A complex formulation for topical treatment, consisting of melatonin, hyaluronic acid, tetracycline, and metronidazole mixture is proposed as periodontal disease adjunctive treatment. In order to follow the structural aspects of the active mixture, spectrophotometric methods: UV-Vis and fluorescence, have been applied. The results obtained complete our previous studies. By adding metronidazole to the mixture composed of: melatonin, hyaluronic acid, and tetracycline, the conjugated effect of the active compounds, combining antimicrobial action with anti-inflammatory and immunomodulatory effect, is expected to significantly improve supportive therapy in moderate forms of periodontitis.

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

Periodontal disease is a multifactorial inflammatory disorder determined by the bacterial biofilm and the host immune response; an inappropriate host response to microorganisms and their products causing the majority of periodontal tissue destruction and ultimately teeth loss [1].

The disruption of bacterial biofilm by scaling and root planing together with supportive therapy are considered, according to the principles of evidence based dentistry, the gold standard procedures for treating and preventing recurrence of periodontal disease [2,3].

The adjunctive treatment should focus on the complex bi-directional host-microbial interaction comprising bacterial destruction agents, but also host-modulating therapeutic factors, in order to address periodontitis.

Recently, a significant number of studies paid particular attention to establishing bi-directional links between periodontal health and systemic conditions, leading to the periodontal medicine concept [2]. Several mechanisms, such as bacteremia, endotoxaemia, and release of inflammatory mediators from periodontal tissues, have been incriminated for the association of periodontal disease to cardiovascular diseases [3], diabetes [4], cancer, chronic kidney diseases, chronic obstructive pulmonary disease, or rheumatoid arthritis [5], among others.

Due to infection nature of the periodontal disease, adjunctive antibacterial therapy is administered to eliminate or reduce the pathogenic bacteria in deep pockets, root furcation and concavities, areas not accessible to mechanical removal by hand or power-driven instruments [6].

The proteolytic enzymes released by the predominant pathogens involved in periodontitis, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Tannerella forsythia, Eikenella corrodens, and Treponema dentocila, cause host tissue destruction resulting in gingival inflammation, loss of gingival attachment, periodontal pocket formation, alveolar bone resorption and teeth loss [7,8]. Once the periodontium is detached from the alveolar bone, and pockets are formed, the lesions become irreversible [8].

In healthy conditions, the multispecies biofilm, present on all surfaces in the oral cavity, form the resident oral microbiome, which generally exists in harmony with the host, delivering important benefits that contribute to overall health and well-being [9]. Pathological changes may occur within the microbial ecosystem, caused by a change in the relationships between the microbes and the host, with an increased number or acquisition of virulence in the bacterial population, leading to dysbiosis [10].

Due to a great number of side effect from nausea, vomiting, gastrointestinal discomfort, to severe life treating bacterial resistance, the systemic antimicrobial therapy is recommended to be administrated with caution, mainly in aggressive periodontitis, if the periodontal infection needs to be rapidly suppressed, or in patients with uncontrolled diabetes [6].

Local antibiotic treatment has several advantages over the systemic administration, such as narrow effective range distribution, a high level at the treated site with lower level elsewhere, not requiring patient's compliance [11].

Among the antibiotics recommended as an adjunctive treatment for periodontal disease, tetracycline class (Tetracycline, Minocycline, Doxycycline) and metronidazole (MZ) are mostly used [6]. Tetracycline (T) is a bacteriostatic antibiotic with a broad spectrum of activity against both Gram-positive and Gram-negative species but with modest clinical improvements in probing depth reductions compared with scaling and root planing alone [12].

MZ has a broad-spectrum antimicrobial activity against protozoan infections and obligate anaerobic bacteria
efficiently inhibiting anaerobic microorganisms (such as P. gingivalis, F. nucleatum, among others) in the periodontal pockets [13].

However, local antibiotic therapy alone may also cause dysbiosis. Therefore, alternate topical active compounds have been used as adjunctive treatment of chronic periodontitis, such as chlorhexidine (CLX), a bisbiguanide class cationic agent with antimicrobial, bactericidal, or bacteriostatic effects (depending of dosage) and lack of systemic toxicity but with several side effects including extrinsic pigmentation, possible irritation of the mucosa, and taste changes. Nevertheless, a systematic review with the meta-analysis by da Costa and co-workers evidenced lower benefit in probing depth (PD) reduction and negligible effect on clinical attachment level (CAL) gain, by using topical CLX [14].

Beside the antibacterial effect, for reducing inflammatory parameters such as bleeding on probing (BOP) and PD, the use of hyaluronic acid (HA), melatonin (MEL) or the recently combined MEL and HA in a complex active compound, as adjunctive therapy after scaling and root planing (SRP) in periodontal compromise patients, was also considered [15–17].

The present paper will focus on improved local treatment of periodontal disease by using, for the first time, four active components with antibacterial and immunomodulatory properties mixed in a complex compound proposed to be employ as a topical application in periodontal pockets. The present paper aims to highlight the association between antibacterial therapy and immunomodulators, in periodontal disease, and to evidence the specific behavior and chemical interaction between T, MZ, MEL, HA in a complex compound for topical administration.

**Experimental part**

**Materials**

All chemicals of high purity have been provided by Sigma-Aldrich, Merck, Germany: (C14H21NO11)n - hyaluronic acid (HA); N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-melatonin (MEL), C6H9N3O3 -1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazol, metronidazole (MZ) and C6H11NO (4S, 4aS, 5aS, 12aS)-4-(Dimethyl amino)-3,6,10,12,12a-penta-hydroxy-6-methyl-1,11-dioxo-1,4,4a,5,6,11,12a-octahydropytetacen-2-carboxamid-tetraacyline (T) (Sigma-Aldrich, Merck, Germany). There were also used organic solvents as: ethanol and ethanol (Merck KGaA, Darmstadt, Germany). The water used was deionized (0.158 µS/cm).

The method applied for formulation mixture preparation

The formulation mixture complies with the safe posology of each compound [6,18–21]: MEL – 3 mg, MZ – 3 mg, T – 3 mg and HA – 100 mg [OSIM patent pending]. The mixtures have been prepared and ultrasonicated for homogenization. All prepared solutions were maintained in cool (8°C) and dark conditions. The sequential procedure applied to obtain the formulation mixture supposed to dissolve the melatonin, metronidazole, and tetracycline. Then, after their homogenization, the necessary amount of hyaluronic acid was gradually introduced under continuous ultrasonication at room temperature.

Investigation methods

The applied investigation methods were spectrophotometric, namely FT-IR analysis (Bruker Tensor 27 with ATR device and using OPUS NT 7.0 software), UV-Vis analysis (UV-Vis spectrophotometer Varian Cary® 50) and fluorescence determinations (Perkin Elmer spectrophotometer).

The FT-IR determinations were performed on the solid mixture. The FT-IR spectra against the background spectrum were recorded using 4 cm–1 spectral resolution over a wavenumber range 4000 – 500 cm–1. Both UV-Vis and fluorescence analysis have been run over 200 – 800 nm wavelength range. The samples were introduced in 10 mm quartz cells.

The derivative spectrophotometry has been applied [16] for strong evidence of the overlapping signals characteristic for MEL, T, HA, and MZ. This procedure allowed differentiating the presence of each compound in the analyzed mixture.

The fluorescence spectroscopy supposed initially to establish the best excitation wavelength. If there were no provided information, we used the absorption spectrum to estimate the optimum excitation wavelength. After obtaining the absorption spectra, the maximum absorbance was used as an initial excitation wavelength. The starting wavelength was set with about 30 nm above the excitation wavelength. Such condition allows avoiding Rayleigh scattering for the emission spectrum. The spectra were recorded up to 800 nm, at slow scan speed (100 nm/ min). The excitation and emission slits were set to 10 nm.

A microscope, Leica DM 3000 LED, equipped with an MC 190 HD camera and fluorescence metal-halide lamp Leica EL6000, allowed the acquisition of fluorescence microscopy images for the complex mixture.

**Results and discussions**

**UV-Vis spectrophotometry investigations**

The structural studies had as a primary purpose the highlight of possible changes of the absorption characteristic bands of each pharmaceutical component when mixed. Figure 1 shows the metronidazole spectrum. The specific spectra of the other components of the topical treatment mixture were presented in our previous work [16, 17]. As noticed from the individual spectra, there are overlapping of some absorption maxima for HA and MEL, MEL and T, MZ and MEL.

In consequence, the usage of the first or second derivate of UV-Vis curve offers the possibility to distinguish the preservation of each component individuality [16].

Figure 2 introduces the UV-Vis spectra of the compound mixture. If there were no provided information, we used the absorption spectrum to estimate the optimum excitation wavelength. After obtaining the absorption spectra, the maximum absorbance was used as an initial excitation wavelength. The starting wavelength was set with about 30 nm above the excitation wavelength. Such condition allows avoiding Rayleigh scattering for the emission spectrum. The spectra were recorded up to 800 nm, at slow scan speed (100 nm/ min). The excitation and emission slits were set to 10 nm.

A microscope, Leica DM 3000 LED, equipped with an MC 190 HD camera and fluorescence metal-halide lamp Leica EL6000, allowed the acquisition of fluorescence microscopy images for the complex mixture.
FT-IR spectrophotometric investigations

The FT-IR analysis allowed to obtain the mixture - MEL, HA, MZ, T spectrum, which has been compared with the individual spectra of each component (MEL, HA, MZ, and T). Figure 3 illustrates the FT-IR spectrum of the complex mixture. This spectrum put in evidence the presence of the major bands for functional groups characteristics of each active component of the studied mixture.

In figure 4 different wavelength ranges from the whole FT-IR spectrum, are presented, to evidence the presence of the specific absorption bands for each compound included in the mixture.

The FT-IR spectrum of the mixture is complex, combining the functional groups’ absorption bands characteristic for each pharmaceutical compound [17].

Comparing the overall detailed spectrum - fig. 4, with the individual spectrum of each component, it can be observed that the structural integrity of the active participants - MEL, HA, MZ, and T is preserved. The important absorption bands were identified with the aid of the experimental data from the FT-IR spectra for the MEL-HA-MZ-T mixture. Table 1 introduces the vibration characteristic of absorption bands. The experimental data presented prove that each component of the active mixture is preserving its specificity.

Supposing that we have our sample in an excited state, following its irradiation with the appropriate excitation

![Fig. 2. UV-Vis spectra for the melatonin, hyaluronic acid, metronidazole and tetracycline mixture (a) and the second derivative of UV-Vis spectrum (b).](image)

![Fig. 3. Complete FT-IR spectrum for the melatonin, hyaluronic acid, metronidazole, and tetracycline mixture](image)

![Fig. 4. Details of FT-IR spectrum - covering different wavelength ranges for the melatonin, hyaluronic acid, metronidazole, and tetracycline mixture](image)
wavelength, it could give off photon through relaxation phenomenon. Such released photon is characterized through a different wavelength compared to the excitation one. Much more, this wavelength presents a larger value compared to the excitation wavelength. On spectra, this is displaced towards the red region. Figure 5 presents the fluorescence spectra for melatonin at various concentrations between $10^{-5}$ and $8 \times 10^{-5}$ mg/mL.

The fluorescence spectra recorded for complex formulation and binary mixtures are presented in the figures 6, and 7. The fluorescence spectroscopy proved that we could evidence the preservation of the functional and structural characteristics of each compound when they are present in a complex mixture.

The following equation could give the fluorescence intensity:

$$F = k (I_0 - I)\quad (1)$$

where $k$ represents the fluorescence process’ rate constant, $I_0$ stands for the incident radiation, while $I$ is the intensity that exists the sample. It results that the difference $(I_0 - I)$ represents the quantity of the absorbed radiation. Lambert-Beer relationship was applied:

$$A = \varepsilon l c = \log \left( \frac{I}{I_0} \right)\quad (2)$$

where $\varepsilon$ is the absorption coefficient at the exciting wavelength used, $l$ stands for the optical path and $c$ is the sample concentration. After replacing in the previous equation the last relationship, we obtain:

Table 1

| Wavenumber (cm⁻¹) | Functional group | Vibration type |
|-------------------|------------------|---------------|
| 3383              | O-H              | Associated O-H groups (H bonds) |
| 3325              | O-H              | Associated O-H groups (H bonds) |
| 3108              | N-H              | Asymmetric stretching |
| 3049              | N-H              | Symmetric stretching |
|                   | C-H              | Aromatic C-H stretching |
| 2922              | C-H              | Aromatic C-H stretching |
| 2915              | CH₃              | Asymmetric stretching |
| 2866              | CH₃              | Symmetric stretching |
| 2776              | C-H              | Methyl group stretching |
| 2674              |                 |               |
| 1743              | C=O              | Stretching |
| 1666              | Aromatic ring    | Stretching |
| 1552              | Aromatic ring    | Stretching |
| 1452              | Aromatic ring    | Stretching |
|                   | CH₃              | Asymmetric bending |
| 1358              | Terminal geminal dimethyl | Symmetric bending |
| 1234              | C-N              | Stretching |
| 1178              | C-C              | Stretching |
| 1159              | C-O              | Stretching |
| 1137              | C-C              | Stretching |
| 1112              | C-H              | In-plane Bending |
| 1037              | C-N              | Stretching |
| 1002              | C-O              | Stretching |
| 939               | C-N              | Stretching |
| 771               | Aromatic C-H     | Out-plane Bending |
| 678               |                 |               |
| 641               | C-C              | In-plane Bending |
| 567               | C-H              | Out-plane Bending |

Fig. 5. Fluorescence spectra for the melatonin at various concentrations

Fig. 6. Fluorescence spectra for the MEL-HA-MZ-T mixture and individual spectrum.
to access deep pockets, surface irregularities, and debridement has limitations, mostly regarding the inability
of the etiological factors contributing to inflammation and subsequent attachment loss. However, mechanical
disruption of the plaque biofilm is the primary treatment of periodontal disease based on the consideration
disruptions, and the tissue destruction seen in periodontitis, can be attributed to dysregulation of inflammatory pathways
and inadequate immune responses to the presence of bacteria [23].

As noticed from the above relationship, there is an exponential type dependence between the fluorescence
intensity and the sample’s concentration. Consequently, the fluorescence does not have a linear dependence on
concentration. Upon using different mathematical approximations involving a logarithm and factorial
development, and considering that the term (2.302 - \ln \epsilon) is less than -0.05, a linear type dependence of
fluorescence for low concentration applies:
\[ F = k_0 (1 - 10^{-\epsilon}) \]  
(3)

The last equation could be expressed as simpler as:
\[ F = kIc \]  
(4)

Such an equation allows the evaluation of the quantum
yield for the studied samples.

Figure 8 introduces the microscopic fluorescence image
for the complex mixture containing all four components.

The main goals of the periodontal therapy are to
preserve, improve, and maintain the natural dentition and
periodontal tissues in order to achieve health, comfort,
esthetics, and function [22]. Although the primary etiologic
role of bacteria is well documented, it has become clear,
lately, that the tissue destruction seen in periodontitis, can be attributed to dysregulation of inflammatory pathways
and inadequate immune responses to the presence of bacteria [23].

A significant number of studies have demonstrated favorable long term outcomes and avoidance of tooth
extraction after periodontal treatment if patients practice
good oral hygiene and are included in a regular maintenance
care program including mechanical debridement and
supportive therapy [23,24].

It is widely accepted that SRP leading to mechanical
disruption of the plaque biofilm is the primary treatment
of choice in periodontal disease based on the consideration
of the etiological factors contributing to inflammation and subsequent attachment loss. However, mechanical
debridement has limitations, mostly regarding the inability
to access deep pockets, surface irregularities, and
furcation areas, therefore using adjunctive antimicrobial agents, to eradicate or reduce the numbers of pathogenic
bacteria, is mandatory [6]. Besides the antibacterial effect, immunomodulatory and anti-inflammatory agents,
addressing periodontal tissues, in conjunction with SRP, could significantly improve CAL and lead to PD reduction
[15].

The goal of the current work was to associate, in a complex
formulation, the antibacterial effect of tetracycline (T) and
metronidazole (MZ) with two anti-inflammatory and
antioxidant agents, melatonin (MEL), a natural hormone
that is generated by the pineal gland, and hyaluronic acid (HA), found mostly in the extracellular matrix of connective
tissue.

In order to assess the benefit of the cumulative effect of
each active compound of the proposed mixture, the
chemical interaction between the components has been
investigated.

The rationale for the association of the above-mentioned
active compounds was based on the individual effect of
topical/systemic administration of each active compound.

Tetracycline (T), effective against most spirochaetes,
and many anaerobic and facultative bacteria, access
bacterial cells by the combined processes of passive
diffusion through outer membrane pores and active
transfer, utilizing an energy-dependent pump in the inner
membrane, and ultimately inhibits protein synthesis on the
surfaces of the ribosomes, arresting polypeptide synthesis
[25].

Beside its bacteriostatic effect with broad activity against
both Gram-positive and Gram-negative species, it was also
found to inhibit host-derived collagenolytic matrix

Metalloproteinases activity, preventing connective tissue
destruction, including periodontium, even when
administered in lower dose [17,26].

Metronidazole (MZ), a nitroimidazole compound,
primarily developed in France for addressing protozoan
infections, enters aerobic and anaerobic bacteria by
diffusion and the nitro group of the compound is reduced
by nitroreductase (a ferrodoxin-like electron transport
protein). At low oxidation-reduction potentials, associated
with anaerobic conditions, the reduction precipitates
release toxic products such as nitro, nitroso, nitroso-free
radicals, and hydroxylamine derivatives, interfering with
DNA synthesis and causing disruption of the helical
structure of the molecules, leads to death of the involved
micro-organisms [27]. Due to this specific action, the
antibacterial effect is specific for obligate anaerobic
bacteria, usually found in deep periodontal pockets.

T and MZ are the most frequently used antimicrobial
agents in the management of the periodontal disease; both
drugs can be given individually or combined, systemically,
or applied topically into the periodontal pocket [25]. Topical administration is preferred, ensuring a higher concentration with significantly reduced side effects. MZ was found to be more effective when combined with the T group in the management of refractory and other rapidly progressive forms of periodontitis [28].

The natural hormone Melatonin (MEL) has important antioxidant, anti-inflammatory, anti-angiogenic role, also stimulating bone formation. The administration of MEL or synthetic analogs, in periodontal disease, either as systemic supplement [29] or topical cream [30], after SRP, improved periodontal parameters comparing to SRP alone [15].

Hyaluronic acid (HA), has opposite physiological effects, depending on the molecular weight. The high molecular weight HA is anti-angiogenic, anti-inflammatory (inhibits endothelial cell growth, binds fibrinogen, reduces the recruitment of inflammatory cells, the levels of inflammatory cytokines and the migration of stem cells) and has a beneficiary role in tissue injury repair, wound healing and immunosuppression. The low molecular weight HA is pro-inflammatory and has pro-angiogenic activities [15,16,31]. However, both high and low molecular HA have bacteriostatic (especially against S. aureus and A. actinomycetemcomitans) [32] and fungistatic actions (against C. albicans) [33].

HA has proved beneficiary effects upon local administration for the periodontal disease [34–36]. In our previous papers, we have proposed and analyzed, with promising results, a complex mixtures of MEL and HA [15,16] and also a complex formulation containing MEL, HA and T [17]. By including MZ, the active complex mixture and also a complex formulation containing MEL, HA have bacteriostatic (especially against S. aureus and A. actinomycetemcomitans) [32] and fungistatic actions (against C. albicans) [33].

Conclusions

The results obtained by using spectrophotometric methods and fluorescence microscopy for analyzing the proposed complex mixture comprising: tetracycline, metronidazole, melatonin and hyaluronic acid, highlights the conjugated effect of the active compounds, combining antimicrobial action with anti-inflammatory and immunomodulatory effect for improved supportive therapy, in a moderate form of periodontitis. Further, in vivo investigations are needed for assessing the effect on periodontal parameters.

By obtaining new complex pharmaceutical formulations, for local administration, with reduced side-effects, the paradigm of periodontal therapy may be shifted from a predominantly surgical approach to the greater use of medicinal/pharmacologic strategies, for the benefit of our patients.

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