast cell lines (NIH/3T3) and malignant fibroblast cells transformed by murine sarcoma virus (NIH-MuSV) cells. Principal component analysing (PCA) methods followed by LDA calculations used to differentiate between the NIH/3T3 and NIH-MuSV cells based on their Raman shift spectra with a success rate of 80-85%. The results suggest that Raman spectra have the best for differentiation obtained from the rich membrane sites.

Luo et al., (2013) have used near-infrared (NIR) Raman spectroscopy (RS) to differentiate premalignant lesions and cancer tissues from normal gastric tissues. They observed significant difference between normal, adenomatous polyp and adenocarcinoma in the respective intensities 936, 1003, 1032, 1174, 1208, 1323, 1335, 1450, and 1655 cm\(^{-1}\) of Raman spectra of gastric tissues. Based on cross-validation method, they obtained better discriminative values with a superior sensitivities (96.3%, 96.9% and 96.9%) and specificities (93%, 100%, and 95.2%) of normal, adenomatous polyp and adenocarcinoma gastric tissues. The results suggest that NIR RS
associated with multivariate statistical algorithms has the potential for early diagnosis of gastric premalignant lesions and cancer tissues at the molecular level.

Krishnakumar et al., (2013) have investigated the biomolecular changes in chemopreventive response of prepared naringenin-loaded nanoparticles (NARNPs) in comparison with free NAR against DMBA-induced oral carcinogenesis by FT-Raman spectroscopic technique. Raman spectra differed significantly between the control and tumor tissues, with tumors showed higher percentage signals for nucleic acids, phenylalanine and tryptophan and a lower in the percentage of phospholipids. Moreover, oral administration of free NAR and NARNPs significantly increased phospholipids and decreased the levels of tryptophan, phenylalanine and nucleic acid contents. On a comparative basis, NARNPs was found to have a more potent antitumor effect than free NAR in completely preventing the formation of squamous cell carcinoma and in improving the biochemical status to a normal range in DMBA-induced oral carcinogenesis. The results suggest that Raman spectroscopy provides a non-destructive, rapid and sensitive monitoring of drug-response studies and this non-invasive technique can be expanded to in vivo applications for monitoring the biomolecular response to a chemopreventive agent.

Duraipandian et al., (2014) have characterized the Raman spectroscopic properties of cervical tissues associated with the multi-stage progression of cervical pre-carcinogenic sequence. Significant Raman biochemical differences were observed among benign, low-grade squamous intraepithelial lesions (LSIL) and high grade squamous intraepithelial lesions (HSIL) cervical tissues. The results suggest that Raman spectroscopy in conjunction with a semi-
quantitative biochemical model of cervical tissues provide new insights into biomolecular origins responsible for prominent tissue Raman spectral features and their variability with progressive dysplasia. The results further suggests that the Raman spectral biomarkers have identified for monitoring the multi-stage cervical pre-carcinogenesis, forming the foundation of applying NIR Raman spectroscopy for the early diagnosis of cervical precancer in vivo at the molecular level.

Christian et al., (2014) have used shifted-excitation Raman difference spectroscopy (SERDS) for differentiation of cancerous from healthy tissues in ex vivo oral squamous cell carcinoma identification. The results revealed that the approach of spectral acquisition by SERDS yields reproducible and significantly different results in terms of molecular composition in benign and malignant oral tissue- in particular in regions of proteins and lipids. The results suggest that SERDS setup has a prospect to complement the standard approach of histopathological diagnosis.

Lloyd et al., (2014) have used non-consensus pathology measurements to improve the diagnosis of oesophageal cancer by Raman spectroscopic probe. They demonstrated that a fully semi-supervised approach has improved the sensitivity and specificity from 73% and 78% (PC-LDA) to 78% and 84% (semi-supervised) for discriminating between intestinal metaplasia and dysplasia and from 44% and 66% (PCA-LDA) to 63% and 72% (semi supervised) when discriminating between intestinal metaplasia and low grade dysplasia. The results suggest that semi-supervised algorithms allows larger training set databases to be utilised resulting in more robust diagnostic models with improved performance.
From the above review of literature, it is inferred that with the help of Raman spectroscopic technique, it is possible to monitor the biochemical changes associated with tumor progression. In this situation, it has been programmed in the present study to investigate the biomolecular changes in antitumor response of prepared SILNPs relative to efficacy of free SIL during DMBA-induced oral carcinogenesis by near-infrared Raman spectroscopy.

### 7.3. Raman spectroscopy

Raman spectroscopy was discovered in the first quarter of the 20th century by Sir Chandrasekhara Venkata Raman. The scattering or “bouncing” of photons by various media had long been studied by Rayleigh in 1871 (Strutt, 1871), Einstein in 1910 (Einstein and Hopf, 1910) and others. Most light is scattered without a change of wavelengths, which is called “Rayleigh elastic scattering” of light. It occurs when light travels through transparent solids and liquids, and it is most prominently observed in gases. For example, the Rayleigh scattering is responsible for the blue color in the sky: as Rayleigh scattering is inversely proportional to the fourth power of the wavelength and the wavelength of blue light is shorter than red light, blue light will scatter more which results in the blue color of the sky at day-time.

The phenomenon of Raman scattering was discovered by Sir Chandrasekhara Venkata Raman in 1928. When a photon is incident on a polarizable molecule it can excite (de-excite) vibrational modes of the molecules, resulting in scattered photons with decreased (increased) energy by the amount of the vibrational transition energies. Raman scattering is an inelastic scattering process as there is an energy
exchange between the molecule and the photon. If the scattered photon has the same energy as the incident photon, the scattering is called Rayleigh scattering which is an elastic scattering process. However, a small fraction of the scattered light (approximately 1 in 10 million photons) is scattered by an excitation, with the scattered photons having a frequency different from, and usually lower than, the frequency of the incident photons. Stokes Raman shift occurs if energy is transferred from the incident light to the molecule, resulting in red-shifted scattered light, with a longer wavelength and lower energy. In the molecule, a ground state electron is excited from a lower vibrational energy level to a higher one. Anti-Stokes Raman shift occurs when energy is transferred to the light from the molecule. The observed light is blue-shifted, with a shorter wavelength and higher energy. A ground state electron in the molecule is excited from a higher vibrational energy level through a virtual state and ends at a lower level. The molecular bonds vibrate at a lower frequency. In either Stokes or anti-Stokes scattering, only one quantum of energy is exchanged. The absolute energy difference between incident and scattered radiation is the same for stokes and anti-stokes Raman scattering.

The Raman effect is weak. Only approximately one in $10^8$ incident photons is shifted. Of these few Raman-shifted photons, even fewer (relative fraction at room temperature) are anti-stokes shifted because fewer molecules start in the required excited state. The Boltzmann distribution describes the relationship between temperature and the fraction of molecules in an excited state. As temperature increases, the relative proportion of ground and excited states changes and the Stokes to anti-stokes intensity
proportion changes accordingly. The rest of those $10^8$ photons are known as Rayleigh scatter. Since the electrons start and finish at the same vibrational energy level, the photons also start and finish at the same wavelength. Everything is unchanged by the encounter with a molecule. A representative vibrational energy level diagram is shown in Figure 7.1 (Ferraro et al., 2003). The challenge of Raman spectroscopy is to detect the Raman ‘needle’ in the Rayleigh ‘haystack.’

![Energy-level diagram illustrating Raman Scattering](image)

**Fig. 7.1 Energy-level diagram illustrating Raman Scattering**

A monochromatic light source, such as a laser, is used to generate enough Raman scattered photons to be detected. A complete Raman spectrum is symmetric about the wavelength of the incident light, with the stokes portion typically presented on the right side and anti-stokes on the left side when plotted on an increasing wavelength axis. However, the Stokes and anti-stokes portions will not be mirror
images of each other because of their relative intensity differences. A complete Raman spectrum of carbon tetrachloride is in theory, the light interaction with a molecule leads to a polarization of the molecule and then the polarized molecule exhibits an induced dipole moment caused by the external field.

The induced dipole moment $P$ is proportional to the electric field $E$ and to a property of the molecule called the polarizability $\alpha$ as shown in the following equation.

$$E = E_0 \cos (2\pi \gamma_0 t) \quad (7.1)$$

$$P = \alpha E \quad (7.2)$$

where, $E_0$ is the maximum amplitude of $E$, $t$ is time and $\gamma_0$ is the frequency of the light wave. The tensor $\alpha$ is called the polarizability and describes the ease by which the electron cloud of a molecule can be distorted by an applied potential field. These expressions are combined to obtain the following expression for the induced dipole moment:

$$P = \alpha E_0 \cos (2\pi \gamma_0 t) \quad (7.3)$$

As this dipole relaxes it is macroscopically observed as scattered light. A molecular vibrational mode $q$ with vibrational frequency $\gamma_{vib}$ is defined below,

$$q = q_0 \cos (2\pi \gamma_{vib} t) \quad (7.4)$$

where $q_0$ is the maximum vibrational amplitude. For vibrational modes to be Raman active there must be a change in polarizability during the molecular vibration $q$, i.e. $(\partial \alpha / \partial q \neq 0)$. If it is assumed that this change
is small and that the distortion is linear, then the first order approximation for Raman polarizability is defined as,

\[
\alpha = \alpha_0 + \left( \frac{\partial \alpha}{\partial q} \right)_0 q
\]  

(7.5)

where \(\alpha_0\) is the polarizability at equilibrium and all distortions are about the equilibrium position. Equation (7.5) can be rewritten in terms of (7.4) as

\[
\alpha = \alpha_0 + \left( \frac{\partial \alpha}{\partial q} \right)_0 [q_0 \cos (2\pi \gamma_{vib})]
\]  

(7.6)

The expression for the induced dipole (Equation 7.3) can be rewritten using (7.6),

\[
P = \alpha_0 E_0 \cos (2\pi \gamma_0 t) + \frac{1}{2} \left( \frac{\partial \alpha}{\partial q} \right)_0 E_0 q_0 \cos (2\pi \gamma_{vib}) \cos (2\pi \gamma_0 t)
\]  

(7.7)

A trigonometric identity for the multiplication of cosines can be used in this equation to arrive at an expression for scattered light from a molecule with a Raman active vibrational mode (i.e. relaxation of the induced dipole moment).

\[
P = \alpha_0 E_0 \cos (2\pi \gamma_0 t) + \frac{1}{2} \left( \frac{\partial \alpha}{\partial q} \right)_0 E_0 q_0 \cos (2\pi (\gamma_0 + \gamma_{vib}) t) + \\
\cos (2\pi (\gamma_0 - \gamma_{vib}) t)
\]  

(7.8)

The first term in (7.8) corresponds to the elastically scattered (Rayleigh) light which has the same frequency \(\gamma_0\) as the incident radiation. The second term involves light that is shifted towards higher frequencies \(\gamma_0 + \gamma_{vib}\), anti-stokes Raman); while the third term describes light that is shifted towards lower frequencies relative to that of the incident light \(\gamma_0 - \gamma_{vib}\), stokes Raman). A spectrum of these wavelength shifts provides information about the molecular vibrational modes of a sample.
7.4. Laser Raman Spectrometer

Raman spectroscopy is a useful technique for the identification of a wide range of substances—solids, liquids, and gases. It is a straightforward, non-destructive technique requiring no sample preparation. Raman spectroscopy involves illuminating a sample with monochromatic light and using a spectrometer to examine light scattered by the sample.

In modern Raman spectrometers (Fig. 7.2), lasers are used as a photon source due to their highly monochromatic nature, and high beam fluxes. This is necessary as the Raman effect is weak, typically the Stokes lines are $\sim 10^5$ times weaker than the Rayleigh scattered component. In the visible spectral range, Raman spectrometers use notch filters to cut out the signal from a very narrow range centred on the frequency corresponding to the laser radiation. Most Raman spectrometers for material characterisation use a microscope to focus the laser beam to a small spot (<1-100 mm diameter). Light from the sample passes back through the microscope optics into the spectrometer. Raman shifted radiation is detected with a charge-coupled device (CCD) detector, and a computer is used for data acquisition and curve fitting. These factors have helped Raman spectroscopy to become a very sensitive and accurate technique.

7.4.1. Light source

The light source used in Raman spectroscopy is mainly lasers because of their higher power output and narrow bandwidth. The choice of wavelength for Raman measurement depends on specific application. For biologic tissues, a NIR laser is commonly used because of its deep penetration depth (700-1000 μm is regarded as the optical windows of biological tissues) and the lower level of tissue autofluorescence under NIR
excitation. For example, 632 nm, 690 nm, 785 nm, 810 nm, 830 nm and 1064 nm lasers have been reported in *in vivo* and *in vitro* Raman study of human tissues (Short et al., 2005; Zhao et al., 2008). Shorter wavelengths are usually used for *ex vivo* thin tissue samples. Both pulsed and continuous-wave lasers are used in Raman spectroscopy. Pulsed lasers are mainly used in time-resolved Raman instrument or time-gated Raman instrument or time-gated Raman spectroscopy to separate fluorescence from Raman scattering. Ultra-short pulsed lasers can be used in coherent anti-stokes Raman spectroscopy (CARS). For conventional Raman spectroscopy, continuous-wave lasers are used most commonly. The critical requirements for the laser source are its intensity and wavelength stability. Solid-state and diode lasers are proper choices for their portable probability.
Raman spectroscopy (RS) is an optical technique that allows for real-time interrogation of biologic tissues with chemical specificity. Using a diode laser, incident photons are scattered on the tissue of interest and the spectral wavelength output is a reflection of the tissues’ molecular fingerprint.

7.4.2. Excitation light delivery

Optical fibers are commonly used for light delivery in medical applications. The main considerations for choosing optical fibers are numerical aperture, core diameter, core material and its transmission properties. Multimode 100-200 μm core diameter fibers are commonly used for excitation light delivery in biological Raman measurements. The choice of the numerical apertures (0.22 or 0.37) depends on the collection capability (numerical aperture matching) of the laser system and the lens system. The transmission properties depend on the core and cladding materials. Raman signals may arise from the fiber’s core material itself and contaminate the tissue signals. Choosing the right optical fiber is therefore important for in vivo Raman spectroscopy.

7.5. Raman Spectroscopic measurement

Spectra were recorded with an HE-785 commercial Raman spectrometer (LabRam, Jobin-Yvon-Horiba, France) using Inphotonics (Inc, Downy St, USA) fiber optic probe. Briefly, this system consists of a diode laser (Process Instruments) of 785 nm wavelength as excitation source, a high efficiency spectrograph with fixed 950 gr/mm/grating coupled with a charge-coupled device-CCD (CCD-1024X256-BIDD-SYN, Synapse). The spectrograph has no movable parts, and spectral resolution is $\sim 4 \text{ cm}^{-1}$. The commercially available Inphotonics (Inc, Downy St. USA) probe consisting of a 105 μm excitation fiber and
a 200 μm collection fiber (NA-0.40) was used to couple the excitation source and the detection system. Samples were placed on a CaF$_2$ window and spectra were recorded at different points with spacing of 1-2 mm using XYZ precision stage. As per specifications of the manufacturer of the Inphotonics probe, the theoretical spot size and depth of field are 105 μm and 1 mm, respectively. The working distance of the probe is 5 mm and therefore, a detachable spacer of length 5 mm was attached at the tip of the probe to maintain focus during all measurements. Prior to each measurement, these spacers were disinfected by CIDEX (Johnson and Johnson, Mumbai, India) solution to avoid inter-subject contamination. Spectral acquisition parameters were: $\lambda_{\text{ex}}$ -785 nm, laser power 52 mW, spectra were integrated for 3 seconds and averaged over three accumulations from each tissue sample.

7.6. Experimental protocol

The local institutional animals ethics committee (IAEC), (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian council of Medical Research, India. A total number of 36 animals were divided into six groups and each group contained six animals. Group I hamsters served as the control and were painted with liquid paraffin alone, three times a week for 14 weeks on their left buccal pouches. Groups II, III and IV hamsters were treated with 0.5% solution of DMBA in liquid paraffin using a No. 4 sable brush, three times a week for 14 weeks on their left buccal pouches (Shklar, 1999). Group II animals received no other treatment. Groups III and IV
hamsters were orally given silibinin at a dose (50 mg/kg body weight/day) and SILNPs (dose equivalent to 50 mg/kg body weight/day of SIL), dissolved in 1 ml of 5% dimethyl sulphoxide (DMSO) and dissolved in distilled water, respectively, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Groups V and VI hamsters received the same dose of SIL (50 mg/kg body weight/day) and SILNPs alone (dose equivalent to 50 mg/kg body weight/day of SIL) as in groups III and IV throughout the experiment period. The dose of silibinin used in this study was chosen based on a dose response study undertaken by us, which demonstrated maximum chemopreventive efficacy at this dose which has been reported in previous studies (Kaur et al., 2010; Sangeetha et al., 2012). The experiment was terminated at the end of 16 weeks and hamsters were sacrificed by cervical dislocation after an overnight fast. The buccal pouches were excised and the tissues were further processed for experiments. One portion of the tissue specimens from each group were frozen in liquid nitrogen and then stored at -80 °C until Raman spectroscopic analysis. The remaining tissue samples were immediately fixed in 10% neutral buffered-formalin and embedded in paraffin. In group V and group VI, the epithelium was normal, intact and continuous as discussed briefly in chapter 4. Therefore, groups I, II, III and IV were chosen for the further Raman spectroscopic analysis.

7.7. Data analysis

Ex vivo spectra from the control and the experimental groups were corrected for CCD response with a NIST-certified SRM 2241 material followed by subtraction of spectral contribution from optical elements. To minimize the influence of slow-moving background, first
Fig. 7.3 Experimental set up of a Raman spectrometer
derivative of preprocessed Raman spectra were calculated (Savitzky-Golay method) (Nijssen et al., 2007; Deshmukh et al., 2011), interpolated in the range of 1800-500 cm\(^{-1}\) (Raman fingerprint region) and vector normalized. Analysis of the preprocessed spectra was carried out using multivariate analysis tool PC-LDA implemented in SPSS-17.0

7.8. Results

7.8.1. Raman spectroscopy

The averaged Raman spectra of the control and the experimental groups (DMBA-alone, DMBA+SIL and DMBA+SILNPs) treated hamster buccal mucosa after normalized with respected to the intensity of the peak at 1458 cm\(^{-1}\) in the spectral range 1800-500 cm\(^{-1}\) are shown in Fig. 7.4. This is an intense peak appearing in the average spectra and it has been used to determine intensity alterations of other bands. This band has been also used generally as a normalization standard (Yao et al., 2009; Akyuz et al., 2011). As seen from Fig. 7.4, the Raman spectra obtained from hamster buccal mucosa are quite complex in nature, consisting of a superposition of large and small biomolecules. Each biomolecules has unique vibrations, corresponding to molecular vibrations such as proteins, nucleic acids, lipids and other specific amino acids. Prominent Raman bands associated with these biomolecules were observed in the buccal mucosa and are listed in Table 7.1 with tentative vibrational assignments. Distinctive Raman spectral features and intensity differences between the control and the experimental groups which can reflect cellular and sub-cellular changes associated with tumor transformation followed by free SIL and SILNPs treatment. The normalized Raman intensities of selected peaks from the control and the experimental buccal mucosa in each group are given in Fig. 7.5.
Table 7.1 Peak positions and tentative assignments of major vibrational bands observed in the control and the experimental groups

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<thead>
<tr>
<th>Control</th>
<th>DMBA</th>
<th>DMBA+SIL</th>
<th>DMBA+SILNPs</th>
<th>Tentative assignments</th>
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<tr>
<td>1740</td>
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<td>1758</td>
<td>1745</td>
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<td>C=O stretching (Amide I, proteins)</td>
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<td>Ring breathing mode in DNA bases (Thymine, Guanine)</td>
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In the present study, the weak Raman band observed at 1740 cm\(^{-1}\) in the control tissues (Group I), assigned to the C=O stretching vibration of phospholipids (Movasaghi et al., 2007; Jachtenberg et al., 2013) disappeared completely in the DMBA-alone (Group II) and the DMBA+SIL (Group III) treated tissues, indicating that the Group II and III treated tissues may be associated with a decrease in the relative amounts of phospholipids. This could be further confirmed by the reduction of intensity of other phospholipid-related peaks such as 1070 cm\(^{-1}\) in DMBA-alone treated tissues. In addition, these bands (~1745 and ~1055 cm\(^{-1}\)) has been observed in the DMBA+SILNPs (Group IV) treated tissues and these results further confirm the presence of phospholipids rich tissues in the control and the nanoparticulate silibinin treated groups.

The band appeared in the region between 1720 and 1600 cm\(^{-1}\) which mainly arise from the amide I vibrational modes of tissue proteins (Movasaghi et al., 2007). Amide I band is a classical Raman marker for protein conformation. As can be seen in Fig. 7.4 and 7.5, the relative Raman intensity at 1663 cm\(^{-1}\) due to the amide I band exhibited higher signal for DMBA-alone induced tumor tissues compared to control tissues. In addition, there is also a relative increase of CH\(_2\)CH\(_3\) bending modes of protein (~1320 cm\(^{-1}\)) in intensity, suggesting that DMBA-alone induced tumor tissues may be associated with an increase in the relative amount of proteins. Further, it is observed that the protein bands at ~1661 and ~1320 cm\(^{-1}\) show significantly decrease in the intensity with respect to tumor tissues (group II), suggesting a decrease in the protein content upon free SIL and SILNPs treatment. The changes in the amide I band could be attributed to conformational changes to the protein structures. Moreover, the DMBA-induced tumor spectra also show significant elevated peaks at 1195, 1001, 621 and 568 cm\(^{-1}\) which might be attributed to the specific amino acids,
phenylalanine and tryptophan, respectively (Su et al., 2012; Jachtenberg et al., 2013; Xue et al., 2014). The increases in intensities of the phenylalanine and tryptophan bands are found to play pivotal roles in tissue classification. This result suggests that tumor progression could be related with an increase in the relative amounts of phenylalanine and tryptophan.

Furthermore, Raman bands associated to nucleic acids at 1577, 778 and 683 cm$^{-1}$ which are respectively due to the ring breathing modes of the DNA bases (Devpura et al., 2012). These relative nucleic acids bands have higher intensities in the Raman spectrum of the DMBA-induced tumor tissues compared to those of control and the silibinin treatment groups. These results indicate that the DNA was higher in tumor tissues. On the other hand, the intensities of several vibrational modes related to nucleic acids decrease in free SIL and SILNPs treated tissues as compared to the DMBA-induced well-differentiated SCC. However, the Raman spectral features of the hamsters treated with SILNPs are much closer to those of control but with significant changes in the band intensities (Fig. 7.4 and 7.5). These intensity variations between spectral peaks of the control and the experimental tissues in each group which further indicate alterations in specific biochemical constituents when the hamster buccal pouch is in different pathological onset. These observable Raman spectral results further confirm that Raman spectroscopy could be used to monitor and predict therapeutic response under free and SILNPs treatments.

It should be noted that these individual peak intensities analysis is not sufficient to resolve complex spectral shifts, because it provides limited peak information. For this purpose, different multivariate statistical algorithms have been extensively investigated. The development of robust algorithms is not only producing a high predicted diagnostic accuracy, but also provide
useful biomolecular diagnostic information from the high-dimensional Raman spectral datasets is highly desirable.

7.8.2 Multivariate analysis

To develop effective diagnostic algorithms for tissue classification between the control and the DMBA-induced treated tissues in each groups, the diagnostically significant PCs are fed into the LDA model together with leave-one-out cross-validation (LOOCV) technique was carried out. Fig. 7.6 shows the two-dimensional scatter plot of the two linear discriminant functions (function 1 vs function 2) based on the PC-LDA model, in which obvious clustering of the tissue types are separated into four different groups. Moreover, LOOCV method was used for evaluation of a performance of the classification models without losing the diversity in the data. In this analysis, 17 out of 20 spectra are correctly classified as the control. Seventeen out of 21 spectra are correctly classified as DMBA-alone induced tumor tissues, whereas 4 out of 21 are misclassified as DMBA+SIL treated tissues. Eleven of 19 spectra are correctly classified as DMBA+SIL treated tissues, whereas 6 of 19 are misclassified as DMBA+SILNPs treated tissues and 2 out of 19 are misclassified as DMBA-alone induced tumor tissues. Thirteen of 20 spectra are correctly classified as DMBA+SILNPs treated tissues, whereas 3 of 20 are misclassified as DMBA+SIL treated tissues and 4 out of 20 are misclassified as DMBA-alone induced tumor tissues and results are shown in Table 7.2.

In order to illustrate the use of PC scores for further diagnostic classification, 2D plots of the first two PC components (PC1 vs PC2) for the entire range (1800-500 cm\(^{-1}\)) are shown in Fig. 7.7. Almost all the control tissues data points are completely separated from the data for the DMBA-alone treated tissues. Moreover, DMBA+SILNPs treated tissues data nearly in the range of the control tissues data points. Pairwise discriminant function
Fig. 7.6  Scatter plot of discriminant (Function 1 vs Function 2) for Raman data from the control and the experimental groups based on LDA
scatter plots for the control and the experimental groups generated using PC-LDA are given in Fig. 7.8. It gives excellent discrimination between the control and the experimental groups. The pairwise discriminant score is observed considerably above the discrimination line for DMBA-alone treated tissues and below the line for the control and DMBA+SILNPs treated tissues. In the present analysis, LOOCV method was used to validate findings of LDA. These values were applied for binary calculations to obtain the performance level of PC-LDA. Using a LOOCV combined with PC-LDA, the sensitivity of 95%, 89%, 100% and 90% and a specificity of 100%, 66%, 85% and 76%, respectively. A high level of sensitivity and specificity was achieved for classifying and differentiating between the control vs DMBA, DMBA vs DMBA+SIL, DMBA vs
DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues. The positive predictive value (PPV) and negative predictive value (NPV) of the prospective data set for the diagnosis of control vs DMBA, DMBA vs DMBA + SIL, DMBA vs DMBA + SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues are 100%, 70.80%, 86.95% and 78.13% and 90.90%, 87.50%, 100% and 85.67%, respectively (Table 7.3). Moreover, accuracy using this position of pairwise discriminant scores is 100%, 77.83%, 92.28% and 87.38%, respectively. In addition, a comparative evaluation of the ROC curves (Fig. 7.9) indicates that PC-LDA based diagnostic algorithm gives more effective diagnostic performances for differentiation of the control and the experimental groups, as illustrated by the improvement in the diagnostic sensitivities and specificities. The integration areas under the ROC curves are 1.0, 0.94, 1.0 and 0.96 for PC-LDA based diagnostic algorithms of control vs DMBA, DMBA vs DMBA + SIL, DMBA vs DMBA + SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues, respectively. These results further demonstrate that ROC curves yield a better diagnostics accuracy than the PC-LDA.

7.9 Discussion

A biomarker is an indicator of a biological state of disease. It is characteristic of a specific state and therefore can be used as a marker for a target disease. Biomarkers can be used to study cellular processes, and monitor or recognize disruption or alterations in the cellular processes of cancer cells. These biomarkers play a very critical role in drug development and treatment as they are the indicators of the normal and cancerous physiologies of an organ. Raman spectroscopy has the potential to identify the biomarkers of a disease. Raman spectroscopy is a vibrational spectroscopic technique capable of providing molecular level information,
Fig. 7.8 Pairwise discriminant score plot based on linear discriminant analysis for the pairs control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs. The discrimination line at 0 gives good discrimination between the groups.
Fig. 7.9 The receiver operating characteristic (ROC) curves showing the diagnostic performance of discrimination results for Raman spectra using the PC-LDA enabling investigation of functional groups and molecular conformations. Molecule-specific spectral bands provide important biochemical information, because each molecule has its own vibrations that can serve as a Raman biomarker. The primary basis for Raman spectroscopic detection is the array of biochemical changes that take place as tissue undergoes tumor transformations and moreover, used not only to further explore pathological processes and their progression, but also to further transform for directing real-time therapeutic intervention. The present study seeks to explore the
potential role of Raman spectroscopy for monitoring the biomolecular changes under free SIL and SILNPs treatment and predicts its therapeutic response against experimental oral carcinogenesis.

The results of this study confirm that there are specific spectral and intensity differences in Raman spectra between the control and the experimental groups, which can reflect changes of cellular and sub-cellular constituents associated with free silibinin and SILNPs treatment during the process of DMBA-induced oral carcinogenesis. The qualitative and quantitative changes of such biochemical constituents are important aspects of tumor transformation. In the present study, Raman signals from phospholipids (\( \sim 1740 \text{ cm}^{-1} \)) which most probably arise from the cytoplasm within the epithelial cells in normal tissues has been found to be lower for the tumor tissues than control tissues. This phenomenon strongly reflects a decreased vibrational stability of lipid chains in DMBA-induced tumor tissues. The lipid content of tumor tissues might be a useful indirect measure of tumor progression. It is well known fact that lipids are the main constituents of cell membrane and required for maintaining cell shape and regulation of various cell processes. A decrease in the phospholipid concentration can be attributed to dysfunction of the cell membrane in malignancy, leading to an increase in phospholipids degradation. The lipid reduction in tumor tissues could be also related to the fast growth of tumor cells which need more energy (Kast et al., 2008). Earlier studies have shown that phospholipids contents were lower in various cancers tissues, including human OSCC (Devpura et al., 2012; Krishnakumar et al., 2013; Singh and Krishna, 2014). Further, there is a relative increase of amide I band (\( \sim 1663 \text{ cm}^{-1} \)) in intensity, suggesting that DMBA-induced tumor tissues may be associated with an increase in the relative amount of proteins. Moreover, the tumor cells utilize certain aminoacids for its protein synthesis, which increases manifold in
tumor for their proper progression and proliferation. This fact is reflected by the significant increase in the relative intensities of tryptophan (1195 and 568 cm\(^{-1}\)) and phenylalanine (1001 and 621 cm\(^{-1}\)) bands. The increased concentration of these specific amino acids, phenylalanine and tryptophan are the common observation in malignant tissues (Huang et al., 2013; Lasalvia et al., 2014). It is strongly believed that the increased content of these amino acids by tumor tissues has been related to inflammation and immune system evasion (Szachowicz-Petelska et al., 2010). The resulting oxidative stress could impair the activity of phenylalanine (4)-hydroxylase (PAH) and tryptophan oxygenase, which then gives rise to increased phenylalanine and tryptophan concentrations. This is in accordance with previous results from other groups who found that the tumor tissues contained higher concentration of these type of amino acids (Munoz-Pinedo et al., 2012; Shafik et al., 2014). Moreover, these specific amino acids vary in the silibinin treated groups, indicating that the tryptophan and phenylalanine content can be restored the control level upon free silibinin and SILNPs treatment (Fig. 7.5).

The frequency of the amide I band is sensitive to proteins conformation and it is very useful for the determination of protein secondary structure. Secondary structure analysis based on the deconvolution of the amide I bands have been extensively reported (Oliveira et al., 2006; Mehrotra et al., 2010; Singh and Krishna, 2014). Some specific frequencies contained in the amide I bands are related to the underlying secondary structure such as helical structure, beta structure and other conformations. In the present study, for the determination of protein secondary structure, the curve-fitting analysis was performed in amide I band (1720-1600 cm\(^{-1}\)). The curve-fitting procedure applied on the protein bands allows the study of conformational changes. The underlying bands of amide I bands as deduced by curve-fitting analysis for the control and the experimental groups are given in
Fig. 7.10. The changes in the amide I band were associated with secondary structure changes related to changes in protein composition could be a useful biomarker for tumor progression. As seen from Fig. 7.10, the peaks at 1687 cm$^{-1}$ arise from anti-parallel β-sheet structure; the peaks observed at 1648 cm$^{-1}$ are assigned to α-helix structure; the peaks at 1670 cm$^{-1}$ arise from β-turn structure; the peak at 1625 cm$^{-1}$ is assigned to aggregated β-sheet structure (Chen et al., 2014). It is observed from Fig. 7.11 that the percentage area of β-sheet and β-turn secondary structure increased in the DMBA-induced oral tumors. Further, the percentage area of α-helix structure decreased. These results suggest that DMBA-induced oral carcinogenesis causes important changes in the protein profile in favor of β-sheet and turn structure. The changes in the protein secondary structure from α to β-conversion might be indication of some important structural alterations in the existing proteins and/or the expression of new types of proteins/aminoacids during tumor development. The appearance of these proteins in the β-conformation may signify more chemical interaction between the proteins and the microenvironment occurring in the cells, which could be related to increase of mitotic activity, one of the cellular alteration characteristics of oral carcinogenesis. Further, the amounts of β-sheet and β-turn secondary structures were decreased in free SIL and SILNPs treatments. Moreover, DMBA + SILNPs treated tissues remarkably reduced the amount of β-sheet and β-turn secondary structure when compared to DMBA + SIL treated tissues. It can be also being seen from Fig. 7.10 that the tumor tissues spectra have increased intensity from peaks associated with nucleic acids and DNA structures. The increased intensity from nucleic structures may suggest an increase in cellular density from the tumor tissues, in agreement with increased cell proliferation. This result suggests that spectral features of nucleic acids may be considered as a sensitive marker for discrimination
Fig. 7.10 The underlying amide I bands in the 1720 -1600 cm\(^{-1}\) region deduced by curve fitting analysis for the control and experimental animals in each group.

Fig. 7.11 Bar diagram of protein secondary structural variations for Control, DMBA, DMBA+SIL and DMBA+SILNPs treated groups. Values are shown as mean ± standard deviation: the degree of significance was denoted as p<0.05, for comparisons of control and experimental groups.
between normal and tumor tissues. On the other hand, nucleic acid contents were reduced upon free SIL and SILNPs treatment. These results may be associated with its antioxidant properties. The higher antioxidative effects produced by SILNPs may be due to the features of nanoparticles, such as higher accumulation in tumor tissue via EPR effects, as well as the features of functionalized silibinin, such as the abilities to effectively inhibit protein synthesis, controlling cell proliferation and tumor growth.

The simplistic empirical analysis above only uses a limited number of Raman peaks or wave band information, for tissue diagnosis; most of the information contained in the Raman spectra has not been utilized. Since biological tissue is complex, it is likely that there are many biochemical species influencing diseases concurrently. Therefore, the complete database was analyzed in order to examine the classification potential of Raman spectroscopy in tandem with advanced statistical techniques. The multivariate statistical analysis (PC-LDA) that utilizes the entire spectrum to determine the most diagnostically significant spectral features may improve the diagnostic efficiency of Raman technique for tissue analysis and classification. Score plots in PCA model provide visualization of the data, whereby the loading of data is an indicator of biochemical similarity.

In this study, canonical discriminant functions, the centroid of each group gives the average of the discrimination value with respect to the concerned groups. Moreover, minimally overlapping clusters belonging to the control and DMBA+SILNPs treated spectra were observed are shown in Fig. 7.6. Average classification efficiency of ~ 82 % was observed. These results based on LOOCV method are also summarized in Table 7.2. In the PCA calculation, the PCs were arranged in descending order related to their weight in building the calculated spectrum. Therefore, first two PCs have the largest weight, and generated in order to differentiate between the control
and the experimental groups. Further, these results suggest that the largest spectral differences between the groups are in the entire range where the differentiation is best, according to PC1 vs PC2 (Fig. 7.7).

Pairwise discriminant function scatter plots between the numbers of samples versus discriminant scores from the linear discriminant function (Fig. 7.8). The differentiation of the control from tumor tissues using PC-LDA yielded overall sensitivity of 89% to 100% and specificity of 66% to 100%. A high level of sensitivity and specificity was achieved for classifying and differentiating between the control, DMBA, DMBA+SIL and DMBA+SILNPs treated tissues. Positive, negative predictive values and accuracy values were obtained using this position of pairwise discriminant score. Furthermore, the best integration areas under the ROC curves are 1.0, 0.94, 1.0 and 0.96 for the control, DMBA, DMBA+SIL and DMBA+SILNPs treated groups. Although the results of the evaluation functions are close, we believe that the AUC is more suitable to assess the diagnostic model.

7.7 Conclusion

In conclusion, the present study has shown that Raman spectroscopy can be used to detect the biochemical composition and structural changes at the molecular level in response to silibinin treatment during DMBA-induced oral carcinogenesis. Specific spectral and intensity differences have been observed in Raman spectra between the control and the experimental groups. A significant increase in the amount of proteins/aminoacids and nucleic acid contents and a decrease in the amount of phospholipids contents are observed in DMBA-alone induced tumor tissues. Further, the composition and secondary structure of proteins were found to be altered during DMBA-induced oral carcinogenesis. Moreover, oral administration of free SIL and SILNPs significantly increased phospholipids and decreased the levels of
proteins and nucleic acid contents. On a comparative basis, SILNPs was found to have a more potent antitumor effect than free SIL in completely preventing the formation of oral carcinoma and in improving the biochemical status to a normal range in DMBA-induced oral carcinogenesis. The present study further shows a great potential of Raman spectroscopy associated with PC-LDA diagnostic algorithms as a complimentary tool for monitoring drug-induced modifications against cancers.