A Card Double Face: Compounds' Functionality and Synergy of a Topical Treatment Proposed for Oral Health Improvement in Periodontal Disease

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The application of an active mixture of melatonin, hyaluronic acid and tetracycline, as antibiotic adjuvant, for scaling and root planing in periodontal disease is exploited. The hyaluronic acid gel has been used as matrix for this topical mixture. The mixture of active ingredients has been structurally studied applying UV-Vis and FT-IR spectrophotometry and fluorescence microscopy. Our target was to put in evidence if melatonin, hyaluronic acid and tetracycline were preserving their specific characteristics when they would be mixed together. The recorded results are encouraging as each active compound maintains its characteristic functional groups imparting their biological action. The improved topical treatment based on melatonin, hyaluronic acid and tetracycline is presenting itself as a promising candidate for improving the oral health for patients with periodontitis.

Keywords: melatonin, hyaluronic acid, tetracycline, oral health, topical treatment

Periodontitis, considered as the sixth most prevalent condition worldwide [1], and one of the major cause of tooth loss, is a multifactorial inflammatory disease based on bacterial biofilm formation, and host immune response, genetically determined and influenced by environmental and life-style factors (tobacco smoking, poor nutrition, stress, alcohol) [2].

The bacterial biofilm, formed by diverse communities of microorganisms resident in the oral environment, generally exists in harmony with the host, and delivers important benefits that contribute to overall health and well-being. However, when systemic or local condition changes, the interactions within the microbial community becomes potentially damaging (dysbiotic), causing diseases in the teeth and their supporting tissues, i.e., dental caries and periodontal diseases [3,4].

Based on complex DNA-DNA hybridization, Socransky and co-workers[5] grouped the oral bacterial species in different colored complexes, based on their typical interspecies associations and sequential colonization: yellow (e.g. oral streptococci, *Actinomyces naeslundii*), violet (e.g. *Veillonella parvula, Actinomyces odontolyticus, Eikenella corrodens*), green (e.g. *Aggregatibacter actinomycetemcomitans* serotype a, and different species of Capnocytophaga), – compatible with periodontal health, orange (e.g. Prevotella species, *Fusobacterium nucleatum*, Eubacterium, anaerobic streptococci, motile Campylobacter) and red *(Porphyromonas. gingivalis, Tannerella forsythia,* and *Treponema denticola*) - involved in development of periodontitis [4,6].

Due to the infectious nature of periodontitis, adjunctive antimicrobial agents, delivered either systemically or locally, are required to eradicate or reduce the numbers of pathogenic bacteria in deep pockets, root furcation and concavities or those residing at or within the periodontal tissues at the biofilm–gingival interface, after applying the standard periodontal treatment consisting of mechanical debridement to remove biofilm and calculus from the affected root surfaces [7].

Systemic antibiotic delivery can lead to unwanted general side effects affecting patient's compliance and may contribute to bacterial resistance [7-9]. On the other hand, local administration allows the application of drugs in the periodontal pocket at a concentration that cannot be reached by the systemic route.

Among the antibiotic for topical usage in periodontal disease treatment, tetracycline (T), part of the group with same name, which includes doxycycline and minocycline, among others, is a bacteriostatic antibiotics that provides a *broad spectrum* of activity against both Gram-positive and Gram negative species (such as *P. gingivalis*, an important periodontal pathogen) with favorable effects on improving clinical attachment level and pocket depth reduction [10].

For a complex disease, such as periodontitis, the beneficial antibacterial role of topical administrated antibiotics could be potentiating by association with other bioactive compounds such as melatonin (MEL) - with important role in bone formation and reduction of bone resorption, and hyaluronic acid (HA) - with proved anti-inflammatory effect [11,12].

Therefore, a pharmaceutical combination of melatonin, hyaluronic acid and tetracycline was prepared to be used as topical treatment periodontitis.

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The aim of the present study was to investigate if each component of the proposed treatment formulation would maintain its specific characteristics and also, to discuss their synergic action.

Experimental part

Equipment and materials

The spectrophotometric analysis has been done on a dual beam UV-Vis spectrophotometer Varian Cary® 50 (Victoria, Australia) for the UV-Vis studies and on a Brucker Tensor 27 (ATR) equipment for the FT-IR investigations. The UV-Vis analysis has been done for a 200 - 800 nm wavelength range with a scan rate of 100 nm/s at a resolution of 1nm using 10 mm quartz cells.

The experimental curves were used to apply the derivative spectrophotometry [12] as the spectrophotometric specific answers of HA and MEL are overlapping.

The applied derived spectrophotometric was offering a versatile method for the correct differentiation of the spectrophotometric answers of the compound analyzed present in the same matrix.

The FT-IR experiments for the solid samples were run for a spectral range from 4000 to 500 cm^{-1} at a spectral resolution of 4 cm^{-1}. At each scan the background spectrum has been recorded. The obtained data were computed with OPUS NT 7.0 (Brucker Optics, Germany) software.

The fluorescence microscopy has been performed on a microscope, Leica DM 3000 LED, equipped with an MC 190 HD camera based on s-CMOS sensor with reduced noise factor, which was used for image acquisition with a HC PL Fluotar 5x/0.15∞ /-/OFN25/C objective. In order to avoid the vignetting effect a field diaphragm was set. A fluorescence metal-halide lamp Leica EL6000 was used.

For all experiments, the working temperature was 25 °C.

The chemical used for all experiments were: N-[2-(5-methoxy-1H-indol-3-yl)ethyl] (melatonin- MEL) (Sigma-Aldrich, Merck, Germany), $(C_{14}H_{21}NO_{11})_n$ - hyaluronic acid (HA) of 300 kDa (Sigma-Aldrich, Merck, Germany), $C_{22}H_{24}N_2O_8$ *-(4S,4aS,5aS,6S,12aS)-4-(Dimethylamino)-3,6,10,12,12a-penta hydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracen-2-carboxamid-* tetracycline (T) (Sigma-Aldrich, Merck, Germany), ethanol (Merck KGaA, Darmstadt, Germany), hydrochloric acid 0.1 M (Merck, Germany) and deionized water (conductivity 128 µS/cm^2).

In Figure 1 the chemical structures of the compounds with pharmaceutical action -MEL, HA and T are introduced. The biopolysacharide HA structure presented in figure 1.b. represents a Haworth projection of the polymer structure consisting of N-acetyl-D-glucosamine and D-glucuronic acid connected through alternating β-(1→3) and β-(1→4) glycosidic bonds[13].

All materials have been of high purity. The deinozed water was obtained with a Milli-Q system (Sartorius GMBH Gottingen, Germany). The samples were homogenised as follows: with an ultrasonic bath (Elmasonic S10 H, Elma Schmidbauer GmbH, Singen, Germany) for solutions, and with a trituration mill (Retsch-PM 100) for solid samples. The solutions for UV-Vis analysis have been disolved in deionised water (conductivity 128 µS/cm^2). After their preparation the solutions have been kept in a dark environement at maximum 8^{0}C. The solution of the active mixture have been obtained dissolving initaially the melatonin (in alcoholic solution) and tetracycline (in acidified with HCl deionized water) and then adding the hyaluronic acid. The mixture was kept afterwards for 60 min in ultrasonic bath at 25^{0}C.

The active matrix of compounds consisted in: MEL - 3mg, HA - 100mg and T - 3mg. The quantities have been normalized for 106 mg total weight. The hyaluronic acid was considered to be the release vehicle for the other active formulations (MEL and T).

Results and discussions

Applying the FT-IR analysis, both the individual spectra of each component (MEL, HA, T) and the complex matrix obtained (MEL+HA+T) were studied.

The FT-IR spectrum of MEL - figure 2 shows the major bands for functional groups at 3306 and 3260 cm^{-1} (vibrations for N-H bending and C-N stretching), 1492 and 1550 cm^{-1} (vibration for C = C aromatic), 1630 cm^{-1} (C = O aromatic bond vibration), 1180 and 1217 cm^{-1} (C-O -bond vibration). These experimental data are in agreement with previous studies [14,15].

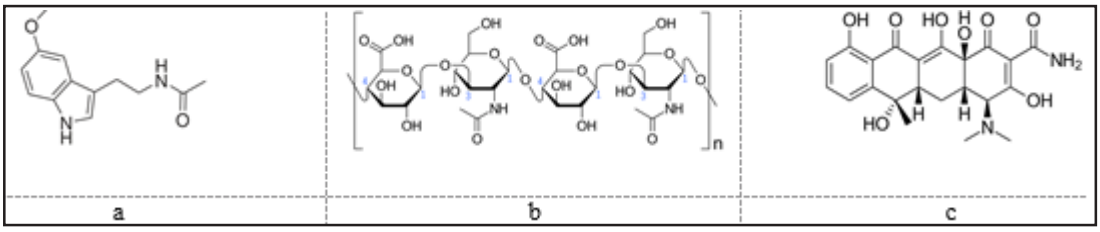

Fig. 1. Chemical structures for: melatonin (a), hyaluronic acid (b) and tetracycline (c).

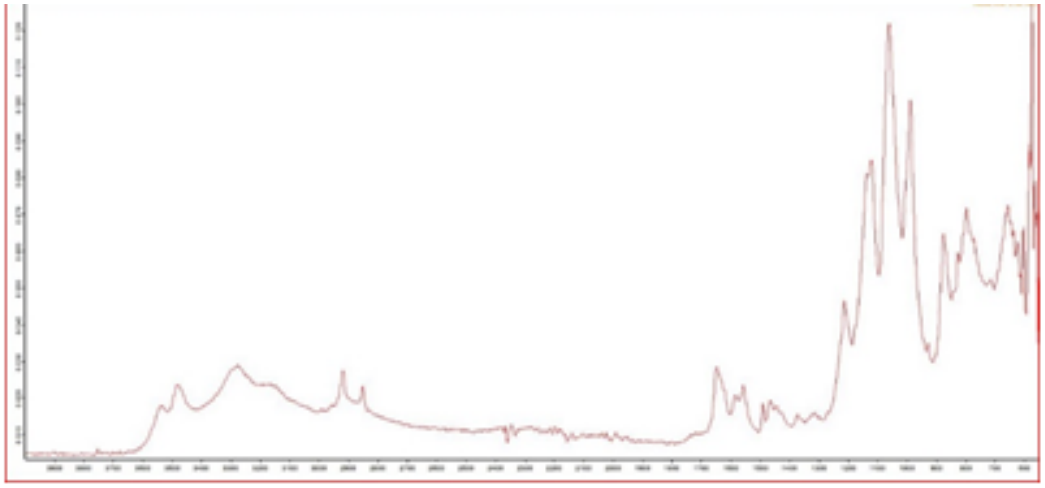

Fig. 2. FT-IR Spectrum for melatonin

Wave number (cm^{-1})	Functional Group	Vibration type
3422	O-H	Stretching symmetric
	N-H	Stretching amide
3016	C-H aromatic	Stretching symmetric
2918	CH_3	Stretching asymmetric
1400	C=C	Stretching symmetric
1300	C-O	Bending asymmetric
1050	C-O (alcohol)	Stretching asymmetric
970	C-C-H	Bending symmetric

Tabel 1
THE MAIN FT-IR ABSORPTION BANDS CHARACTERISTIC OF HYALURONIC ACID

The FT-IR spectrum also presents absorption bands at 1650 and 1550 cm^{-1}, respectively, representing vibrations for the two amide groups I and II, and at 1750 cm^{-1} the vibration of the carbonyl bond stretch appears [16]. At the wavenumbers 2870 and 2960 cm^{-1} the vibration bands correspond to the symmetrical stretch of the -CH_3 bond. The presence of absorption bands at 1477, 3217, 1431 cm^{-1} confirms the occurrence of stretching vibrations for C-N, N-H, C-H, and C-C bonds.

The FT-IR spectrum for hyaluronic acid (fig. 3) highlights the wavelengths at which the characteristic absorption bands appear, as shown in table 1.

From the FT-IR spectra of HA, the stretching band N-H belonging to the amide group on the region between 3200-3600 cm^{-1} is highlighted. An amide band at 1595-1710 cm^{-1} is attributable to the stretch of C=O and the bending of N-H. A specific band for C-H bending is also present in the region 1350-1480 cm^{-1}, and the C-O stretch of the proteoglycan cycle occurs between 985-1140 cm^{-1}.

The FT-IR spectra of tetracycline (fig. 4) allows to track the main constitutional functional groups [17].

The main vibration bands whose signals could be identified in the tetracycline FT-IR spectrum is presented in Figure 4. Compared to the compounds used for the active mixture, tetracycline displays the richest FT-IR spectrum. Most polynuclear aromatic compounds, such as tetracycline, generally have 3-4 absorption bands in the 3000-3100 cm^{-1} region. This is due to the stretching vibrations of the aromatic ring C-H bonds showing medium to strong intensity. Thus, the bands at 3000 and 3049 cm^{-1} are associated with the C-H vibrations of the phenyl nucleus. Several deformations within the plane of the C-H bond occur in the 1300-1000cm^{-1} region, being sharp bands and medium to low intensity. As a number of interactions are possible, increased attention is needed in the interpretation of the absorption-vibration bands in the substituent area to the benzene nucleus, which could increase the intensity of these bands. The stretching vibrations of the C-C bond in benzene occur in the region 1625-1530 cm^{-1}, showing the vibration of the high-intensity benzene skeleton at about 1500 cm^{-1}. In the tetracycline spectrum, vibrations of the aromatic ring at 1452, 1527,

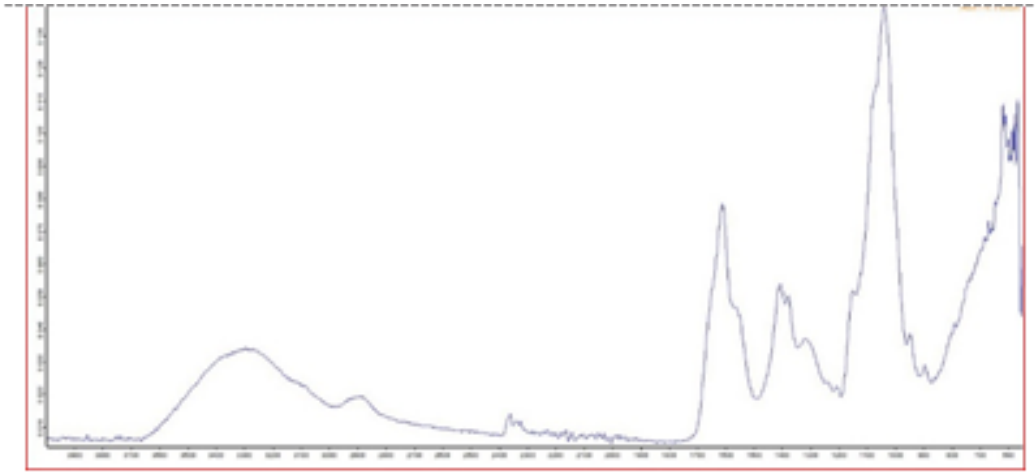

Fig.3. FT-IR Spectrum for hyaluronic acid

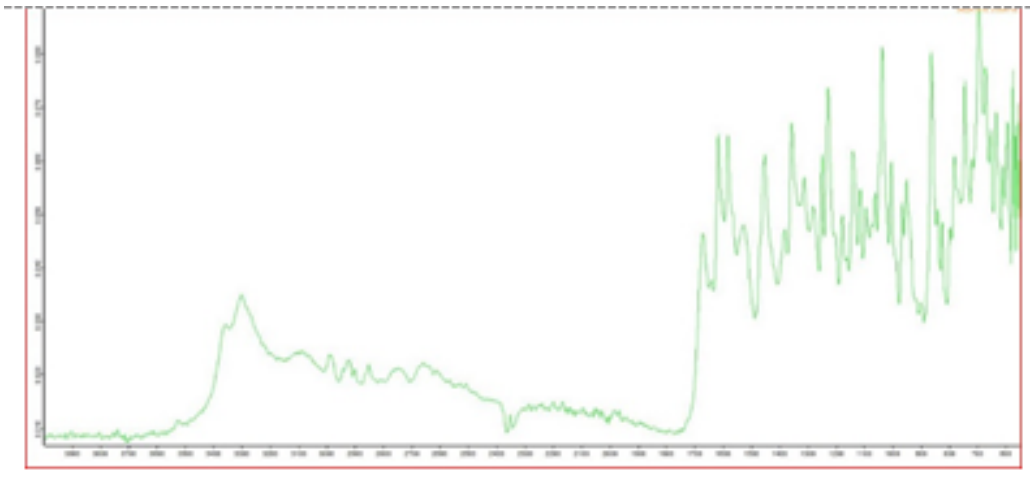

Fig.4. FT-IR Spectrum for tetracycline

1552, 1618 and 1666 cm^{-1} were observed. Further, at 567 cm^{-1}, outside the plane of the aromatic ring an absorption band for deformation can be observe. Methyl groups present two types of stretching vibrations: symmetrical and asymmetric stretching for C-H bonds. Also, the absorption band at 1358 cm^{-1} was attributed to the bending vibration of the geminal terminal dimethyl groups. Frequency of N-H is reduced due to hydrogen bonding. In the specific area for stretching there are overlapping of the N-H and O-H bonds, therefore were mentioned together in the table associated with the tetracycline FT-IR spectrum. The amide groups exhibit two medium intensity vibration bands corresponding to the symmetrical and asymmetrical stretching vibrations of N-H. Due to the hydrogen bonds formed, these absorption bands appear at 3108 and 3049 cm^{-1}.

The qualitative analysis of the individual FT-IR spectra for each component: M, HA and T allowed the identification of characteristic absorption bands. FT-IR spectra of the physical mixture: MEL, HA and T is shown in figure 5. The recorded spectrum exhibits absorption bands in the same regions in agreement with the functional groups identified in the individual spectra (figs. 2, 3 and 4).

The FT-IR spectrum details for the complex matrix, (fig. 6), clearly highlights the presence of the characteristic

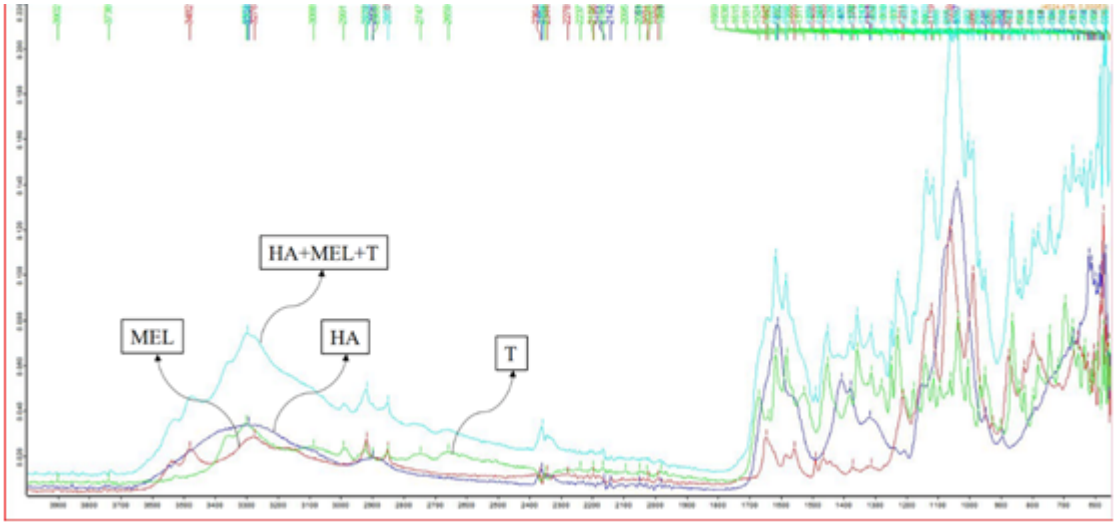

Fig.5. FT-IR Spectrum for the melatonin, hyaluronic acid and tetracycline mixture

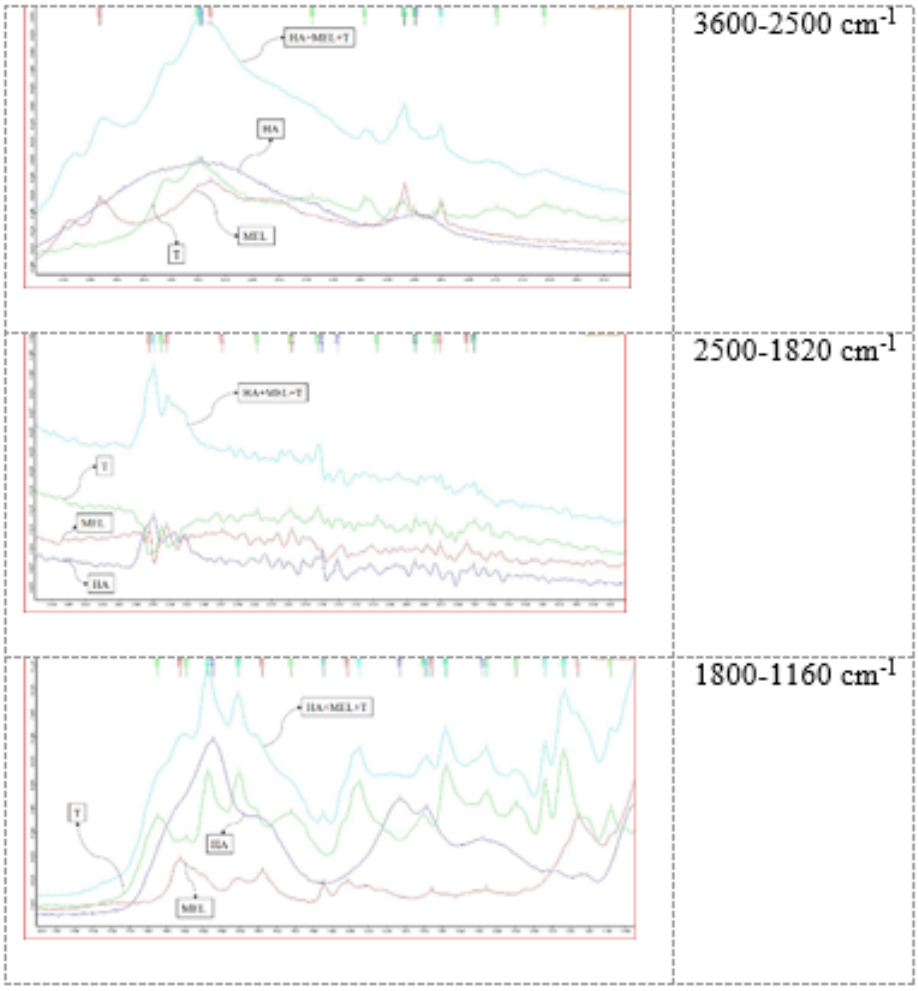

Fig.6. FT-IR Spectrum details for the melatonin, hyaluronic acid and tetracycline mixture

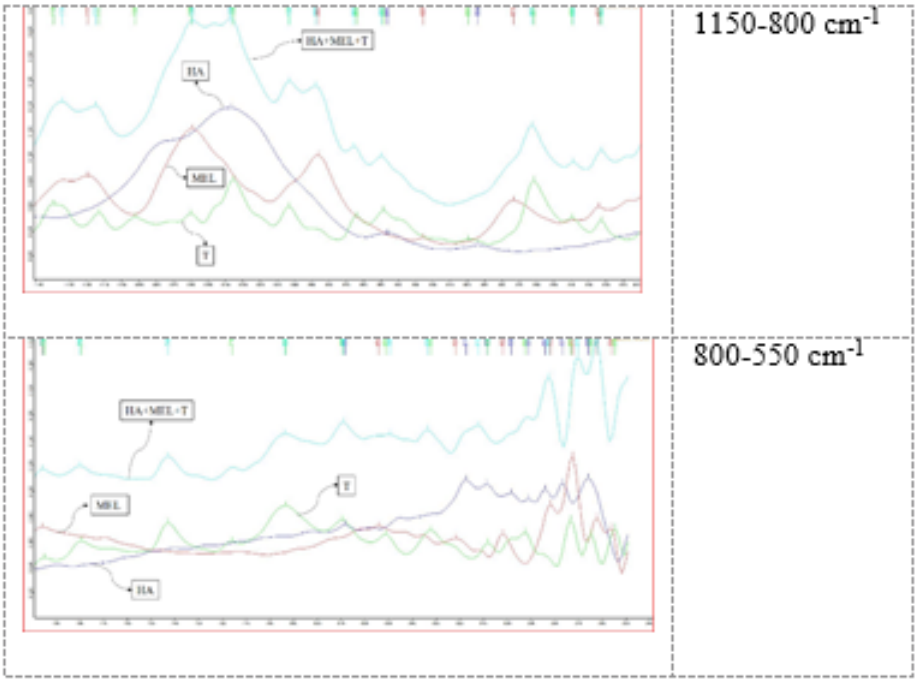

Fig.6. Continuated

absorption bands for each drug used. Overall, the result of the FT-IR structural analysis suggests that the chemical stability of the respective functional groups in HA, MEL and T is maintained as compared to control (individual) spectrum.

It is evident that by achieving a complex matrix of the three compounds a single spectrum is obtained that practically combines the prominent absorption bands for each individual component. Thus, it is confirmed that there are no interactions between the medicinal products used to obtain the complex matrix. The results of our FTIR experiments were able to point out if any structural changes of the MEL, HA or T occurred. The FTIR results of complex mixture highlighted: the intensity absorbance bands of O-H functional group (λ= 3273 cm^{-1} and 3325 cm^{-1}), the presence of absorbance bands of C-H group (at wavenumbers 2855 cm^{-1} and 2922 cm^{-1}), the absorbance band for C=O functional group (λ=1743cm^{-1}) and the intensity of absorbance band for C-O functional group (at wavenumber 1159 cm^{-1}). Analysis of structural changes with FTIR can sustain the preservation of each active compound's individuality (fig. 6).

Structurally complex matrices containing MEL, HA, and T have been developed and studied for tracking if changes and interactions occur between components. The individual UV-Vis spectra, as well as the recorded spectra for the active mixture and the first derivate spectra are presented in (fig.7).

The structural UV-Vis spectrophotometric analysis carried out on the complex matrix have shown unequivocally that MEL, HA and T present simultaneously in the biocompatible matrix is not individualized and do not react with each other, manifesting their specific properties. For this matrix, both the first order derivatives and the 2-order derivatives were obtained, so that where the derivatives of the first order cannot clearly detect the presence of the components with superimposed absorption maxima, it is possible to identify them by applying the second derivative of the spectrophotometric curves (fig. 8). The results of direct and derived UV-Vis spectrophotometric analysis (orders one and two) corroborated with those of the detailed FT-IR investigation are presented in figures 5 and 6.

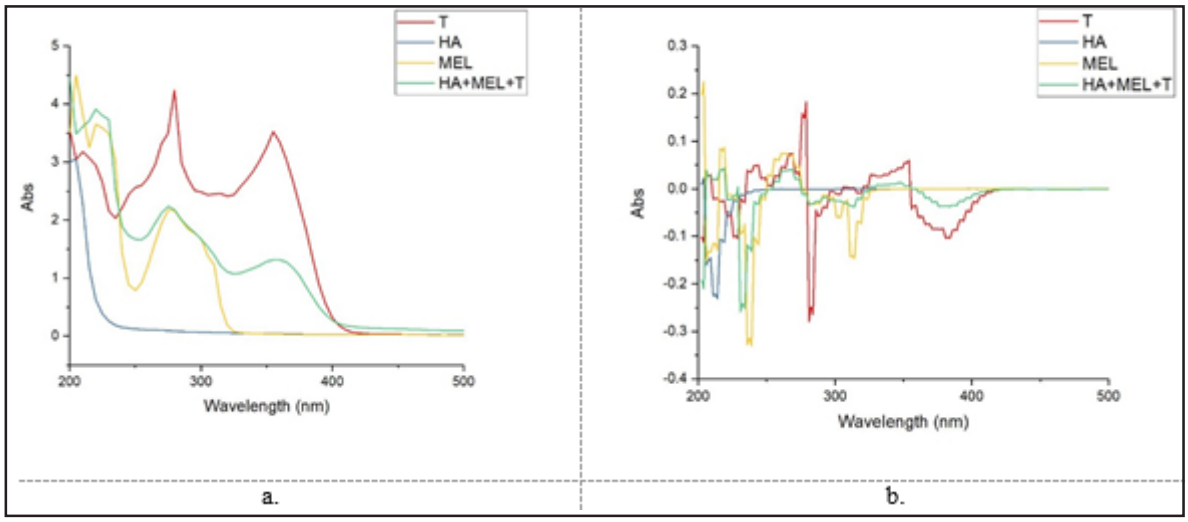

Fig. 7. UV-Vis spectra for the melatonin, hyaluronic acid, tetracycline and their mixture (a); mixture's first derivate spectra (b)

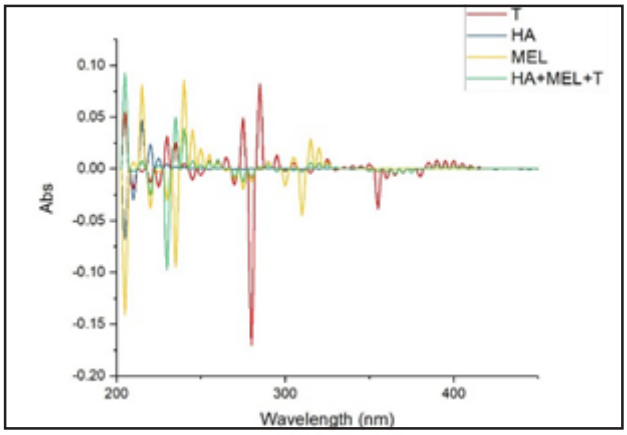

Fig.8. Second order derived UV-Vis spectra for melatonin, hyaluronic acid and tetracycline mixture

The results obtained support the statement that the components of the biocompatible matrix containing MEL, HA and T do not interact with each other, consequently could have a synergistic action in the biological environment.

The fluorescence process is governed by three important events taking place at different moments, separated by several orders of magnitude. The molecular excitation is likely to be produced as a result of interaction with a photon in a time window of femtoseconds order (10^{-15}s), while the vibrational relaxation of the electrons from the excited state to the lowest energy level is much slower and the time is measured in picoseconds (10^{-12}s). The final process, the emission at a wavelength greater than that of the excitation photon and the return of the molecule to the fundamental state, occurs in a relatively long time, nanoseconds (10^{-9}s). Although the entire lifetime of molecular fluorescence, from excitement to emission, is measured in only a few billionths of a second, the phenomenon is amazing, the manifestation of the light and matter interaction creates the basis of fluorescence microscopy in dynamic and steady state [18]. Because of extremely sensitive emission profiles, spatial resolution and high specificity, this new type of investigation become an important tool both in genetics and cell biology as well as in the field of clinical determinations [19,20]. Since both MEL and T show fluorescence, the physicochemical investigations of the proposed mixture for periodontitis were completed with a microscopic fluorescence study. Thus, it is intended to emphasize the preservation of the characteristics and functionality of each component of the proposed complex matrix, for oral topical treatment (fig. 9).

The results of fluorescence microscopy investigations presented in figure 10 prove that the fluorescence of MEL (blue) and T (green) make possible to identify these components in the mixture (fig. 10). At the MEL-specific excitation wavelength (278 nm), a characteristic blue fluorescence (emission at 347 nm) is obtained. For T, at the characteristic excitation wavelength (450 nm), green fluorescence appears (emission at 529 nm). It is interesting to note that, when changing the excitation wavelength of MEL with a radiation having a wavelength of 450 nm, characteristic for T, the blue staining characteristic of MEL disappears.

The fluorescence microscopy made possible the differentiation between the two fluorescent compounds from the active mixture (MEL and T), proving the characteristics preservation for each component.

The three components of the proposed mixture have distinct characteristics and were individually employed in topic applications for periodontitis treatment.

MEL (1 % orabase cream formula) was successfully used, for 20 days, in diabetic patients with periodontal disease, improving clinical periodontal parameters (gingival index and pocket depth) [21].

High molecular level HA has a proved anti-inflammatory effect and also antibacterial action, reducing the recolonization by periodontal pathogens (such as Campylobacter, Prevotella intermedia, and Porphyromonas gingivalis) after SRP and topical administration for 14 days [22].

Tetracycline in embedded in hollow, nonabsorbable fibers was the first topical antibiotic available for periodontal disease [23], with successful clinical results, decreasing pocket depth and increasing attachment level [24]. Moreover, the tetracycline family was also found to inhibit host-derived collagenolytic matrix metalloproteinases (MMP) activity and, consequently, connective tissue destruction in various tissues, including periodontium, by mechanisms unrelated to their antibiotic properties [25]. In this respect, based on this unexpected characteristic of tetracycline, in lower dose, not antibacterial effective, a new pharmacological approach called *host-modulation therapy*, adjunct to SRP, could be further developed [25].

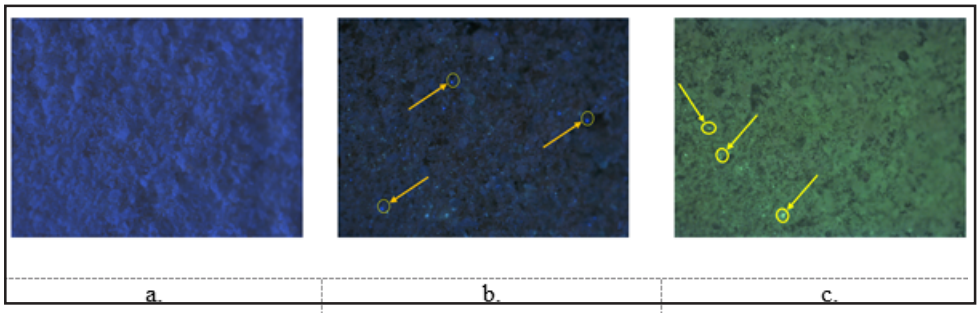

Fig. 9. Fluorescence microscopy image for melatonin, hyaluronic acid and tetracycline mixture (a); λ_{em} = 347 nm -blue spots pointing the presence of melatonin (b); λ_{em} = 529 nm - green spots pointing the presence of tetracycline(c)

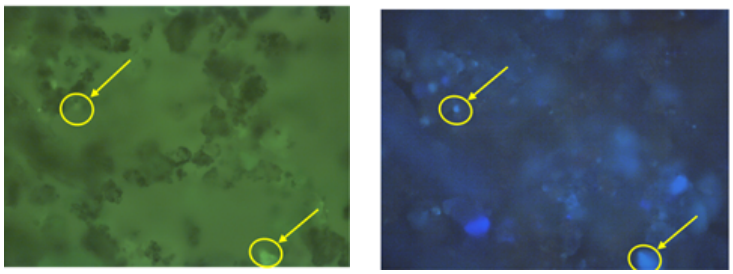

Fig. 10. Fluorescence microscopy image for melatonin and tetracycline mixture in hyaluronic acid gel showing the melatonin florescence behavior when changing the radiation wavelength

Conclusions

An attractive pharmaceutical combination of melatonin, hyaluronic acid and antibiotic with possible applications for improving oral health in periodontal disease has been studied. The aim of the present paper was to investigate if each component of the proposed treatment formulation would structurally maintain their specific characteristics. The experimental results recorded proved that no structural changes occurred at the level of the functional groups of the compounds when FTIR or UV-Vis analysis was used. The fluorescence characteristics for the mixture containing MEL, HA and T were presented. The investigations performed allowed a reliable and reproducible discrimination between the active mixture's components.

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