RESEARCH
O&G Forum 2024; 34 – 2s: 587 - 592

ASSESSMENT OF INFRARED SPECTRAL VARIATION OF AGE AND GENDER OF NORMAL HUMAN SALIVA.

RUNNING TITLE: Infrared spectral variation of normal human saliva.

Kavishri S[1], Dr. Abirami Arthanari[2], Dr. Elangovan Dilipan[3]
[1]Graduate, Saveetha Dental College,
Saveetha Institute of Medical and Technical Science,
Saveetha University,
Chennai 77, Tamil Nadu, India.
[2]Senior Lecturer
Department of Forensic Odontology,
Saveetha Dental College and Hospitals,
Saveetha Institute of Medical and Technical Sciences, Chennai - 600077.
Email:abiramia.sde@saveetha.com
[3]Department of Physiology,
Saveetha dental college and hospitals,
Saveetha institute of medical and technical sciences, Chennai - 600077.
Email:dilipane.sdc@saveetha.com

CORRESPONDING AUTHOR:
Dr. Abirami Arthanari,
Senior Lecturer, Department of Forensic Odontology,
Saveetha Dental College and Hospitals,
Saveetha Institute of Medical and Technical Sciences,
Chennai - 600077.Phone: 6366811772.
Email: abiramia.sde@saveetha.com

Abstract

INTRODUCTION: The composition of saliva can change with age, and these changes may be reflected in the infrared spectral characteristics. Factors such as hormonal changes, overall health, and diet can contribute to these variations.Gender-related differences in saliva composition may also manifest as spectral variations. Hormonal differences between males and females, as well as potential variations in the abundance of certain biomolecules, could contribute to these differences.Molecular identification: FTIR spectroscopy allows for the identification and characterization of different chemical functional groups present in a sample. ATR-FTIR spectroscopy provides enhanced sensitivity to surface layers and thin films due to the interaction of infrared light with the sample at the crystal-sample interface. It allows for the detection and characterization of surface contaminants, coatings, or modifications.

MATERIALS AND METHODS: The investigated group included 4 healthy female volunteers and 4 healthy male volunteers whose salivary samples were freshly collected. Then, about 5 ml of whole saliva into the sterile tubes.Subsequently, all materials were frozen at −20 °C until analysis time.Samples are freeze dried in lyophilizer (to make it as powder or solid form).The freeze dried samples were dissolved in methanol and samples were analyzed in ATR-FTIR.In ATR-FTIR method, the salivary samples were additionally dried on optical slides.

DISCUSSION: The spectral band pattern of human saliva samples showed major variations among male and female samples.Comparing to male samples female samples showed more spectral band variation between 900 to 1750 cm-1.The peak observed at spectral interval between 2800 to 3500 cm-1.Originate from C-H, O-H and N-H stretching modes of proteins while strong banded at 3250 cm-1 is attributed to amide A (N-H) stretching.Nevertheless ,due to the fact that most of the salivary proteins are glycosylated , these peaks can be attributed to glycosylated alpha amylase, mucin or other sugar residue vibrations.

CONCLUSION: Study can be further investigated to a broad scale of different age and time intervals among healthy and unhealthy people.From this study that has been carried out , we can conclude that saliva can be regarded as a first line diagnostic tool in patients who are suspicious of salivary gland tumors.

KEY WORDS:
Infrared spectral variation; Human saliva ; FTIR spectroscopy ; ATR-FTIR method.

INTRODUCTION:

Infrared spectroscopy is a technique that analyzes the interaction of infrared light with matter, providing information about the molecular composition and structure of a sample.While there has been significant research on using infrared spectroscopy for various applications, including disease diagnosis, drug monitoring, and biomarker identification, its application specifically for assessing age and gender from saliva spectra is relatively limited. Nonetheless, I can provide some insights into the general aspects you might consider in such an assessment(1).
Age-related variations: The composition of saliva can change with age, and these changes may be reflected in the infrared spectral characteristics. Factors such as hormonal changes, overall health, and diet can contribute to these variations. To assess age-related spectral variations, you would need to collect saliva samples from individuals across a wide range of ages and compare the infrared spectra between different age groups. Statistical analysis and pattern recognition techniques can be employed to identify any consistent spectral differences(2).
Gender-related variations: In addition to age, gender-related differences in saliva composition may also manifest as spectral variations. Hormonal differences between males and females, as well as potential variations in the abundance of certain biomolecules, could contribute to these differences. To investigate gender-related spectral variations, you would need to collect saliva samples from males and females of different age groups and compare their infrared spectra. Machine learning algorithms or chemometric techniques can be employed to classify the spectra based on gender(1,3).ATR (Attenuated Total Reflection) FTIR spectroscopy is a variation of Fourier Transform Infrared Spectroscopy (FTIR) that is commonly used for analyzing samples that are difficult to study using traditional transmission spectroscopy. ATR-FTIR spectroscopy is particularly useful for analyzing solid samples, liquids, and even semi-solid or gel-like samples(4).The ATR technique involves placing the sample in direct contact with a high refractive index crystal, such as diamond or germanium, which serves as an internal reflection element (IRE). In ATR, the incident infrared light is internally reflected multiple times within the crystal and interacts with the sample at the crystal-sample interface(5). This interaction results in the absorption of infrared light by the sample, which can be detected and analyzed to obtain the infrared spectrum.
Here are some key features and advantages of ATR FTIR spectroscopy:
Sample Compatibility: ATR-FTIR spectroscopy allows for the analysis of a wide range of samples, including solids, liquids, pastes, gels, and powders. It requires minimal sample preparation, as the sample is simply placed in direct contact with the crystal, eliminating the need for complex sample preparation techniques(6).Non-destructive Analysis: ATR-FTIR spectroscopy is a non-destructive technique that does not require the destruction or alteration of the sample. It allows for the analysis of precious or limited samples without the need for extensive sample handling(7).
Surface Sensitivity: ATR-FTIR spectroscopy provides information about the molecular composition of the sample in close proximity to the crystal surface. It is particularly useful for analyzing thin films, coatings, or surface layers, allowing for the detection of surface contaminants or the characterization of surface modifications(8).Rapid Analysis: ATR-FTIR spectroscopy offers fast analysis times, as the measurements can typically be performed within seconds to minutes. This enables high-throughput analysis and efficient screening of samples(9).
Quantitative Analysis: ATR-FTIR spectroscopy can be used for quantitative analysis by correlating the intensity of absorption bands with the concentration of analytes in the sample. Calibration curves or chemometric methods can be employed to establish the relationship between spectral features and analyte concentration(10).
Simplified Sample Handling: Compared to transmission spectroscopy, ATR FTIR spectroscopy simplifies sample handling by eliminating the need for sample preparation techniques such as grinding, dilution, or pellet formation.Salivary diagnostics for oral and systemic diseases are thoroughly identified and the role of salivary gland tissue engineering for future diagnostics (11).

AIM AND OBJECTIVE:

To assess the infrared spectral variation of age and gender of normal human saliva.One common aim is to identify the presence of unknown compounds in a sample.The objective of this research is to assess the infrared spectral variation of normal human saliva and investigate any potential correlations or differences associated with age and gender, aiming to explore the feasibility of using infrared spectroscopy as a non-invasive method for age and gender determination.

MATERIALS AND METHODS:

Saliva sample preparation:

The investigated group included 4 healthy female volunteers and 4 healthy male volunteers whose salivary samples were freshly collected. Then, about 5 ml of whole saliva into the sterile tubes.Subsequently, all materials were frozen at −20 °C until analysis time
Then the saliva samples were kept in a cooling centrifuge 1600 rpm for 15 mins at 4 degree Celsius(to neglect food particles).0.1 percent triton X was added to prevent viral contamination.
200 microliter samples were taken and added in a fresh microfuge tube.Then the samples were kept in an ultrasonicator box for 1 hr at 50 degree Celsius(for breaking the molecules).Samples are freeze dried in lyophilizer (to make it as powder or solid form)(11).

ATR-FTIR Measurements:

Thermo Scientific Nicolet iS5 spectrometer with an iD5 ZnSe ATR attachment was used to capture the ATR-FTIR spectra of saliva samples. The regis- ter spectral signal in the range of 4000 cm-1 to 700 cm-1 was applied to the deuterated triglycine sulfate (DTGS) detector. Before a series of measurements, an appropriate measurement process was created. The ATR crystal was specifically coated with 20 l of fresh saliva and left to air dry at ambient temperature. The spectral resolution was 4 cm1 and there were 64 images total. For each freshly prepared sample, the spectra were taken three times, averaged, and then one spectrum per patient was produced. The amide I band (1647 cm1) was optimized for the spectra.No smoothing technique was applied to enhance the visual quality of any of the given spectra(12).

Data reduction and treatment schemes:

Using the software program OMNIC 9.0, the captured spectra were graphically interpreted and their data reduced. The second derivatives (Savitzky-Golay, 7 points) and curve-fitting analysis (a Gaussian band shape) were performed using the OMNIC 9.0 program.

Real-time PCR study:
In saliva samples from both patients and healthy controls, the level of Bcl-2 gene expression, an antiapoptotic factor that is closely linked to the proliferation process, was assessed. The analyses were done at the steps listed below(2).

RNA isolation:
The RNeasy Mini Kit (Qiagen, Netherlands) was used to extract the total RNA from fresh salivary samples in spin columns with lysis buffers in accordance with the manufacturer's instructions. The Spark 10 M spectrophotometer (Tecan, Switzerland) was used to measure the amount (ng/l) and quality (1.8-2.0) of RNA at 260 and 280 wavelengths(5,12).

Reverse transcription:
The next stage was using a Precision nanoScript kit (PrimerDesign, UK) with a 50 ng RNA template and additional reagents in 0.2 ml PCR tubes. Each sample underwent annealing at 65 °C for 5 min in a thermocycler (Bio-rad, USA), followed by incubation at 42 °C for 20 min and 75 °C for 10 min for the extension stage(12).

Real-time PCR:
cDNA template (25 ng), forward and reverse primers (per 0.25 M), Master MIX SYBR Green (10 l), and RNAse/DNAse free water (up to 20 l) were used in the final phase of the Precision PLUS qPCR technique (PrimerDesign, UK). The reaction's temperature-time conditions were established as follows: Enzyme activation takes place for 2 min at 95 °C, followed by denaturation for 10 s at that temperature and primer annealing for 1 min at 60 °C.

Analysis:
After normalizing to the reference gene, the comparative threshold cycle (CT) was used to measure the relative expression of the Bcl-2 gene.
(GAPDH) using the 2-CT Livak technique. There were three copies of each experiment run(7).

RESULTS:
Fig. 1 shows the ATR-FTIR spectra of saliva samples: spectrum averaged from 4 healthy male and 4 healthy female volunteers samples.

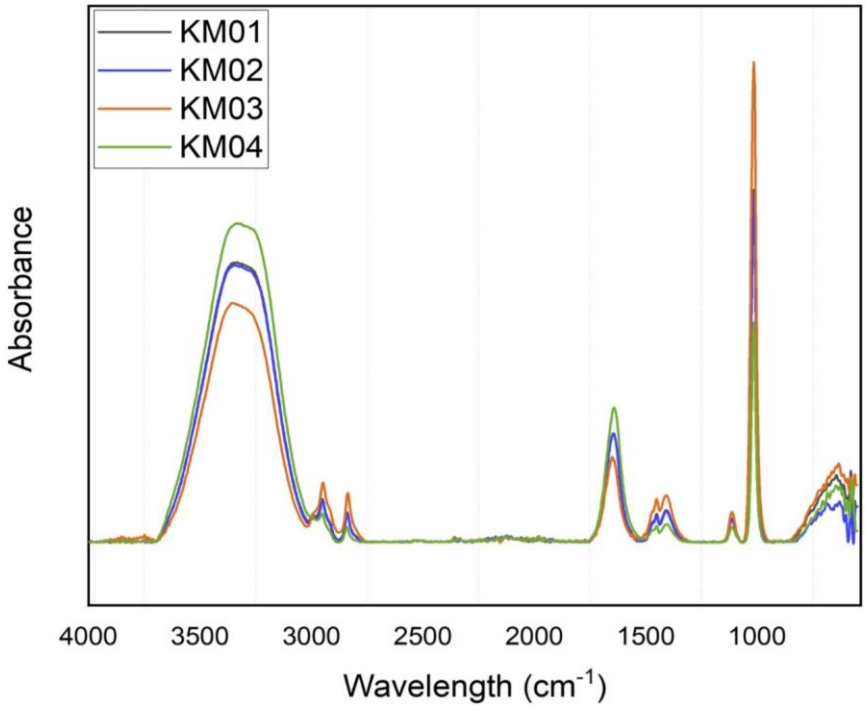

Figure 1: ATR-FTIR spectra characters of male saliva samples.

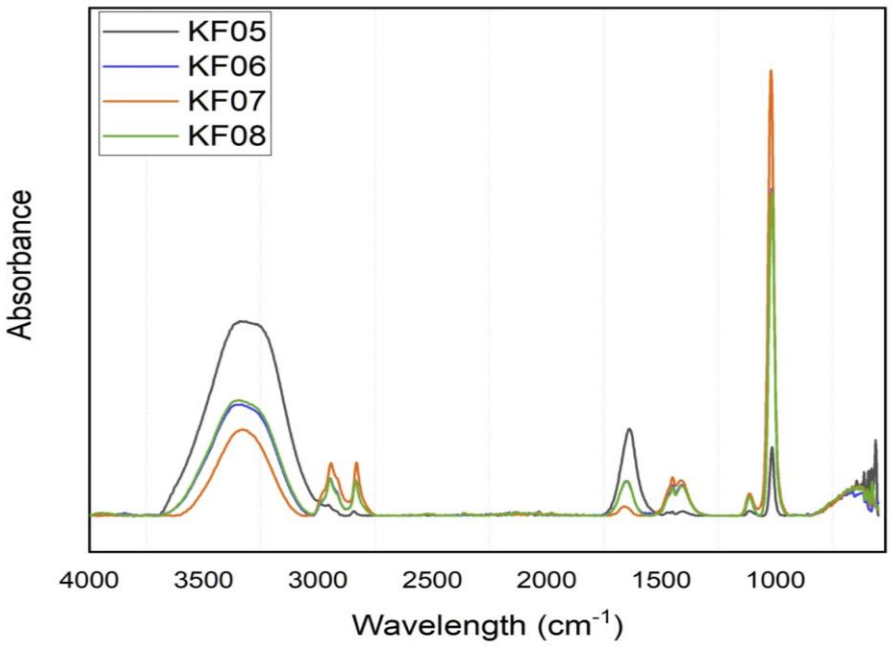

Figure 1: ATR-FTIR spectra characters of female saliva samples.

The spectral band pattern of human saliva samples revealed significant differences between the samples from men and women.Female samples displayed more spectral band fluctuation between 900 and 1750 cm-1 as compared to male ones.

This might be brought on by changes in hormones like estrogen and other metabolic substances.The bands attributed to the components of proteins and lipids dominate the high frequency range.

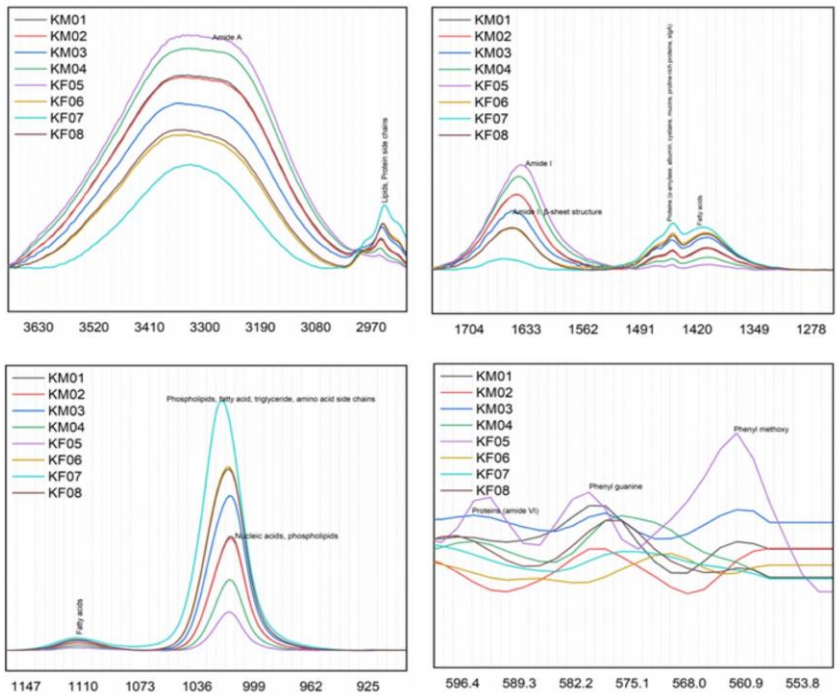

Figure 3: ATR-FTIR spectra characters of human saliva samples. Curve fitting analysis of different wavelength band pattern shows presence of functional groups in saliva samples.

The peak was measured at a spectral range of 2800 to 3500 cm-1.origin from the protein's C-H, O-H, and N-H stretching modes, while the strong band at 3250 cm-1 is attributed to the N-H stretching of the amide A ring.
Furthermore, the C-O stretching vibrations from carbohydrates may possibly be responsible for peaks seen in the 1300 to 1400 cm-1 range.
Consequently, sugar moieties are where the bands at 1480, 1456, and 1431 cm-1 come from.
However, as the majority of salivary proteins are glycosylated, these peaks can be attributed to vibrations of glycosylated mucin, alpha amylase, or other sugar residues.
The characteristics of saliva from healthy people's infrared (IR) spectra were contrasted based on gender and age.

DISCUSSION:
According to the results of my research,the spectra clearly show that the band positions and relative intensities have changed significantly.The spectral band pattern of human saliva samples showed major variations among male and female samples.Comparing to male samples female samples showed more spectral band variation between 900 to 1750 cm-1.This might be due to hormonal variations such as estrogen and other metabolic compounds.The high frequency range is dominated by bands assigned to proteins and lipids constituents.The peak observed at spectral interval between 2800 to 3500 cm-1.Originate from C-H, O-H and N-H stretching modes of proteins while strong banded at 3250 cm-1 is attributed to amide A (N-H) stretching.Furthermore peaks observed within the 1300 to 1400 cm-1 interval may also be attributed to the C-O stretching vibrations from carbohydrates. Consequently the bands at 1480 ,1456 and 1431 cm-1 originate from sugar moieties .Nevertheless ,due to the fact that most of the salivary proteins are glycosylated , these peaks can be attributed to glycosylated alpha amylase, mucin or other sugar residue vibrations.
Based on gender and age, the properties of the infrared (IR) spectra of saliva from healthy individuals were compared. The absorption bands of proteins and lipids are revealed to exhibit statistically significant differences between male and female groups. At the same time, males have higher absorbance in the bands corresponding to proteins and nucleic acids, whereas females have higher absorbance in the bands corresponding to lipids(2).
It was feasible to compare the characteristics of the FTIR spectrum (intensity, area of bands of absorption) with the biochemical composition of the material using the example of human saliva. It has been shown that changes in the conformational shape and isoelectric point of salivary proteins can be inferred from the substantial correlations between the content of -amino acids and chloride ions and the absorption band at 1637 cm1(5).
Saliva from healthy and diseased individuals can be distinguished by changes in a number of key metabolic components, including fat, proteins, glucose, thiocyanate, and carboxylate, as indicated by spectral analysis. Based on infrared spectroscopy, the overall accuracy for the diagnosis of diabetes was 100% on the training set and 88.2% on the validation set. As a result, we have proven that infrared spectroscopy may be used to create complicated biochemical profiles in saliva and identify a number of possible spectral signatures related to diabetes.(13)
Results showed that psoriatic and diabetic patients have different protein secondary structure compositions when compared to the control group. In actuality, the lack of the amide II band and the presence of several secondary protein-structure conformations distinguish the saliva spectra of the control group and those of the palmoplantar psoriasis patients from those of the plaque psoriasis and diabetic patients(14).
The findings show that spectrum signals ascribed to oscillations in inorganic phosphates, carbohydrates, and amide I/II (secondary structure of protein) are the most sensitive to changes related to the advancement of salivary gland cancer. Numerous spectroscopic biomarkers have been suggested as potential indicators of the emergence of salivary gland tumors(15).
Additional data from FTIR spectroscopy may help in a reliable tumor characterisation, however homogenous tissue pretreatment procedures are necessary for measurement comparability for potential future diagnostic (routine) application(12).

CONCLUSION:
Based on the research that has been done it is conceivable to draw the conclusion that saliva can be used as a primary diagnostic tool in patients who are suspicious of salivary gland diseases.Examination of proteins, lipids, and DNA structural changes by FTIR spectroscopy, has several medical applications.Study can be further investigated to a broad scale of different age and time intervals among healthy and unhealthy people.

CONFLICT OF INTEREST:
There is no conflict of interest.

ACKNOWLEDGEMENT:
We extend our gratitude to Saveetha Dental college and hospital for their constant support and encouragement and hereby helping in successful completion of this research work.

FUNDING:
1.Saveetha institute of medical and technical sciences
2.Saveetha dental college and hospital
3.Saveetha university
4.Sri Srinivasa Clinic

ETHICAL CLEARANCE:
Ethical clearance is not required.

REFERENCE:
1. *Belskaya LV, Sarf EA, Kosenok VK. Analysis of Saliva Lipids in Breast and Prostate Cancer by IR Spectroscopy. Diagnostics (Basel) [Internet]. 2021 Jul 23;11(8). Available from: http://dx.doi.org/10.3390/diagnostics11081325*
2. *Belskaya LV, Sarf EA, Solomatin DV. Age and Gender Characteristics of the Infrared Spectra of Normal Human Saliva. Appl Spectrosc. 2020 May;74(5):536–43.*
3. *Karthikeyan S, Mata-Miranda MM, Martinez-Cuazitl A, Delgado-Macuil RJ, Garibay-Gonzalez F, Sanchez-Monroy V, et al. Dynamic response antibodies SARS-CoV-2 human saliva studied using two-dimensional correlation (2DCOS) infrared spectral analysis coupled with receiver operating characteristics analysis. Biochim Biophys Acta Mol Basis Dis. 2023 Oct;1869(7):166799.*

4. *Attie JN. Tumors of Major and Minor Salivary Glands: Clinical and Pathologic Features. 1981. 100 p.*
5. *Parker F. Applications of Infrared Spectroscopy in Biochemistry, Biology, and Medicine. Springer Science & Business Media; 2012. 602 p.*
6. *Rodrigues LM, Magrini TD, Lima CF, Scholz J, da Silva Martinho H, Almeida JD. Effect of smoking cessation in saliva compounds by FTIR spectroscopy. Spectrochim Acta A Mol Biomol Spectrosc. 2017 Mar 5;174:124–9.*
7. *Rodrigues RP, Aguiar EM, Cardoso-Sousa L, Caixeta DC, Guedes CC, Siqueira WL, et al. Differential Molecular Signature of Human Saliva Using ATR-FTIR Spectroscopy for Chronic Kidney Disease Diagnosis. Braz Dent J. 2019 Oct 7;30(5):437–45.*
8. *Ni D, Smyth HE, Gidley MJ, Cozzolino D. Towards personalised saliva spectral fingerprints: Comparison of mid infrared spectra of dried and whole saliva samples. Spectrochim Acta A Mol Biomol Spectrosc. 2021 May 15;253:119569.*
9. *Orphanou CM, Walton-Williams L, Mountain H, Cassella J. The detection and discrimination of human body fluids using ATR FT-IR spectroscopy. Forensic Sci Int. 2015 Jul;252:e10–6.*
10. *Bayne S, Carlin M. Forensic Applications of High Performance Liquid Chromatography. CRC Press; 2017. 276 p.*
11. *Streckfus CF. Advances in Salivary Diagnostics. Springer; 2015. 204 p.*
12. *Stefanakis M, Bassler MC, Walczuch TR, Gerhard-Hartmann E, Youssef A, Scherzad A, et al. The Impact of Tissue Preparation on Salivary Gland Tumors Investigated by Fourier-Transform Infrared Microspectroscopy. J Clin Med Res [Internet]. 2023 Jan 10;12(2). Available from: http://dx.doi.org/10.3390/jcm12020569*
13. *Scott DA, Renaud DE, Krishnasamy S, Meriç P, Buduneli N, Cetinkalp S, et al. Diabetes-related molecular signatures in infrared spectra of human saliva. Diabetol Metab Syndr. 2010 Jul 14;2:48.*
14. *Bottoni U, Tiriolo R, Pullano SA, Dastoli S, Amoruso GF, Nisticò SP, et al. Infrared Saliva Analysis of Psoriatic and Diabetic Patients: Similarities in Protein Components. IEEE Trans Biomed Eng. 2016 Feb;63(2):379–84.*
15. *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-attributable Disease : a Report of the Surgeon General. 2010. 728 p.*
16. *Sneka S, Preetha Santhakumar. Antibacterial Activity of Selenium Nanoparticles extracted from Capparis decidua against Escherichia coli and Lactobacillus Species. Research Journal of Pharmacy and Technology. 2021; 14(8):4452-4. doi: 10.52711/0974-360X.2021.00773*
17. *Vishaka S, Sridevi G, Selvaraj J. An in vitro analysis on the antioxidant and anti-diabetic properties of Kaempferia galanga rhizome using different solvent systems. J Adv Pharm Technol Res. 2022 Dec;13(Suppl 2):S505-S509. doi: 10.4103/japtr.japtr_189_22.*
18. *Sankar S. In silico design of a multi-epitope Chimera from Aedes aegypti salivary proteins OBP 22 and OBP 10: A promising candidate vaccine. J Vector Borne Dis. 2022 Oct-Dec;59(4):327-336. doi: 10.4103/0972-9062.353271.*
19. *Devi SK, Paramasivam A, Girija ASS, Priyadharsini JV. Decoding The Genetic Alterations In Cytochrome P450 Family 3 Genes And Its Association With HNSCC. Gulf J Oncolog. 2021 Sep;1(37):36-41.*