


**Research Article**

## Confocal Raman Spectroscopy Analysis of Biochemical Modifications in Diabetic Nails Induced by Photobiomodulation

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### Abstract

Chronic hyperglycemia in diabetic patients can affect all body tissues, including peripheral tissues such as nails and hair. Nails are vital protective structures for the feet and hands, particularly in diabetic individuals who often experience impaired wound healing. Therefore, maintaining healthy nails is crucial for protecting these parts of the body. It is well-documented that the nails of diabetic patients undergo physicochemical changes due to the glycation of keratin. In this context, the objective of the present study is to utilize photobiomodulation (PBM) therapy to restore the biochemical integrity of healthy nails, thereby enhancing protection for diabetic patients. For this study, 30 healthy participants and 30 participants with type II diabetes (DM2) underwent PBM therapy twice a week, totaling 10 sessions. The point contact technique was used at two points located in the medial and lateral regions of the base of the nail on the fifth finger of the left hand. PBM was performed using low-intensity laser equipment, class B, with a Gallium-Aluminum Arsenide Diode (GaAlAs) semiconductor (808 nm; 100 mW; 4 J; 40 sec.; continuous). Nail fragments from the distal region were collected before (T0) and after (T2) therapeutic treatment. Biochemical changes attributed to PBM were measured using Confocal Raman Spectroscopy, with a Horiba confocal Raman spectrometer, model Xplorer, coupled to a laser with a wavelength of 785 nm and a power of approximately 15 mW. Spectral changes related to the biochemical components of the nail, such as keratin, disulfide bonds, and tyrosine, were evident after PBM. Additionally, changes were observed in the peak areas of 1654 cm<sup>-1</sup> ( $\alpha$ -helix) and 1622 cm<sup>-1</sup> ( $\beta$ -sheet), which are protein markers for the groups investigated.

**Keywords:** Diabetes mellitus; Photobiomodulation therapy; Raman spectroscopy; Fingernails.

### Introduction

Diabetes Mellitus (DM) is a multifactorial metabolic disorder that arises from alterations in the secretion and/or action of insulin, leading primarily to elevated blood glucose levels (hyperglycemia) [1]. The repercussions of hyperglycemia extend beyond mere glycemic control, exerting direct effects on various organs including the eyes, kidneys, heart, nerves, and blood vessels [2]. DM is a widely recognized condition highlighted as major contributor of morbidity and mortality. It is estimated that approximately 50% of diabetic individuals are unaware of their condition [3]. This is an emerging public health problem, regardless of its development level. Around 69% of diabetes cases are observed in developing countries, where a surge of diagnoses is

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expected to occur in the coming decades, particularly in young individuals [4, 5]. The estimated prevalence of DM among individuals aged 20-79 years has increased from 151 million to 463 million today. Without effective mechanisms to stop this escalating trend, projections indicate that the number of people with DM will reach 578 million (10.2% of the population) by 2030, eventually reaching 700 million (10.9%) by 2045 [6]. The severity of diabetes mellitus arises from systemic hyperglycemia, which significantly increases the likelihood of glucose and amino acids reactions, thereby inducing functional alterations in proteins – a phenomenon known as protein glycation. Diabetic patients exhibit elevated levels of glycation in collagen, hemoglobin and keratin [2]. For this reason, they commonly exhibit glycation in the keratin of their hair and nails. Glycation in nails leads to their deterioration, thereby limiting their protective function. This role is particularly crucial in diabetic feet, as it is essential to avoid wound formation due to healing challenges inherent in these patients. Furthermore, weakened and biochemically altered nails are associated with a higher prevalence of onychomycosis compared to healthy nails.

In addition, it was found that the nails of diabetic patients present disparities in calcium content, density, porosity, roughness, hardness, protein conformation and disulfide bond content [7]. Such differences can serve in the diagnosis of diabetes; however, they should also be looked at carefully in search of mechanisms for obtaining healthy tissue. Usually, patients with nail damage administer calcium and silicon-based supplements, as these elements play a strengthening role in the nails. [8, 9] However, Strømme et al argue that supplementation could have a systemic effect, suggesting that the calcium and silicon concentrations reaching the nail would be small [10, 11]. Therefore, the authors suggest a topical nail treatment aimed at increasing calcium and silicon levels in the nails. The proposed product offers the advantage of incorporating the elements within the enamel itself. [10]

The present study takes a different approach from the mentioned authors, as it focuses on the application of photobiomodulation therapy to increase and preserve non-glycated keratin in the nail. To achieve this goal, PBM was applied to both diabetic and non-diabetic patients and the resulting biochemical changes in the nails were analyzed by using Confocal Raman Spectroscopy.

## Materials and Methods

### Study location

The photobiomodulation procedures and nail collection were conducted at Centro Integrado Lineu Araújo of the Municipal Health Foundation of Teresina – PI, in September 2019. Optical spectroscopy analyzes were carried out at the Biomedical Vibrational Spectroscopy Laboratory

of Universidade Brasil. The study was submitted to the Research Ethics Committee of Universidade Brasil, with CAAE code 01968718.6.0000.5494, and authorized under code 3.066.001.

### Sampling

Sixty (60) participants were selected, including thirty (30) non-diabetics, classified in the study as healthy (HEA), and thirty (30) type II diabetics (DIA). Participants, both male and female, were aged between 45 and 65 years, with type II diabetes confirmed through blood glucose and glycated hemoglobin examinations.

All research participants presented intact nails in the tested region, agreed to participate in the entire procedure and signed the Informed Consent Form. Participants were advised not to use any type of nail polish or product on their nails, not cut them and keep them clean and intact.

### Low-intensity laser photobiomodulation (PBM) therapy

Prior to the beginning of the PBMT, volunteers were instructed regarding the procedure and provided with protective equipment. They were then placed comfortably, had the measurement areas cleaned and underwent PBMT. The measurements were conducted by a RIWT/DMC laser, with a 808 nm wavelength, 4 J/cm<sup>2</sup> energy density, 100 mW power, 40 seconds irradiation time at each point and an area equivalent to 0.028 cm<sup>2</sup>. The application was punctual and precise, with the pen held perpendicular to the surface, targeting two (2) points, located in the medial and lateral region of the base of the nail of the fifth finger of the left hand. PBM sessions were held twice a week, for a total of 10 sessions.

### Nail collection

After nail cleansing with water and neutral soap to reduce any possible contamination, nail fragments were removed from the fifth finger of the left hand. After collecting the nails, they were properly packaged in Eppendorf-type tubes and then sent for analysis.

### Acquisition of Raman spectra

Raman measurements were taken on the distal region of the nail fragments, using a Horiba Confocal Raman Spectrometer, model Xplorer, coupled to a 785nm wavelength and 5 mW power laser. The Raman signal was collected by a Charge Coupled Device (CCD) camera and recorded by a computer. Data acquisition occurred in the spectral region between 400-1800 cm<sup>-1</sup>.

### Data processing

After Raman spectra acquisition, data pre-processing was conducted, which included baseline correction utilizing

polynomial adjustment (degree 7), removal of specific noise arising from cosmic rays and smoothing of the spectra using the Savitzky-Golay filter (size 9, grade 5). These procedures were performed using Labspec software (Horiba JobinYvon, France). After these steps, the spectra were vector normalized to eliminate systematic differences between measurements.

### Data analysis

Before starting the statistical analysis, all data underwent hierarchical cluster analysis to verify data homogeneity and to identify possible outliers. After this process, spectral averages were computed for each group, enabling the visualization of the main biochemical characteristics between the groups. To evaluate the differences in the secondary conformation of the proteins, deconvolution analysis was performed, involving the region from 1580 to 1720  $\text{cm}^{-1}$ , attributed to amide I. The deconvolution analysis was performed for DIA T0, DIA T2, HEA T0 and HEA T2 data. To obtain the final area values of the 1622  $\text{cm}^{-1}$  ( $\beta$ -sheet) and 1654  $\text{cm}^{-1}$  ( $\alpha$ -helix) peaks, 10 repetitions were performed at each evaluation. As a criterion for checking whether the deconvolution analysis was carried out correctly, negative area values were not considered and the  $R^2$  value, referring to the deconvolution adjustment, was always above 0.95.

The results were considered statistically significant for a p-value  $\leq 0.05$ , indicating a significance level of 5%. The procedures to obtain this comparison included: evaluation of data distribution using the normality test, evaluation of medians and quartiles using boxplots and application of the Student's T or Mann Whitney test.

## Results and Discussion

### Profile of biochemical exams of research participants

Regarding the age group, among the sixty (60) participants – 30 diabetics and 30 healthy individuals – an average age of 48.4 ( $\pm 5$ ) years was observed for healthy participants, while diabetic participants had an average of 55.7 ( $\pm 8$ ) year. It is noteworthy that in both the diabetic and healthy groups studied in this work, there was a prevalence of female individuals at 70% compared to 30% of males. This difference in gender distribution within the study group was also described in the study performed by Flor and Campos (2017), where a higher prevalence of diabetes mellitus in female individuals was also observed [3]. Regarding blood glucose and HbA1c values, it

was observed that there were no significant changes among healthy participants after PBMT. In diabetic participants, HbA1c showed a slight decrease, although not statistically significant. However, for these same individuals, blood glucose decreased from 193.35 to 185.58 mg/dL after PBMT (Table 1). It is worth noting that diabetic participants in the present study can be classified as uncontrolled diabetics, which may lead to complications related to diabetes, such as microvascular changes and neuropathies [12]. The values obtained for glycated hemoglobin and fasting blood glucose align the estimates described by Nathan et al. [13].

### Assessment of biochemical composition

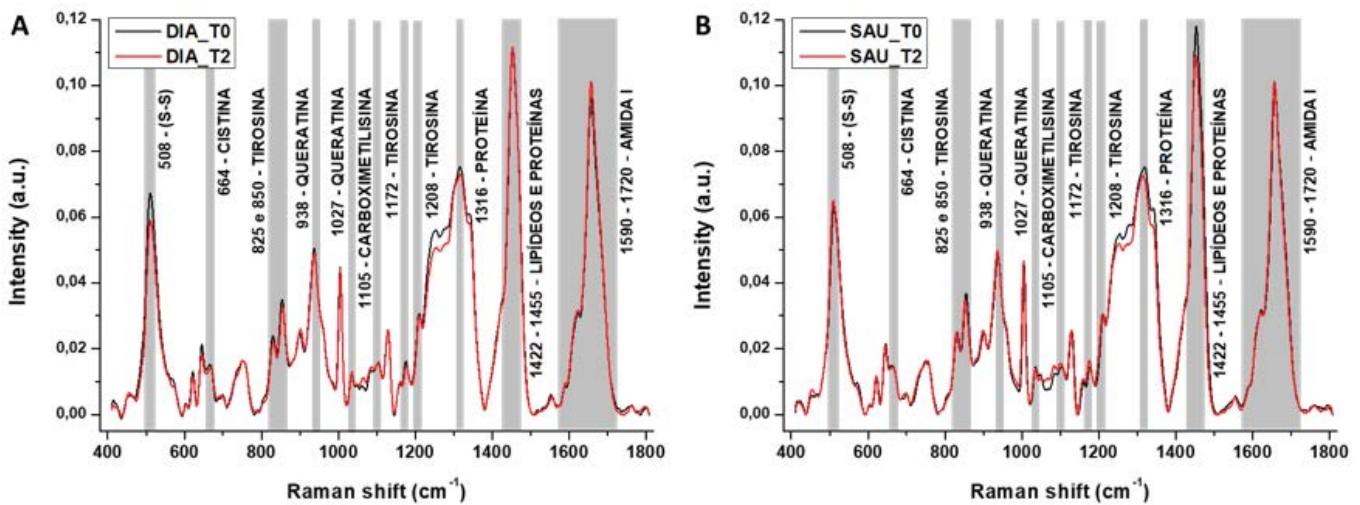
After all data processing procedures, means were generated for the two groups evaluated: healthy and diabetic, before (T0) and after (T2) the application of PBMT. For the average spectrum of the diabetic group after therapy (DIA T2), it can be observed that peaks marked in the graph at Figure 1 showed a decreased intensity after PBMT (with exception of peaks 1027 and 1656  $\text{cm}^{-1}$ , which showed a decrease in intensity, and peak 1453  $\text{cm}^{-1}$ , which presented an intensity equal to that given before therapy (DIA T0)). However, when evaluating the average spectra of the healthy group after therapy (HEA T2), it is noticed that, for most peaks, an opposite trend was noticed for the DIA T2 group. In other words, peaks 508, 664, 938, 1105, 1172, 1208 and 1656  $\text{cm}^{-1}$  had an intensity increase in comparison to the healthy group before therapy (HEA T0). However, peaks 1027, 1316 and 1453  $\text{cm}^{-1}$  exhibited intensity decrease, while peak at 825  $\text{cm}^{-1}$  exhibited an intensity similar to the HEA T0 group (Figure 1 A-B).

### Significance analysis of marker peaks

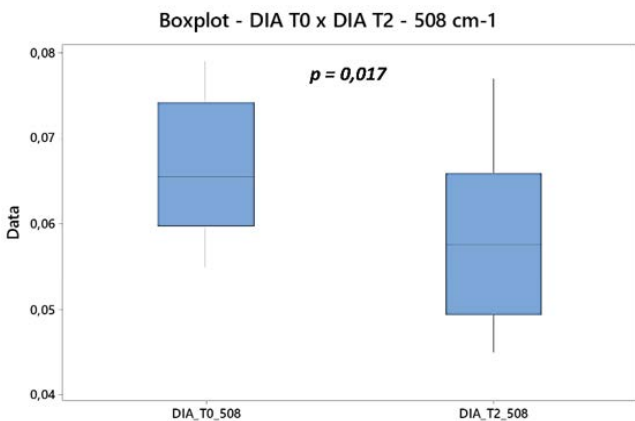
Statistically significant differences were demonstrated based on 508 $\text{cm}^{-1}$  peak intensity of the DIA T2 group in relation to DIA T0, with a p-value equal to – 0.017 (Figure 2). This intensity decrease confirms that PBMT may have influenced disulfide bonds in the nails of diabetic patients, improving protein structure. Similar results were identified by Shin et al. (2016), who reported that the primary alterations in the biochemistry of diabetic nails after laser therapy occurred in the region of disulfide bonds (S-S) [14]. In the aforementioned study, an intensity decrease of the disulfide band in post-therapy diabetic nails in relation to untreated diabetic nails was noted, suggesting that the structure of the nails in this region assumed a more stable conformation.

**Table 1:** Mean and standard deviation of fasting blood glucose and glycated hemoglobin (HbA1c) values of diabetic and healthy research participants.

Participants Exams	Health (n=30)		Diabetics (n=30)	
	Before laser	After laser	Before laser	After laser
Glucose (mg/dL)	89,56 (5,6)	90,37 (5,4)	193,65 (96,07)	185,58 (80,17)
HbA1c (%)	5,14 (0,23)	5,17 (0,23)	8,93 (1,99)	8,25 (1,79)



**Figure 1 A-B:** Comparison between the DIA and HEA groups before and after PBMT. The marked peaks are the main changes evidenced and related to the structure of the nails.



**Figure 2:** Boxplot of the comparison between the DIA T0 x DIA T2 groups for the individual intensity of the 508 cm<sup>-1</sup> peak.

The comparison between HEA T2 and HEA T0 groups showed that intensity changes were more significant. The significance level was noted for peaks 938 cm<sup>-1</sup> ( $p = 0.0009$ ), 1105 cm<sup>-1</sup> ( $p = 0.005$ ), 1172 cm<sup>-1</sup> ( $p = 0.001$ ), 1208 cm<sup>-1</sup> ( $p = 0.009$ ) and 1453 cm<sup>-1</sup> ( $p = 0.003$ ) (Figure 3). The observed changes in the nails of non-diabetic participants classified as healthy in this study demonstrate that the therapy may have improved the structure of the nails. This greater number of peaks with statistically significant changes may be attributed to a rearrangement of the biochemical components of the nails of healthy participants when compared to diabetics, allowing structural improvements to occur more easily.

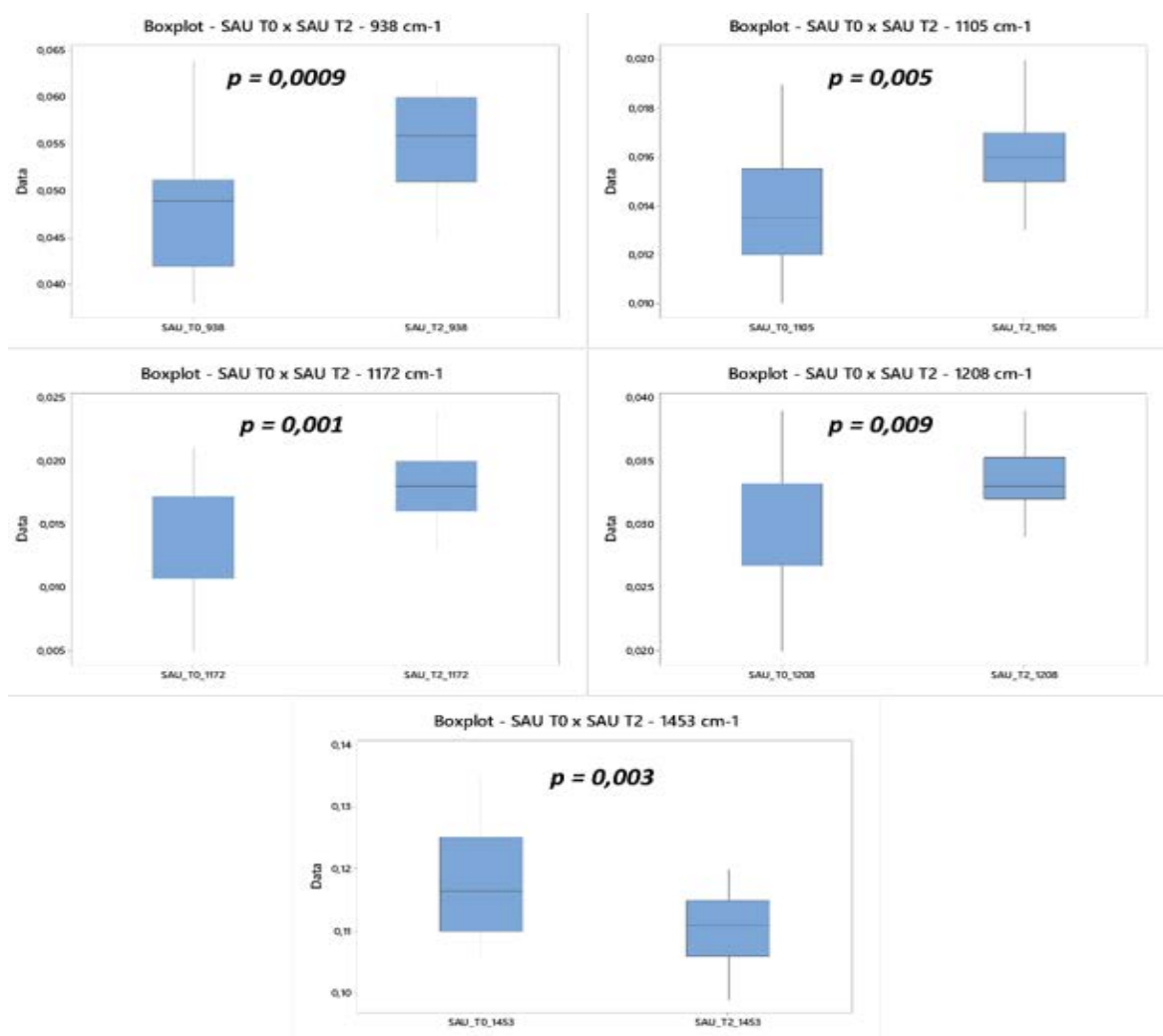
### Deconvolution Analysis – Amide I

The deconvolution analysis of the spectra of the experimental measurements was carried out aiming for protein conformation verification in the nails of both diabetic and healthy participants. The nail evaluation before PBMT showed that the diabetic group (DIA) and the healthy group

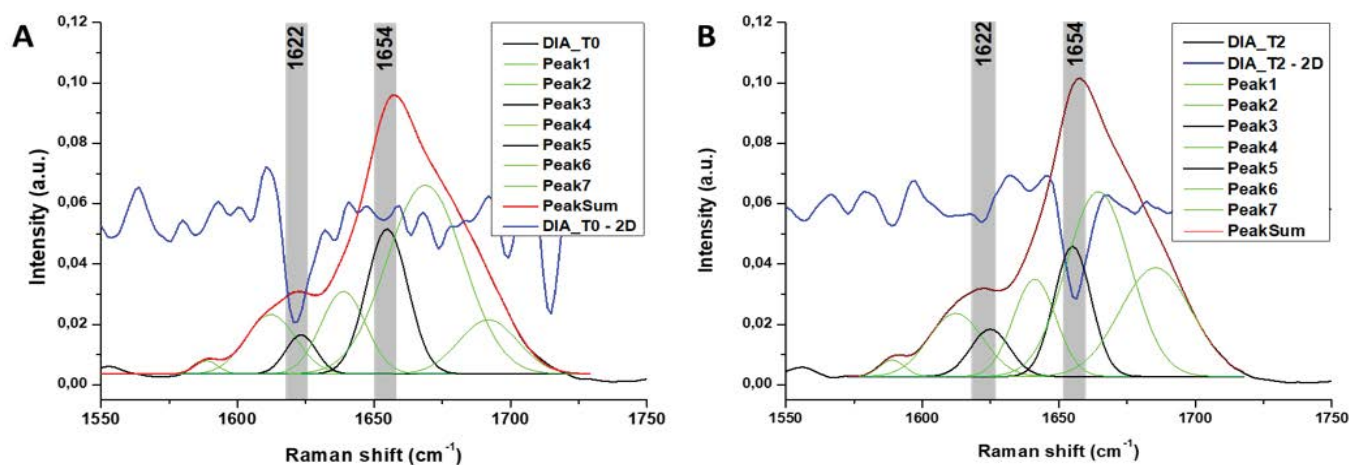
(HEA) presented a higher intensity and a larger area of the 1654 cm<sup>-1</sup> peak ( $\alpha$ -helix) in relation to the 1622 cm<sup>-1</sup> peak ( $\beta$ -sheet). Thus, in the healthy group, the intensity ratio of the 1654/1622 peak is 1.76, whereas in the diabetic group, the ratio is approximately 3.18. It should be noted that the higher intensity of the 1654 cm<sup>-1</sup> peak ( $\alpha$ -helix), when compared to the 1622 cm<sup>-1</sup> peak ( $\beta$ -sheet) for the HEA group, may indicate protein damage [15]. When analyzing the deconvolution for the DIA T0 x DIA T2 group, inferences can be drawn about the 1654 cm<sup>-1</sup> ( $\alpha$ -helix) and 1622 cm<sup>-1</sup> ( $\beta$ -sheet) conformations. Both the intensity and the area of the 1654 cm<sup>-1</sup> peak decreased after PBMT, whereas for the 1622 cm<sup>-1</sup> peak, both parameters showed an increase post therapy.

Considering the area values, the variation percentage of the 1622 cm<sup>-1</sup> peak post therapy was 88.23%, while for the 1654 cm<sup>-1</sup> peak, -1.23%. The area decreases of the 1654 cm<sup>-1</sup> peak, attributed to the  $\alpha$ -helix conformation after laser therapy, corroborates the results obtained in the evaluation by Shin et al. (2016) [14]. Based on the intensity, after PBMT, it was noticed ratio of peaks 1654/1622 decreased from 3.18 at T0 to 2.5 at T2 (Figure 4). This proportion (1654/1622) closely resembles that observed in the healthy group, suggesting an improvement in nail structure.

Deconvolution analysis of the spectra of experimental measurements was carried out aiming of verifying the behavior of protein conformation in the nails of diabetic (DIA) and healthy (HEA) participants. When checking the comparison between the HEA T0 and HEA T2 groups, small changes were observed, in both intensity and area of the marker peaks of the investigated proteins in this study, specifically at 1654 cm<sup>-1</sup> ( $\alpha$ -helix) and 1622 cm<sup>-1</sup> ( $\beta$ -sheet). Regarding the area values, the percentage of variation for the 1622 cm<sup>-1</sup> peak post therapy was -20% while for the 1654



**Figure 3:** Boxplots of the comparison between the HEA T0 x HEA T2 groups for the individual intensity of the peaks 938, 1105, 1172, 1208 and 1453 cm<sup>-1</sup>.



**Figure 4:** Deconvolution plots of the amide I region within the range between 1580 to 1720 cm<sup>-1</sup> at times T0 and T2. A: diabetic group at time T0 and B: diabetic group at time T2. Black lines represent the peaks of the regions of interest (1622 and 1654 cm<sup>-1</sup>) and the blue lines refer to the spectrum of experimental measurement.

$\text{cm}^{-1}$  peak, it was 1.75%. At time T0, before the application of PBM, the intensity ratio of peaks 1654/1622 was 1.76 and after PBMT (T2), the ratio value increased to 1.85, indicating stabilization in the proportion of alpha and beta structures over time, without significant variation (Figure 5).

It is understood that the mechanical properties of individuals' nails can undergo changes due to various factors, including changes in vascularization, oxygenation, water content, as well as endocrine and metabolic factors, including diabetes mellitus [16]. Thus, it can be inferred that, typically, diabetics nails will present greater protein disruption, in addition to possible changes in the keratinization and growth process, when compared to a healthy individual. Therefore, considering that the normal nail structure is maintained by protein organization, the results shown indicate that TFBM allowed to bring diabetic and healthy nails closer when considering the ratio of 1654/1622, indicating better protein structure. Therefore, given that the normal nail structure relies on adequate protein organization, the results found in this study indicate that PBMTT was able to bring biochemical convergence between diabetic and healthy nails. In other words, by considering the 1654/1622 ratio, the biochemical structure of DIA nails improved in ways of reaching the structure observed in healthy individuals.

Furthermore, after PBMT, it can be concluded that for the  $\beta$ -sheet conformation ( $1622\text{ cm}^{-1}$ ), a decrease was noted in the HEA group, indicating a disruption in proteins caused by the therapy. Also, an increase in this same conformation was observed for the DIA group, indicating an improvement in the protein structure of the nails. As to the  $\alpha$ -helix conformation ( $1654\text{ cm}^{-1}$ ), just a variation was observed for both groups (DIA and HEA). However, the intensity of this conformation is noticeably higher for the DIA group, a fact that can lead to a condition in which the proteins are loosely or destructurally linked [14,15]. Furthermore, after PBMT HEA group showed intensity decrease in peak  $1622\text{ cm}^{-1}$  – beta-sheet conformation – indicating protein disruption, while DIA group presented intensity increase in the same peak. This last observation in the DIA group suggests an improvement of protein structure of the analyzed nails.

## Conclusion

Low intensity laser photobiomodulation therapy (PBMT) was administrated to all research participants. Following this process, distinct spectral differences were observed between the groups before therapy (T0) and after therapy (T2). The main biochemical components that showed changes after therapy included: disulfide bonds, cystine, tyrosine, keratin, lipids, and proteins. For diabetic nails, the element that showed a statistically significant variation and that can be considered a marker for nail improvement after PBMT is

disulfide bonds ( $508\text{ cm}^{-1}$ ). In healthy nails, the components were keratin ( $938\text{ cm}^{-1}$ ), tyrosine ( $1172\text{ cm}^{-1}$ ), tyrosine and phenylalanine ( $1208\text{ cm}^{-1}$ ) and proteins and lipids ( $1453\text{ cm}^{-1}$ ).

Furthermore, it was possible to identify changes in the protein markers  $1654\text{ cm}^{-1}$  ( $\alpha$ -helix) and  $1622\text{ cm}^{-1}$  ( $\beta$ -sheet) between the groups investigated after PBMT. An intensity increase in  $\beta$ -sheet conformation was noted for the post-therapy diabetic group, which indicated an improvement in the protein organization of the nails. Few changes were evident in the  $\alpha$ -helix protein conformation, indicating that PBMT did not cause major effects on this conformation.

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