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EFFECT OF ESSENTIAL OILS AS ADJUTANTS ON THE TREATMENT OF SUBJECTS WITH PERIODONTITIS: ASSESSMENT OF METABOLIC VARIABLES AS EFFECT MODIFIERS

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ABSTRACT

Background and objectives:

The increasing scientific data indicate on a double cause-effect relationship between periodontitis (PD) and cardiovascular disease (CVD), including metabolic syndrome (MetS). Such interaction can be mediated by inflammation mediators, but also by the effect of bacteria for which the periodontium may represent an entry into the circulation. Several plant extracts have a beneficial effect on periodontal disease progression thought different mechanisms. Such component also may improve different MetS-related parameters including lipids, that further may influence the general inflammatory status, as well as PD. Furthermore, a direct anti-inflammatory effect on the periodontium is not excluded. The present thesis aimed to analyze the effect of a nutraceutical composed of several plant extracts in subjects with PD and different levels of risk for the MetS. Specifically, it has been evaluated: 1) if the responses of some periodontal clinical variables were more effective in subjects treated with the extract compared to controls; 2) if the effect on MetS-related variables in the subjects treated with the extract could be more favourable compared to controls; and 3) to evaluate the effect of selected plant extracts on inflammatory markers and determine if those were associated with a worse periodontal condition, and specific periodontal variables. Some parts of the study, which aimed to assess inflammatory markers were not completed due to COVID-19 pandemic.

Materials and Methods:

The clinical study was a randomized, controlled, double-blinded clinical trial. 62 patients were divided in 2 groups: 1) test group who received the extract in form of irrigation solutions in the periodontal pockets during regular periodontal treatment. They have continued at home during the follow-up period, taking the essential oils (EOs) as a rinse twice per day, and as a spray when regular toothbrush could not be performed (n = 30); 2) control group patients that followed the same protocol, but using regular irrigation and a placebo spray at home (n = 32). Periodontal clinical variables of all participants were gathered, a blood sample was drawn from each subject.

Results:

Some periodontal variables achieved the statistically significant differences between 2 groups: BP5T0, BP6T0, NBOLT0, BOLTOTT0, PISIMT0, SPIM3MT0 and BP6T1, while there were no differences in assessed cardiometabolic variables, except glycemia that decreased significantly in both groups. A slight increase in systolic, diastolic blood pressure and pulse were observed in the test group, as well as a slight decrease in body weight, body mass index and waist circumference. On the other hand, in the control group body weight, body mass index, systolic blood pressure, pulse and waist circumference increased, while diastolic blood pressure decreased.

Conclusions:

The preliminary results obtained indicate that nutraceutical based on EOs as an adjuvant therapeutic agent for periodontal treatment might have some beneficial effect on PD variables when used as an irrigation solution or mouthwash. It might be associated with improvements in some MetS-related variable, and this could have a protective effect against periodontal disease progression, but also CVD. Future long-lasting studies are required to confirm these encouraging preliminary data and elucidate the underlying mechanisms.

Keywords: Cardiovascular disease, Carvacrol, Essential Oils, Eucalyptol, Inflammation, Limonene, Linalool, Metabolic syndrome, Periodontitis, Plant extract, Terpineol, Thymol.

1. BACKGROUND

The present project that was proposed, evaluated and then financed (as a part of PON 2017project), concerns the execution of experiments that could lead to the creation of an industrial path for the identification of a plant extract which acts on the prevention of dental plaque formation in subjects at risk of both PD and the MetS.

The incidence of PD has continuously increased all over the world, but still there is difficulty of finding effective and minimally invasive treatments, once it is diagnosed at an advanced stage, that further encourage basic and applied research in order to identify functional solutions, for both its prevention and early diagnosis. The evidence accumulated through epidemiological studies suggests that PD is associated with systemic diseases such as cardiovascular disease (CVD) and diabetes [1, 2]. Furthermore, a double cause-effect relationship has been hypothesized, where some systemic pathologies may affect the onset and progression of PD, while the presence of PD may have an effect on systemic health. This interaction can be mediated by the release of inflammation mediators with potential systemic effects, but also by the effect of bacteria for which the periodontium may represent an entry into the systemic circulation [3, 4]. Examining the data of almost 14.000 subjects who participated in the American study called NHANES III (Third National Health and Nutrition Examination Survey), it was noted that the prevalence of metabolic syndrome increased with the severity of periodontal disease [5]. Scientific papers published by several research groups have hypothesized that cytokines such as tumor necrosis factor- alpha (TNF-α) and intrleukin-6 (IL-6) [6], oxidative stress[7] or abdominal obesity [8] can represent the basis of the association between PD and the metabolic syndrome. A strong role of small dense (sd) proatherogenic low-density lipoprotein (LDL) subclasses in the future cardiovascular risk of patients with the metabolic syndrome has been also demonstrated [9] and a later study has shown that this phenotype of LDL, with the predominance of sdLDL, is common in subjects with mild untreated PD [10]. Moreover, in the above mentioned study, it was shown that subjects with PD have high levels of heat shock protein 60 (Hsp60) compared to controls, with a strong correlation between sdLDL and periodontal disease that is in accordance with another research study [11]. In this regard, it has been suggested that Hsp60 may represent the link between PD and atherosclerosis [12]. Consequently, the aim of the present research was to find solutions to identify the risk of PD early, all that can be easily evaluated with economic and minimally invasive techniques using the most modern knowledge and molecular methods. This project is consistent with one of the National Smart Specialization (SNSI) technological strategies approved by the European Commission and published on the website of the Research Italy Health subgroup; in the “Nutraceutical, Nutrigenomics and Functional Food” trajectory and within this, the project is part

of the theme “New Diagnostic Systems", “Nutritional Disorders, dietary strategies for the prevention of diseases associated to the diet, metabolic diseases” and “Foods calibrated on the nutritional needs of specific groups of people (proxy-personalized)”.

Currently nutraceuticals are getting important attention due to both nutrition and therapeutic potentials [13]. In addition, as reported in 2017 by Dr Aronson in *the British Journal of Clinical Pharmacology* [14], there is no a specific definition of nutraceuticals in order to distinguish them from other food-derived categories, such as functional foods, food supplements, herbal products, pre- and probiotics, and fortified foods. Briefly, we are summarizing what the term nutraceutical and some related words refer to.

Nutrient is defined as *“a nutritious substance”*, indicating the role of nutrients as functionally active components of the diet (in the Oxford English Dictionary (OED) defined as *“any substance which nourishes by promoting the growth or repairing the waste of animal bodies”*).

The term “nutraceutical” was invented by Stephen L. Defelice in 1989, who established The Foundation for Innovation in Medicine in 1976 [15]. Furthermore, the nutraceuticals are defined as the phytocomplex (derivatives of vegetal origin), and as the pool of secondary metabolites (derivatives of animal origin), concentrated and administered in a suitable pharmaceutical form. Beyond basic nutritional functions of nutraceuticals, they improve well-being and life quality by reducing the disease risk or beneficially affecting target functions; thus, they can be used for the prevention and/or the treatments. Finally, nutraceuticals seem to have the same or even more anti-inflammatory and antimicrobial effect compared to the conventional therapy without adding any chemicals, although there is not enough scientific evidence on this topic [16].

The term *“preparation”* can refer to the pure substance itself (prepared from a plant resulting in often crude extracts - herbal products) or to the pharmaceutical formulation [14]. Considering all these definitions and using the approaches of Dr Aronson, the plant extract used in our study could belong in the group "Herbal products". Having in mind the definition of a herb in the OED (“*a plant of which the stem does not become woody and persistent (as in a shrub or a tree) but remains more or less soft and succulent, and dies down to the ground (or entirely) after flowering”*; spec. applied to plants of which the leaves, or leaves and stem, are used for food or medicine, or in some way for their scent or flavour), it is clear that a lot of herbal products are obtained from plants that are not rigidly speaking herbs [14]. In this context, there is no internationally recognized definition of a herbal medicine [17]. However, there are official definitions for terms related to herbal products proposed by the European Union (EU): “herbal substances (or herbal drugs)”, “herbal preparations”, and “herbal medicinal products” [18, 19]. *A herbal substance* is usually in an unprocessed, dried form, sometimes also fresh consisting of mostly whole, fragmented, or cut plants, plant parts, algae, fungi, or lichen. *A herbal*

preparation is obtained by subjecting herbal substances to different treatments such as extraction, purification, concentration, expression, fractionation, distillation, or fermentation. *A herbal medicinal product* is any medicinal product entirely containing as active ingredients one or more herbal substances or herbal preparations, or herbal substances in combination with one or more such herbal preparations [19]. Furthermore, *"the product may include vitamins or minerals with well-documented evidence for safety, providing that the action of the vitamins or minerals is ancillary to that of the herbal active ingredients regarding the specific claimed indications"*.

The World Health Organization has described the constituents of herbal medicines as those that include *"herbs, herbal materials, herbal preparations and finished herbal products"* and *"in some countries by tradition, natural organic or inorganic active ingredients that are not of plant origin (e.g. animal and mineral materials)"* [20].

1.1 ORAL CAVITY

The tooth represents a complex composition of accurately patterned, mineralized matrices and soft tissues. Mineralized tissues consist of enamel (produced by the epithelial cells called ameloblasts), dentin and cementum (produced by mesenchymal cells called odontoblasts and cementoblasts, respectively), while soft tissues include the dental pulp and the periodontal ligament along with the invading nerves and blood vessels [21]. In recent years, the role of healthy teeth, including the impact of oral health on general well-being, has become increasingly evident. It should be mentioned that tooth loss, caused by different reasons, such as tooth decay, trauma, periodontal diseases, congenital malformations as well as age-related changes, is generally replaced by artificial materials which lack several of the essential biological characteristics of the natural tooth. On the other hand, given that human teeth have very low to almost absent regeneration potential, as a result of early loss of stem cells with regenerative capacity, considerable effort has been made over the past few decades to identify and characterize tooth stem cells, and to clarify the complex processes which these cells follow during the tooth development [21, 22].

The oral cavity is surrounded by the lips anteriorly, the cheeks laterally, the floor of the mouth inferiorly, the oropharynx posteriorly (positioned between the hard and the soft palate, and behind the circumvallate papillae of the tongue), and the palate superiorly [23]. Humans possess two sets of teeth: 20 primary teeth and 32 permanent teeth and they are classified as central and lateral incisors, canines, premolars, and molars. Different designation/identification systems may be used to classify permanent teeth, but the most common method is the Universal numbering system [24, 25] where teeth are counted from 1-32 beginning with the maxillary right third molar (#1) to the left maxillary third molar (#16), continuing to the left mandible ant the left mandibular third molar (#17) and ending in the right mandible and the right mandibular third molar (#32) **(Fig. 1).**

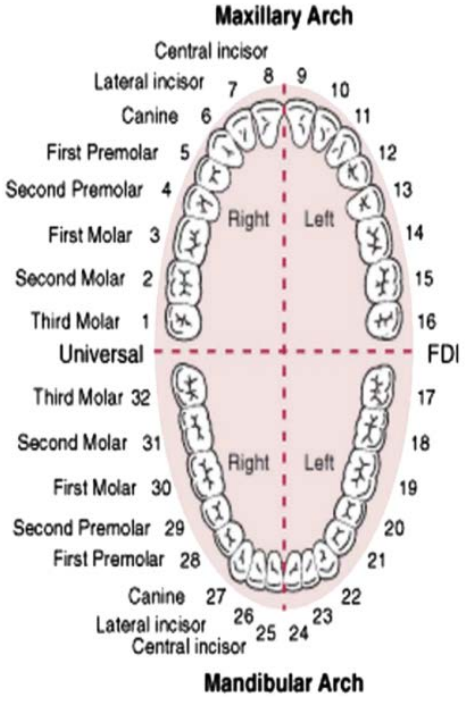


Fig. 1. Universal numbering system for permanent teeth as recommended by the Federation Dentaire Internationale (FDI). (From [23])

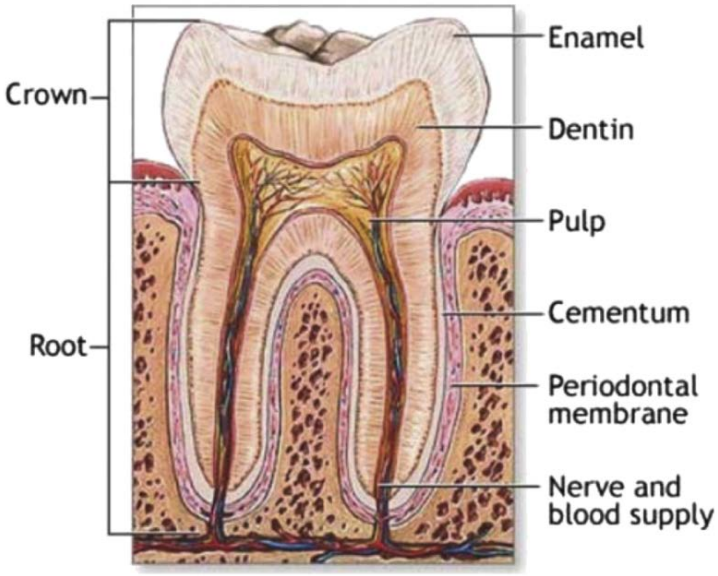


Fig. 2. Anatomy of a tooth. (From [23])

Having very specific functions each tooth has a specific location and contains specific shapes that support its function. However, although different in shape, all teeth have the same anatomical parts and each tooth can be divided into 2 parts: the crown and the root(s). Enamel, the first line of protection for the tooth, covers the outer portion of the crown, represent the hardest substance in the body, mostly made of calcium phosphate, a rock-hard mineral, and is somewhat translucent. Dentin, is immediately beneath the enamel layer, and lies between the enamel and the cementum in the tooth forming the bulk of the tooth that can be sensitive if the enamel is lost. The cementum is a very thin substance that covers the root of the tooth and is not as hard as the enamel but has a similar hardness to bone - contains microscopic tubes and aids in attaching the tooth to the bony socket **(Fig. 2).** The soft tissue including the blood and nerve supply to the tooth (pulp) is housed within the dentin, extending from the tip of the root - the pulp canals to the crown - the pulp chamber. In case that the pulp area becomes exposed to decay, a bacterial infection can occur and root canal therapy may be needed in order to save the tooth. Supporting structures of the teeth (periodontium) consists of the periodontal ligament, gingival tissue, bone, blood, and nerves. The periodontal ligament is made up of thousands of fibers, which connects the cementum to the bony socket and alveolar surrounding bone, in both the maxilla and mandible in humans and act as shock absorbers for the teeth, which are exposed to heavy forces during function. Also, these ligaments have a function as sensory, nutritive,

and remodelling structures surrounding the roots. Teeth and bone are covered by gingival tissue which protect them and provides an easily lubricated surface. The alveolar portions of the mandibular and maxillary bones contain sockets to support the roots of the teeth. Each tooth and periodontal ligament have a nerve supply (the trigeminal nerve) which make the teeth sensitive to various stimuli, while the vitality of the tooth is maintained by the blood supply.

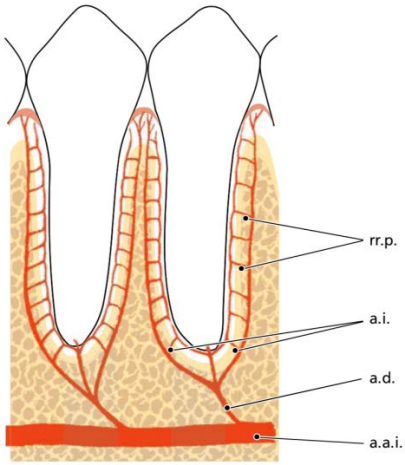


Fig. 3 The schematic drawing depicts the blood supply to the teeth and the periodontal tissues (From [26])

The blood supply to the teeth and the periodontal tissues. The dental artery (a.d.), which is a branch of the superior or inferior alveolar artery (a.a.i.), dismisses the intraseptal artery (a.i.) before it enters the tooth socket. The terminal branches of the intraseptal artery (rami perforantes, rr.p.) penetrate the alveolar bone proper in canals at all levels of the socket. They anastomose in the periodontal ligament space, together with blood vessels originating from the apical portion of the periodontal ligament and with other terminal branches from the intraseptal artery (a.i.). Before the dental artery (a.d.) enters the root canal it puts out branches which supply the apical portion of the periodontal ligament [26].

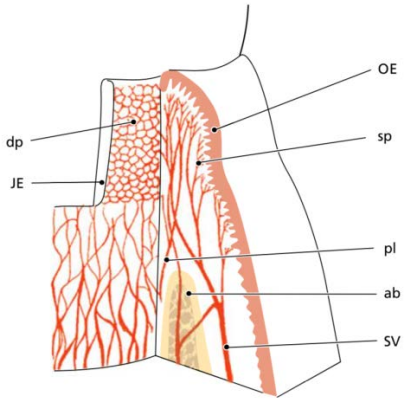


Fig. 4 Schematic drawing of the blood supply to the free gingiva (From [26])

On the figure above the blood supply to the free gingiva is presented schematically.
The main blood supply of the free gingiva derives from the supraperiosteal blood vessels (SV) which, in the gingiva, anastomose with blood vessels from the alveolar bone (ab) and periodontal ligament (pl). To the right, the oral epithelium (OE) is depicted with its underlying subepithelial plexus of vessels (sp). To the left beneath the junctional epithelium (JE), the dentogingival plexus (dp) can be seen, which, under normal conditions, comprises a fine-meshed network without capillary loops.

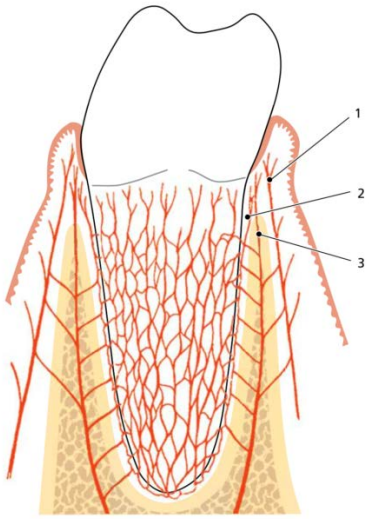


Fig. 5 The schematic drawing of the blood supply of the periodontium (From [26])

On the figure above the blood supply of the periodontium is presented schematically.
The blood vessels in the periodontal ligament form a polyhedral network surrounding the root. It is important to note that the free gingiva receives its blood supply from (1) supraperiosteal blood vessels, (2) the blood vessels of the periodontal ligament, and (3) the blood vessels of the alveolar bone.
A general name for disease of the teeth is tooth decay that also includes cavities.
Cavities (caries) usually occur on molars and premolars; it is caused by a combination of several factors (bacteria, sipping sugary drinks and poor cleaning of the teeth) and represent permanently damaged areas in the hard surface of the teeth (the enamel and deeper structures).
Poor oral hygiene may also induce PD - inflammation of the deeper structures of the teeth (periodontal ligament, jawbone, and cementum). Inflammation of the surface portion of the gums, around and between the crowns of the teeth is called gingivitis and untreated gingivitis can progress to PD. It may be caused by the presence of plaque (a sticky, color less film made of bacteria and the substances

they secrete) and tartar (plaque mixed with minerals in a harder substance). Plaque can be without difficulty brushed off, while tartar necessitate a professional cleaning to be removed.

1.2 BLOOD VESSELS

The vascular system maintain cellular homeostasis through a complex network of arteries, capillaries and veins [27]. Regarding the vessel organization within the cardiovascular system, there are essentially two components in the circulatory system: the cardiovascular system (consisting of the heart, vessels, and blood with all cellular components within the blood) and lymphatic systems (consists of lymphatic microvessels (capillaries) and larger lymph vessels).

The most blood vessels usually consist of three histologically distinct regions with variable amounts of smooth muscle cells and elastin [28]. The variability in cellular constituents is based on the physiological function that the vessel serves to an organ or tissue and it is determined early in the development. Each region of a blood vessel is called a "tunic" (latin term meaning "*a membrane or related structure covering or lining a body part or organ*"). Anatomically (from the lumen of the blood vessel outward), these regions are termed the tunica intima, the tunica media, and the tunica adventitia (**Fig. 6**).

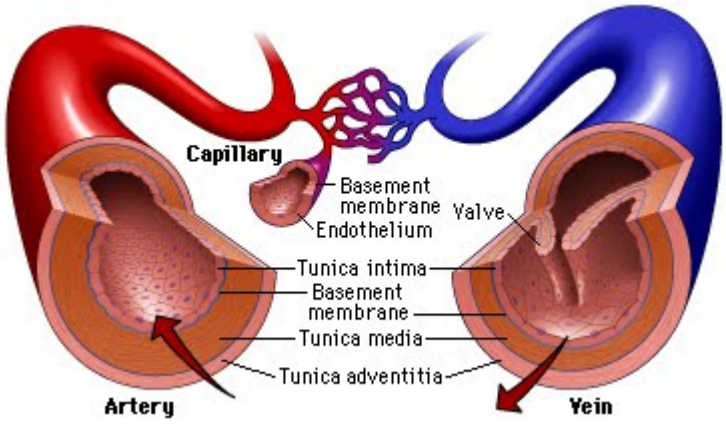


Fig. 6 Blood Vessel Structure (from https://slidetodoc.com/histology-of-the-circulatory-system-the-cardiovascular-system/)

The thinnest constituent layer, ***the tunica intima***, consists of a single layer of endothelial cells mounted on a basement membrane (basal lamina). Below there is a subendothelial, fibroelastic connective tissue layer and an organized layer of internal elastic lamina that provides flexibility and stability for endothelial cells [29]. The endothelial cells, being in direct contact with the blood, have a critical role in all aspects of tissue homeostasis. They regulate vascular tone by an interaction with components of the peripheral nervous system and also are involved in coagulation processes and thrombolysis as well as in inflammatory and immunological processes, and, consequently are

implicated in atherosclerosis and occlusive vascular disorders [30, 31]. The subendothelial layer of intima consists of vascular smooth muscle cells (VSMCs), arranged in several layers, and the extracellular matrix (ECM) that is rich in longitudinally oriented elastic fibers, proteoglycans and also dendritic cells are present [32, 33]. In response to proatherogenic stimuli, VSMCs proliferate and produce a high quantity of modified ECM, forming pre-pathological intimal thickening [29, 32] that could further lead to the early stage of atherosclerosis, when, under a layer of VSMCs, lipids are accumulated in the ECM, forming lipid pools, rich in proteoglycans and hyaluronan [34]. In this part of the subendothelial layer, the ECM proteoglycan biglycan facilitates binding, retention, and deposition of LDL-C. ***The tunica media*** contains mainly VSMCs cells and elastin fibers, collagen, and proteoglycans [31]. In larger arteries these cell layers tend to be more highly organized owing to the function that these vessels play in the movement of large volumes of blood. Another layer in this region of the blood vessel provides structural support and it is an external elastic lamina. These interrupted layers of elastin separate the media from fibroelastic connective tissue which makes the last and outermost layer called ***the tunica adventitia***. In the adventitia both lymphatic and nerve plexi are observed along with the *vaso vasorum* which represents a network of small thin-walled blood vessels and provides these larger arteries and veins with adequate oxygen and nutrients playing a major role in normal vessel wall biology and pathology [28]. It has been suggested that the leading cause and the initial event of atherosclerosis is specifically the injury of vasa vasorum - disruption or occlusion which lead to ischemia and ischemic necrosis of the cells in the subintimal layers [35]. Fibroblasts are the main cells in the adventitia which also contains progenitor and immune cells (including macrophages, T cells, B cells and dendritic cells [36, 37]. Fibroblasts differentiate into myofibroblasts in response to injury, migrate to the intima, secrete factors that regulate the growth of endothelial cells, VSMCs and recruit inflammatory and progenitor cells to the vessel wall [30, 38, 39].

It is known that changes in the vascular wall environment are often the main trigger for CV events [40], including the presence of atherosclerotic plaque, e.g. more than 86% of acute myocardial infarction occurred in those subjects with unstable plaques associated with a stenosis <70% of the vessel lumen [41]. Further, it is known that circulating cholesterol particles (LDL particles) are transported from the vascular space into the arterial wall and retained in the extracellular matrix, where they are prone to be oxidized. LDL oxidation plays a significant role in atherogenesis, oxLDL represent an early event in atherosclerosis, that and further contributes to formation of atherosclerotic plaque [42]. In addition to increased oxidation, increased arterial entry and arterial retention, as well as decreased clearance by LDL receptors, the main mechanisms include also endothelial dysfunction, foam cell formation, SMCs migration and proliferation and induction of platelet adhesion. PD also

may affect lipoprotein metabolism and all lipoprotein classes. The effects of PD or its bacterial signatures may be involved not only in elevated storage of proatherogenic lipids but also in attenuation of the anti-atherogenic processes, thus increasing the overall risk of atherosclerosis [43]. Direct and indirect mechanisms have been proposed to be involved in putative PD -induced atherosclerosis [43]; as a consequence of oral inflammation, these patients are continuously exposed to dysbiotic oral bacteria and their virulence factors that cause and maintain systemic low-grade inflammation. In susceptible subjects, bacteremia and endotoxemia linked with immune dysfunction produce proatherogenic responses and CV risk factors. The association between chronic apical PD and atherothrombotic CVD has been reported too [44].

1.3 THE PATHOPHYSIOLOGY OF ATHEROSCLEROSIS AND PERIODONTITIS AND THE CORRELATIONS BETWEEN THE TWO DISEASES

Some authors suggested that PD should be considered as a public health problem [45], because of its high prevalence as well as its consideration as a risk factor for CVD. In this term, the European Society of Cardiology describes PD as a disease which increases the CVD risk [46]. Similarly, the joint Workshop of the European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) reported in 2012 that there is a strong evidence that PD increases the risk for atherosclerotic CVD" [45], that also has been concluded by the American Heart Association stating that PD is associated with vascular atherosclerosis, independently of known confounding factors highlighting that the need to uniform criteria in the measurement of PD, as well as a need for long term, well designed controlled intervention studies involving treatment-response protocols [47]. The PAROKRANK study is one of the biggest epidemiologic studies that reported a significant increased risk for acute myocardial infarction among subjects with PD (about 30 % compared to controls) after adjusting for confounding variables (diabetes mellitus, smoking habits, years of education, and marital status) [48]. Also, several other authors indicated a similar risk range (25-50%) for the development of CVD in subjects with PD compared to healthy subjects [49, 50] and some even indicate such risk by 65 % [51]. Furthermore, PD is associated with endothelial dysfunction, a known surrogate marker of atherosclerosis further associated with decreased flow-mediated dilation of the brachial artery, probably as a result of an elevated thickening of the intima-media layer of the vessel [52-55]. On the other hand, systematic review by including 14 randomized clinical trials, indicate that periodontal therapy may improve endothelial function [56].

Periodontal bacteria or inflammatory mediators could cause such dysregulation in the endothelium and later lead to atheroma plaque formation. Interestingly, periodontal treatment can improve endothelial function [57-60]. Both improvements in periodontal status, clinical (probing pocket depth

and attachment loss measurements at 6 sites per tooth) and microbiological (quantitative assessment of some known periodontal pathogens by DNA-DNA checkerboard hybridization) was correlated with less progression of atherosclerosis (assessed by carotid intima-medial thickness (CIMT)) after 3-year median follow-up period [61], supporting the previously published evidence that faster atherosclerosis progression could explain mechanistically the link between PD and CVD, and also highlighting the possible importance of primary periodontal care in the prevention of CVD. However, the available evidence is not sufficient to establish such a causal relationship.

Direct infection of the atheromatous plaque by periodontal bacteria [62, 63]; and the systemic inflammatory state caused by PD are 2 main pathways which stimulated the relationship between PD and atherosclerosis. Some evidence indicates that periodontal bacterial species directly contribute to the atherogenic process [64], while some studies have reported the presence of periodontal pathogens in the atheroma plaque [65-68]. The periodontal pathogens most commonly identified are the following *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, and while *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Prevotella nigrescens*, *Eikenella corrodens*, *Fusobacterium nucleatum* and *Campylobacter rectus* have been less often identified [69], although the prevalence of these bacteria varies among the different studies, but the authors usually found more than one bacterial species in plaques analysed, resulting in the hypothesis that the endothelial dysfunction is caused by the action of several bacteria and not a single pathogen [63].

The access of these bacteria to vascular tissue is described by the production of bacteraemia through the ulceration of the periodontal pocket, during habitual therapeutic procedures or even while brushing or chewing, in subjects with PD or gingivitis [3, 70]. Yet, transcellular mechanism (the passage of bacteria through the cells of the periodontal pocket to the capillary system) is other proposed mechanisms [71], as well as phagocytosis by leukocytes, inside which the pathogens avoid lytic processes and remain alive, and then depart from these cells in a different part of the organism [72].

When PD is accompanied with an ulcerated sulcular epithelium, bacteria from the subgingival biofilm can simply go through the lamina propria and also through the endothelial cells of the subgingival inflamed vessels and, consequently, access the blood stream. A huge evidence indicate that *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* invade epithelial, endothelial and immune cells, such as dendritic cells and monocytes [73].

In addition, it is known that an altered lipid profile may be induced by bacteria of periodontal origin, including increased low-density lipoprotein (LDL) and decreased anti-atherogenic capacity of high-density lipoprotein (HDL) [10, 74]. *P. gingivalis* is able to oxidize the LDL [75], which may also form lipopolysaccharide (LPS)-LDL complex, bonding to LPS, the cell wall components of *P.gingivalis*. Additionally, these forms of LDL favor its deposit in the vascular wall, creating a pro-inflammatory

and pro-atherogenic environment, and inducing the transformation of macrophages to “foam cells” [76]. Given that only certain strains of periodontal pathogens have manifested an invasive capacity and a pro-coagulating effect [77], the decisive role of the bacterial strain in both the invasion and survival of *P. gingivalis* within the cells of the vascular wall has been highlighted [78]. In this context, knowledge about the mechanisms how this bacterium interplays with the vascular endothelium may result in a development of a therapy for lowering the risk of CVD.

The group of Prof. Mesa [79] proposed an explicative model of the interaction between periodontal pathogens and the atherogenic process. Once the periodontal bacteria gain entry to the bloodstream has the capacity to invade the endothelial cells and such invasion leads to the activation of the endothelial cells, which express cell surface adhesion molecules (VCAM-1), and to their apoptosis, allowing the entry of microorganisms into the vascular wall. This environment generates the arrival and activation of previously phagocytized monocytes, which harbor live periodontal pathogens, and their penetration into the vascular wall, where they will be transformed into macrophages; these would undergo apoptosis as a result of the action of the bacteria, and then be released to the medium. Then, the oral bacteria in the medium my affect LDL, leading to its oxidation into oxidated LDL (ox-LDL), which consecutively could induce the transformation of macrophages into “foam cells” and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs). Gingipain from *P. gingivalis* caused a selective proteolysis a main component of LDL particles, apoB-100, and the binding of LDL to cell receptors, an essential step for the progress of atherosclerosis [80]. The production of ROS, caused by PD, could contribute to an increase in the lipid peroxidation, generating an atherogenic lipid profile [81].

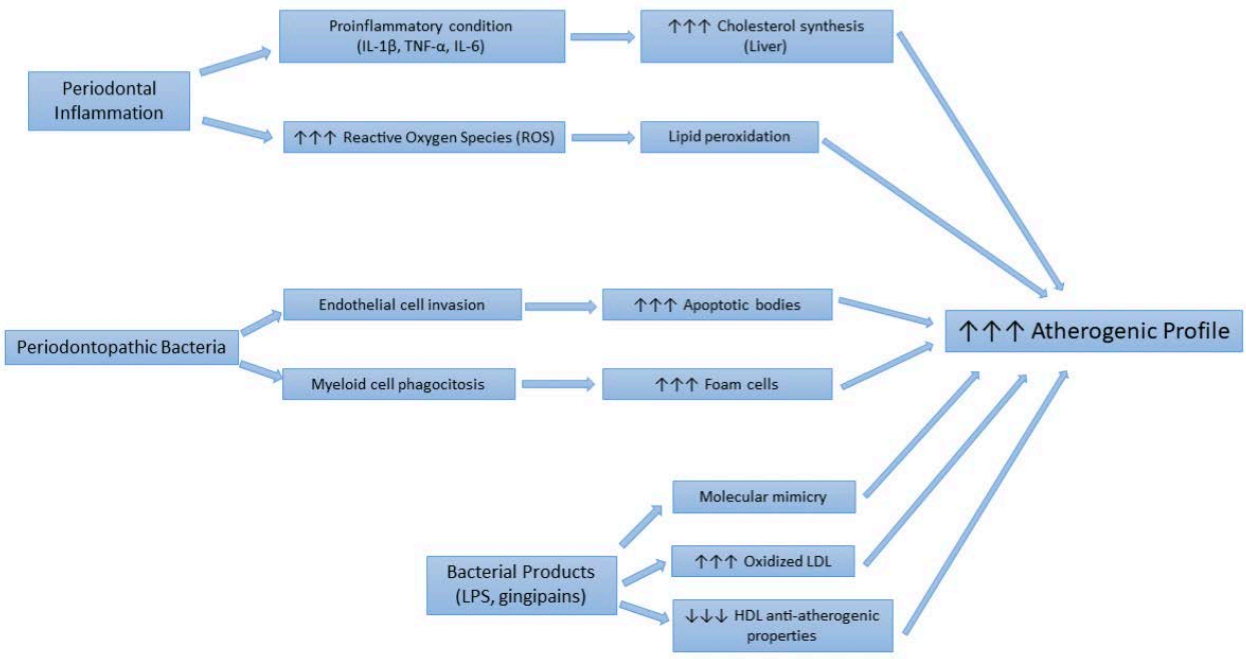


Fig. 7 Model of the interaction between periodontitis and atherosclerosis (From [79])

LPS, a glycolipid endotoxin, is the main component of the membrane and considered as the most important surface antigen, responsible for maintaining of the membrane structure, molecular mimicry, antigenic variations, antibody inhibition, immune system activation and mediation of the adherence to host cells. Lectin receptors on the cell surface and physicochemical interactions have been proposed as possible mechanisms of adherence of LPS, although the exact mechanism remains unknown [82]. In addition, LPS-induced endotoxemia has been suggested as possible molecular link between PD and CVD. Furthermore, it is known that PD is associated with a low-grade systemic inflammation that could contribute to a higher CVD risk, especially in combination with other risk factors [83].

Different toxins may be produced by periodontal pathogens (adhesins, lectins and proteases) that regulate bacterial biofilm and may inhibit host immune response like such as the protease gingipain from *P. gingivalis*, which may inactivate and/or degrade several interleukins (1β, 6 and 8) by a process called "localized chemokine paralysis", or surface receptors from both non-immune and immune cells [84, 85]. Recently, it has been reported that Korean patients with PD have increased number of circulating platelets compared with controls [86]. Also, in studies performed on platelet-rich plasma, *P. gingivalis* induced an increased platelet aggregation [87].

Molecular mimicry or cross-reactivity between bacterial components and the host have been reported. The homology between bacterial and human heat shock proteins (HSPs) may induce atherosclerotic changes [88]. Bacterial HSPs can be recognized as host HSPs and provoke an autoimmune response that contributes to the atherosclerotic processes. In atherosclerotic condition, endothelial cells express HSPs, while cross-reactive T-cells are present in peripheral blood and in the arteries of patients affected by this disease [89, 90]. Cross-reactivity between human HSP60 from endothelial cells and HSP60 from *P. gingivalis* has been determined by specific antibodies. Also, T lymphocytes reacting against HSP60 from *P. gingivalis* have been detected in peripheral blood from subjects with atherosclerosis [91]. Finally, it has been showed that untreated patients with mild PD had elevated levels of serum HSP60 and small, dense LDL particles, compared with controls matched by age and body mass index, supporting the link between both atherogenic dyslipidemia and increased levels of HSP60 with PD [10]. Small, dense LDL are particularly atherogenic with a little affinity for the apoB/E receptor of hepatocytes, that delay their blood clearance, and a high affinity for vascular glycosaminoglycans, which keeps them in the vascular artery wall [92, 93].

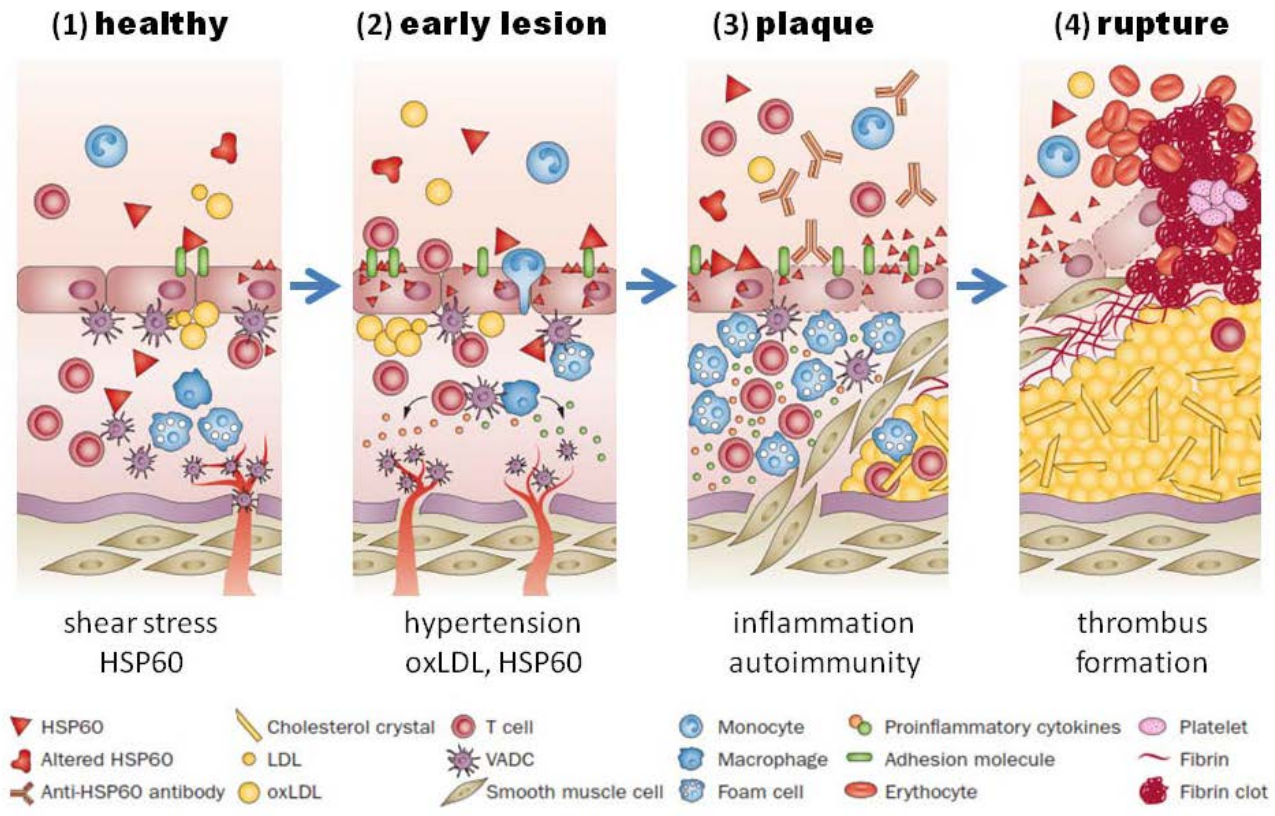


Fig.8 HSP60-induced atherosclerosis (From [90])

As dyslipidaemia is associated with a state of systemic inflammation, lipid alterations could also increase the susceptibility to PD [94], that further suggests a possible bi-directional association between PD and dyslipidaemia [95].

1.4 PERIODONTAL DISEASE

Periodontal disease is classically defined as a chronic inflammatory lesion and gingivitis and PD are the most common diseases derived from periodontium involvement. PD is a complex chronic inflammatory disease caused by gram-negative anaerobic bacteria, located in the subgingival biofilm [96], which can induce the production of inflammatory mediators, causing the destruction and loss of dental bone support [97].

In the initial phase there are no clinical signs, thus the presence of inflammation cannot be observed. However, when the lesion progresses, vasodilation occurs locally due to the action of bacterial metabolic products including cytokines [98]. Such initial lesion continues to progress, and a leukocyte infiltrate (mostly lymphocytes and neutrophils) is produced towards the site of inflammation. Crevicular fluid increase occurs and clinical signs of inflammation appear [99]. In the next phase, or established injury, an inflammatory infiltrate, consisting of T and B lymphocytes, plasma cells, and neutrophils, appears followed by an increase in collagenolytic activity and more collagen-producing

fibroblasts. This stage corresponds to moderate to severe gingivitis [99]. The final phase or advanced lesion is distinguished by an unresolved process, fibrosis and an irreversible loss of bone structure, characterized by clinical and histological patterns [96]. Also, a dense inflammatory infiltrate in connective tissues and predominantly neutrophils in the epithelium are noticed, while, on the other hand, an apical migration of plasma cells to the junctional epithelium occurs to try to defend or keep the epithelial barrier intact and, consequently there is a continuous loss of collagen and connective tissue. Finally, if the lesion extends deep, the osteoclasts cause a resorption that affects the alveolar bone [73].

It should be highlighted that PD is a multifactorial disease that requires interdisciplinary treatment concepts and the selection of a therapy that affects the microbiological nature of the disease [100]. In this regard, the recently introduced classification of periodontal diseases [101] aims to identify well-defined clinical entities using clear criteria that are able to link diagnosis with prevention and treatment, thus moving towards precision and individualized dentistry [102].Interestingly, in the last two decades EOs were extensively tested regarding their beneficial properties against a broad spectrum of bacteria [103], that indicate their use in the files of dentistry and periodontal diseases.

1.5 CHRONIC CARDIOVASCULAR DISEASES

Chronic CVDs such as coronary heart disease, myocardial infarction, and ischemic stroke are still one of the leading causes of death worldwide [104]. According to the World Health Organization (WHO) in 2016 it accounted for about 17.9 million deaths representing about 31% of the total of deaths globally [105]. Atherosclerosis represents one of the most important underlying contributor of the CVDs as a pathological condition characterized by the plaque formation in the inner lining (intima) of the arterial walls through the accumulation of lipids, cells, and extracellular matrix [106]. In addition, it is very well known that increased LDL-cholesterol levels are the major risk factor for coronary heart disease and together with high cholesterol and triglycerides are the main risk factors for atherosclerosis [107]. The current clinical approaches against atherosclerosis are mostly focused on the prevention of plaque growth and destabilization by controlling of different risk factors (such as hypertension, cholesterol level, diabetes, cigarette smoking, etc.) through lifestyle modifications (healthy diet, exercise, smoking cessation, etc.) and medication. Recent understanding of atherosclerosis at a molecular level revealed that cholesterol and lipid deposition are not the only causative factors of this disease. Systemic and chronic inflammation plays critical roles at both stages, at the initiation and the progression of atherosclerotic disease [108, 109]. During atherogenesis, monocytes are recruited from blood to the inflamed arterial vascular wall and locally differentiate into inflammatory macrophages and lipid-laden foam cells, which further drive the local inflammatory process and stimulate plaque development and thrombosis [110, 111]. IL-10, a known broad-spectrum immunoregulatory cytokine with powerful anti-inflammatory properties [112], play a protective role against formation of atherosclerotic lesion inhibiting the production of multiple inflammatory mediators from activated macrophages and dendritic cells [113].Of interest, intramuscular injection of IL-10 encoding plasmid DNA in IL-$10^{-/-}$ mice increased noticeably the cytokine level and inhibited the plaque formation by 60% [114].These preclinical findings clearly suggest IL-10 as a promising therapeutic molecule in the management of atherosclerosis [115]. As mentioned above, inflammation plays a major role in the genesis and progression of atherosclerosis [116]. Evidence suggests that subjects with higher levels of circulating C reactive protein (CRP) have a greater risk of suffering of an acute myocardial infarction or cerebrovascular event [117]. On the other hand, the presence of inflammation, the composition of atheromatous nuclei and the thinning of the fibrous cape that cover the nuclei are the main determinants of atheroma plaque vulnerability [47]. It has been reported that the presence of inflammation at the level of the atheromatous plaque interacts with the formation of the fibrous cap, and produces apoptosis and degradation of the extracellular matrix by activation of metalloproteinases and further augmenting the risk of plaque

rupture and consequent development of cardiovascular events [47, 118]. In such situations, certain alternative therapeutic approaches such as the use of nutraceuticals and dietary supplements may be reasonable [119]. In a recent, 6 months prospective study, the effects of *Citrus bergamia* juice (known as Bergamot) extract on cardio-metabolic risk was investigated in subjects with moderate hypercholesterolemia, showing a significantly reduced plasma lipids, improved the atherogenic lipoproteins (reducing atherogenic sdLDL subclasses) and subclinical atherosclerosis (reducing carotid intima-media thickness (cIMT)) [120]. Other recent randomized, double-blind, placebo-controlled study using the supplement containing chlorogenic acid and luteolin (known as Altilix) [121] showed the beneficial effect of this artichoke extract on the two early atherosclerotic markers: cIMT and flow-mediated dilation (FMD), highlighting the clinical importance of such supplementation in subjects with the metabolic syndrome, as well as its nutraceutical properties and benefits on vascular function including favorable action on the cardiovascular system and also hepatoprotective activity. It is known that the main causes of mortality of subjects with non-alcoholic fatty liver disease (NAFLD) are CVDs. Although the available data are not numerous for a final conclusion and relatively few nutraceuticals have been adequately studied for their effects on NAFLD, several nutraceuticals have been shown to contribute to the improvement of lipid infiltration of the liver and of the related anthropometric, and/or biochemical parameters [122]. However, such their positive effects are associated with well-chosen dose, supplementation for a medium-long period, as well as lifestyle changes.

1.6 PERIODONTITIS AND CARDIOVASCULAR RISK

PD is also described as an infectious disease which affects the tooth-supporting tissues and leads a numerous clinical, microbiological and immunological symptoms, associated with and, probably, induced by progressive interaction among infectious agents, host immune responses, hazardous environmental exposure and genetic predisposition [123]. Anaerobic bacteria are considered as periodontal pathogens, and the following have been highlighted: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella spp*., *Bacteroides forsythus*, *Eikenella*, and *Capnocytophaga*. However, it is important to highlight that those bacteria are mandatory for the disease development but are not enough and do not account for all cases of PD. The results of one survey in the USA, indicate that chronic PD affects about 46% of the adult population, with a higher prevalence among the elderly population [124]. This prevalence refers to the cohort of young adults according to the World Health Organization (WHO), aged from 35 to 44years. Interestingly, forms of PD that occur at younger ages (before the age of 30 years), have other characteristics in addition to age and are known as aggressive PD with the prevalence ranging from 0.2% in Caucasians to 2.6%

in Afro-Americans [125].

It is known that the microbiota of the human oral mucosa together with other anatomical locations in the body constitute the human and that an equilibrium between these microorganisms and the host response has a crucial role in both health and development of disease. Unfavourable modifications in the composition of the microbiota are known as dysbiosis [126], that is seen in both cases, PD and CVD. In case of PD, antiseptics and antibiotics such as Chlorhexidine or Metronidazole, are delivered locally in addition to scaling and root planning procedures, in order to eradicate the subgingival microbes, therefore creating a healthy subgingival environment. However, the evidence in the literature is still inconclusive [127], and future clinical trials with strict methodological criteria that will allow a more precise evaluation of the efficacy of local antimicrobials in the treatment of chronic PD, are required. In addition, the interest in the application of natural products has been increased in the last years. Several natural products and herbs have suggested to have better properties and less side effects compared to chemical agents for irrigation. Furthermore, the use of natural extracts and EOs as an irrigation agent for ultrasonic instrumentation has shown to benefit slight adjunctive effect compared to chlorhexidine or water [128]. Yet, the use of natural extract in subjects with a more severe degree of PD was associated with a greater improvement compared with controls [129]. Natural products in forms of oral spray have shown to be efficient against common oral pathogens, but also safe, without significant cytotoxicity in an *in vitro* study [130]. Thus, nutraceuticals might have the potential to prevent the infections and may be used as an adjunctive treatment to conventional therapy as they seem to have the same or even more anti-inflammatory and anti-microbial effect without adding any chemicals. However, still there is not enough scientific evidence on this topic [131, 132].

Numerous mechanisms have been suggested as possible links between PD and atherosclerotic cardiovascular diseases (ASCVD), but the most important include systemic inflammation, molecular mimicry and direct vascular injury interfered by pathogens [47].Several systematic reviews and meta-analyses have been published suggesting an association between periodontal disease and ischaemic heart disease [133-137]. Some authors have suggested that in clinical examination PD and CVD have a weak association and that actually systemic bacterial exposure from PD could be a more trustworthy risk factor. In this context, Mustapha and colleagues [136] reported that PD with increased markers of systemic bacterial exposure (periodontal bacterial burden, PD specific serology and CRP) is associated with a greater risk of coronary heart disease compared with subjects without PD [117]. Also, it has been shown that subjects with PD have higher levels of other inflammatory markers (tumour necrosis factor (TNF), interleukin (IL)-1, 6 and 8) [138].The short-term adaptive response of inflammation is essential for the integration of injury response and repair in cells and tissues, while

the long-term consequences of prolonged inflammation are often not beneficial [139]. On the other hand, it is known that low-grade and chronic features of inflammation are recognized in metabolic diseases such as obesity, insulin resistance, type 2 diabetes, and CVDs [140, 141]. This is referred to an atypical immune response known as metabolically triggered inflammation "metaflammation," which is triggered by metabolic surplus, leading to the activation of different molecules and signalling pathways involved in inflammation [140]. It should be mentioned that both metabolic and immune systems are regulated by the same cellular machinery through numerous hormones, cytokines, and bioactive lipids included in metabolic and immune responses. These metaflammatory pathways can be activated not only by extracellular mediators such as cytokines and lipids particularly saturated fatty acids, but also by intracellular mediators such as endoplasmic reticulum stress and elevated production of reactive oxygen species derived from mitochondria. Fatty acid-binding proteins (FABPs), a family of lipid chaperones, have molecular and cellular links between FABPs and metaflammation, particularly in the context of metabolic diseases such as obesity, diabetes, and atherosclerosis [139].

There is growing data in the literature demonstrating the beneficial effects of nutraceuticals in metabolic diseases and showing significant impact on different cardiometabolic risk factors (including inflammatory markers) and CVD risk. However, more randomized trials as well as observational studies with specific CVD endpoints are needed.

1.7 PLANTS AND INFLAMMATION

Chronic inflammation and oxidative stress are associated with the most of the common chronic disorders and diseases [142, 143]. It is known that the normal functions of biological molecules (such as proteins, lipids, and DNA) are destabilized by oxidative stress sustained by free radicals (ROS, NRS), that also affects many inflammation-related signalling pathways, thus influencing the cellular and tissues homeostasis. On the other hand, chronic inflammation is characterized by the production of pro-inflammatory cytokines and chemokines which lead to pain, redness, and swelling of the involved tissue [144]. In traditional medicine EOs have been used for the treatment of inflammatory processes [145] as they possess many beneficial properties due to the presence of several anti-oxidant and anti-inflammation compounds such as terpenes, the main class of compounds, and especially monoterpenes [146]. They are also present in numerous pharmaceutical products [147].

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is an acyclic monoterpene found in EOs of hundreds of plants widely spread worldwide and principally in *Lamiaceae* family [148]. Several *in vitro* and *in vivo* studies demonstrated different anti-inflammatory effects of this monoterpene interfering also with the mediators of the inflammation pathways. In details, in RAW 264.6 monocyte/macrophage-like cells linalool decreased the generation of lipopolysaccharide (LPS)-induced TNF-α and IL-6 and inhibited the activation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [149]. Also, in animal models it has been shown that linalool attenuated acute lung inflammation by reducing TNF-α, IL-6, IL-8, IL-1β and monocyte chemoattractant protein-1 (MCP-1) production [150], further supporting linalool as a promising tool to treat inflammatory related diseases.

Terpineols are isomers of monocyclic monoterpene alcohol, naturally present in different plants, among which the most common are α-terpineol and terpinen-4-ol [151]. In particular, the latter has been shown *in vitro* to suppress inflammatory mediator production by activation of monocytes [152], to inhibit inflammatory cytokine generation in LPS-stimulated human macrophages [153], but also in animal models to attenuate inflammation in dextran sulphate sodium-induced colitis [154], prevent LPS-induced acute lung injury by decreasing LPS-induced NF-κB activation and trigger peroxisome proliferator-activated receptor gamma (PPAR-γ) [155].

Limonene (1-Methyl-4-(prop-1-en-2-yl) cyclohex-1-ene), a cyclic monoterpene and one of the most common terpenes in nature as well as the main constituent of citrus EOs. Its anti-inflammatory effects are principally linked to the modulation of cytokines and the interference with the inflammatory-related pathways as demonstrated by *in vitro* and *in vivo* assays. Limonene decreases leukocytes infiltration and neutrophils migration, as well as the levels of TNF-α in cell derived from the

peritoneal cavity and in the peritoneal exudate of zymosan-induced peritonitis BALB/C mice [156]. In LPS inflammation-induced RAW 264.7 macrophages, limonene reduced in a dose-dependent manner the levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, together with the reduced expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX) and prostaglandin E2 (PGE2) [157]. Similarly, *in vitro* model of osteoarthritis with IL-1β-stimulated human chondrocytes, limonene negatively modulated nitric oxide (NO) production by decreasing iNOS, matrix metalloproteinase (MMP)-1 and MMP-13expression, besides NF-κB and p38 activation [158].

Carvacrol (5-isopropyl-2-methylphenol) is a cyclic monoterpene mainly present in the EO of plants from *Lamiaceae* family. In an experimental rat model of periodontal disease (EPD), carvacrol maintain alveolar bone resorption and decreased tissue lesion at histopathology, with preservation of the gingival tissue demonstrating also anti-inflammatory and antibacterial activities [159]. In addition, *in vitro* in murine macrophages carvacrol (1, 10, and 100 μg/mL) reduced the LPS-induced nitrite production as well as *in vivo* in a model of carrageenan-induced pleurisy with a pre-treatment 50 or 100 mg/kg; i.p., carvacrol reduced the levels of TNF-α and suppressed leukocytes recruitment in pleural lavage [160]. In human macrophage-like U937 cells, carvacrol suppressed LPS-induced COX-2 expression activating PPARγ indicating on its anti-inflammatory properties [161] and with no selectivity for both COX-1 and COX-2 enzyme isoforms [162]. Furthermore, it has been shown that carvacrol induces Nav blockade in DRG neurons [163, 164] and Gonçalves *et al.* reported also significant analgesic activity and dose-dependency of *T. capitatus* EO, it is suggest that it is the main active molecule behind the antinociceptive effects of *T. capitatus* through peripheral nervous excitability blockade [165]. Other study showed that carvacrol and thymol have the most potent antimicrobial activity against *Escherichia coli, Sta. aureus, Str. epidermidis, Enterococcus faecalis, Yersinia enterocolitica, Candida albicans, Bacillus cereus, Listeria monocytogenes, Salmonella typhimurium* and *Saccharomyces cerevisiae*, with the exception of *Pseudomonas aeruginosa* [166]. Considering that *T. capitatus* fractions are characterized by the presence of carvacrol as dominant constituent, the antibacterial properties may be attributed to this oxygenated monoterpene.

Thymol (2-isopropyl-5-methylphenol), a monoterpene phenol, is a typical compound in EOs from thyme species. Thymol also ameliorates inflammation *in vitro* in LPS-induced inflammation in murine macrophage cells [167] as well as in LPS and interferon (IFN)-γ induced macrophage inflammation, besides inhibition of the iNO RNA expression in J774A.1 cells [168]. Thymol may also modify prostaglandin catalysed biosynthesis by the inhibition of COX-1 and COX-2 isoforms [169]. Moreover, in mouse mammary epithelial cells, LPS-induced inflammatory response was decreased after thymol treatment (40 μg/ml) by the down regulation of MAPK and NF-κB signalling pathways [170].

Eucalyptol or 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) is a bicyclic monoterpene isolated from EOs from numerous plants [171]. Its anti-inflammatory properties have also been investigated in human and animal models of respiratory diseases such as asthma, Chronic Obstructive Pulmonary Disease (COPD) and bronchitis [172-174]. *In vitro* studies on LPS-induced human lymphocytes and monocytes showed a reduced expression of cytokines including TNFα, IL-6 and IL-1β accompanied with a decrease in NFκB activated form [175, 176]. On the other hand, given that the interaction between natural polysaccharides and proteins such as mucin and the polar head of membrane phospholipids is known, they may have a protective effect once they replace hydrogen bonds of water molecules, generating and increasing local viscosity [177]. Indeed, polyphenols from *Olea europaea* and natural polysaccharides from *Opuntia ficus indica* had been used as mucoprotective agent due to their ability to form a protective layer on mucosal surface and to accelerate the re-epithelization of dermal wound [178, 179]. Furthermore, the coexisting of both extracts leads to a very significant reduction of intercellular adhesion molecule (ICAM)-1 protein on Caco-2 cells (an immortalized cell line of human colorectal adenocarcinoma cells).

2. AIM

The available scientific evidence shows an association between PD and CVD, including the fact the American Heart Association and the European Society of Cardiology have added PD as one important risk factor in their guidelines. However, more *in vitro* and clinical studies are needed to deep the knowledge and to better understand a relationship between PD and CVD; thus, further research is highly encouraged. It is also evident that CVD remain the main and the first cause of mortality worldwide, so the importance of the role of PD in the development and/or progression of CVD may make it inevitable to incorporate periodontal treatment and care in the public health system. Traditional CV risk factors including genetic variants, but also behavioural and socio-economic factors are undoubtedly, at least in part, responsible for the association between PD and altered lipid metabolism. However, it remains to be clarified whether PD induces higher lipids or higher lipids imply PD [180]. Understanding of this direction would make clear if reduction in periodontal disease would mean CVD prevention too. However, it has been documented that improvement in periodontal health may influence lipid levels and could help in the standard care of hyperlipidaemia [181]. In addition, improved oral hygiene as well as standard non-surgical periodontal treatment are efficient in lowering ox-LDL, and consequently in reducing oxidative stress [81] and inflammation markers, which are well-known factors associated with an increased CV risk and the CVD development [182]. However, the underlying mechanisms are unclear.

In subjects suffering of PD, PD treatment might stabilize impaired lipid metabolism, while increasing public knowledge about the importance of daily oral hygiene might *per se* reduce development of PD and consequently CVD.

The hypothesis of the present study is that subjects with the MetS tend to have greater prevalence of PD and worse both periodontal and metabolic clinical variables. Several plant extracts have been documented to exert protective role against PD reducing inflammation, but also lipids and other the MetS-related parameters.

The general aim of the present study was to analyse the effect of a nutraceutical composed of several plant extracts in subjects with PD and different levels of risk for the MetS.

The specific aims include:

1. to evaluate if the response of some periodontal clinical variables was more effective in subjects treated with the extract compared to controls;
2. to evaluate if the modifier effect of MetS-related variables in the treatment outcomes of the subjects treated with the extract could be more favourable compared to controls;
3. to evaluate the effect of plant extracts on several inflammatory markers.

The realization of these objectives would help to increase the knowledge in this field, and it is expecting that the obtained results would help in the prevention of PD and, consequently CVD, based on the increasing evidence indicating the relationship between these two diseases. In addition, the results would shed a light on a new therapeutic approach for the treatment of PD as a monotherapy or as add-on to existing therapeutic approaches. Such a natural approach could further strengthen the action of standard therapeutic approaches, but also decrease the side effects seen with the use of some of them (e.g., chlorhexidine).

It should be emphasized that a "healthy" periodontium is one that is comfortable and free from both functional and aesthetic problems [183] and participating in the present study would increase awareness of the importance to maintain the oral hygiene and health. It was also planned to study cardio-metabolic risk of such subjects that would contribute to elucidating the known relationship between PD and CVD. In addition, investigation of different inflammatory cytokines would provide better understanding of the underlying mechanisms.

The new formulation of mouthwash/irrigation solutions and nebulizer spray containing several plant extracts is expecting to have favourable effects on both periodontal and metabolic variables, thus further support the use of nutraceuticals in the treatment of PD, preventing its worsening, but also as an additional approach in the treatment of MetS parameters, and in both cases, consequently decreasing CVD risk. Yet, its ease way of administration, benefits for public health as well as low cost should be emphasized.

Although future investigation is needed to confirm its contribution to better treatment outcomes of PD in terms of clinical and metabolic biochemical variables, we believe that an adjutant of a nutraceutical composed of several plant extracts to therapy in subjects with PD and different levels of risk for the MetS would result in distinct favourable effects.

Fifty-four patients with PD were studied during a 3-month follow-up period.

Due to the COVID-19 pandemic we were at a disadvantage for continuing and finalizing the present study. Actually, at the moment of the state of alarm to limit viral transmission (lock down) the subjects should not come to perform the last visit by protocol. The dental clinics were closed and there was no possibility to see the subjects at the cite and to perform activities scheduled for the last visit including taking biochemical variables and final blood and periodontal samples. Consequently, missing data at the end of the treatment (after 6 months) are the main limitation of the study together with relatively short time frame of 3 months. However, the main strength of the present study includes the control (placebo) group, the double-blinded randomization, the good adherence to the treatment, as well as that all parameters were measured in a blinded manner. The preliminary data indicates on beneficial effects of the tested extract on several PD variables, highly supporting future research work

in this field. Longer follow up period, with a bigger cohort of subjects is recommended in order to confirm such effects of the nutraceuticals, strengthen analyses of the hypothesis and to increase the statistical power to detect such effects.

3. WORK PROGRAM

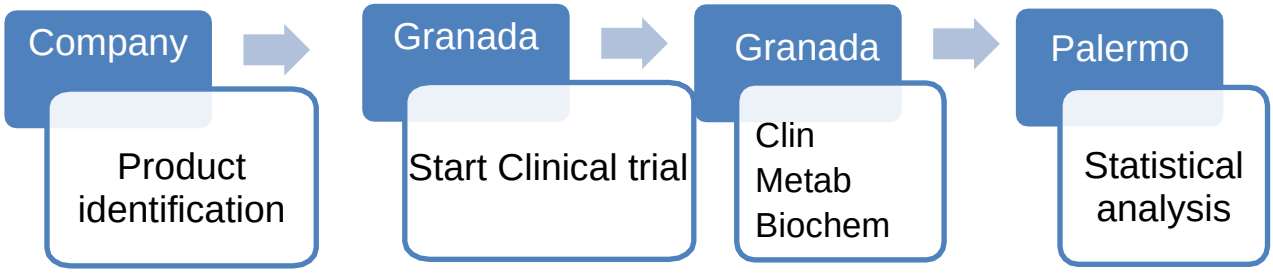
The project has been planned to be performed in a period of three years with the following milestones: Mainly the milestones have been respected, but some changes in the work program become necessary especially following the outbreak of the pandemic. At the beginning we took few months in order to carefully establish the milestones of the project including the study protocol. Then, the period of enrolment has been expanded (from 6 to 18 months) including the total number of the participants (62), as we obtained very interesting preliminary data. Consequently, the period I have stayed in Spain has been prolonged **(Attachment 1-2).** Then, unfortunately, as a consequence of the COVID-19 pandemic, the trial has been interrupted. In details, the modified work program looks like the following:

1. April 2018 – April 2019: my Staying in Italy, at the company and at the University of Palermo. Identification of natural extracts to be tested (experience in the company) and evaluation of collected data in order to design the clinical study (drafting and submission of the protocol to the ethics committee of Granada, Spain); advanced training (courses of Spanish, English language and biostatistics at University of Palermo); contracting order of experimental products and placebo, their labelling, inventory and shipping to the University of Granada.
2. April 2019 – April 2020: my Stay in Spain, at the University of Granada. Management of different issues related to the experimental product, materials necessary for laboratory analyses and organization of the trial in Spain (at University of Granada); screening and enrolment of the subjects in the trial; further bibliographic research looking for the latest published data, improvement of Spanish language; a procedure of the cotutelle (joint supervision) of my doctoral thesis has been created, and the agreement between two Universities has been made.
3. May 2020 – Sep 2021: Smart working as the first COVID-19-related emergency decree due to ongoing COVID-19 pandemic; participating to webinars, Bioinformatics course, improving of the English language; further bibliographic research looking for the latest published data and writing of the review article; statistical analysis of all data collected; writing of the thesis and manuscript preparation for publication.

In detail, the stages of the clinical study were:

1. Study registration, ethical approval, and planning of all procedures related to its performing: December 2018 – July 2019;
2. Study enrolment, allocation, samples and data gathering. Follow-up period: September 2019 – April 2020;
3. Statistical analysis of all data: May 2020 – September 2020.

4. Manuscript preparation and publication process: October 2020 - Sep 2021.



3.1 EXPERIENCE IN THE COMPANY: PRODUCT IDENTIFICATION

During the 6months in the company useful data for a subsequent analysis were collected, highlighting the potentialities and criticalities, both scientific and technical, as well as economic leading to a possible future development of the idea, the main object of the present research.

We searched PubMed and Scopus listings for relevant publications using combinations of the following keywords: “nutraceuticals in periodontal”, “periodontitis”, “anti-inflammation”, “cardiovascular risk and periodontitis”, “metabolic syndrome and periodontitis”, “periodontal therapy”, “essential oils”, “distillation essential oil”, “gas chromatography”, “antimicrobial activity”, “*Streptococcus mutans*”, “*Lactobacillus species*”, “*Olea europaea*”, “*Opuntia ficus indica*” and “*Propolsave*”, “*Rosmarinus officinalis*”, “*Lavandula x intermedia*”, “*Thymus capitatus*”.

Particular attention was paid to the techniques for the design and production of potentially patentable and marketable plant extracts useful in the prevention of dental plaque in subjects at risk of PD and the MetS.

3.1.1 CHOICE OF VEGETABLE MATRICES AND EXTRACTS

3.1.1.2 FOCUS ON ESSENTIAL OILS, PROPERTIES AND USES

In medicine, EOs have been researched for their antibacterial, antifungal, antiviral, insecticidal, anticancer, and antioxidant properties [184, 185]. Plant-based active compounds are considered as a significant source of new chemical substances with potential therapeutic effects [186]. EOs from aromatic and/or medicinal plants, which constitute the odorous, volatile products of an aromatic plant’s secondary metabolism, are complex mixtures of volatile and semi volatile organic compounds originating from a single botanical source [187, 188]. They are formed from mixtures of terpenes [189] and very volatile oxygenated compounds and being very concentrated they are used in dilution with different solvents. Furthermore, the stereochemical properties of EOs can vary and depend upon

the method of extraction [190]. However, extraction products may also vary qualitatively and quantitatively in their composition [191].

3.1.1.3 EVALUATION OF ANY EXTRACTS TO BE USED AS ADDITIVES TO THE PRODUCT: FOCUS ON EXTRACT OF *OPUNTIA FICUS INDICA OLEA EUROPAEA* AND *PROPOLSAVE*

Olea europaea, Opuntia ficus indica and *Propolsave* extracts are a micro-encapsulated dry powder containing purified substances obtained from *Olea Europaea* leaves, *Opuntia ficus indica* cladodes and propolis. During production, *Opuntia* is added to the extract of *Olea* to obtain a micro-encapsulated product able to create a multi-effect called *Mucosave*. The adhesive mucous properties of *Mucosave* act as a protective barrier for the mucous membranes [192] and the purified polyphenols of *Olea* intervene in anti-inflammatory responses and in bacterial protection. *Propolsave* exerts anti-bacterial activity and contains a multi-acting combination of highly purified and characterized polyphenols derived from propolis, and a natural mucoprotective extract from the *Opuntia ficus indica* [129].

3.1.2 EXTRACTION METHOD AND TESTS

3.1.2.1 STUDY OF THE TECHNIQUE AND THE TIMING OF REALIZATION

There are different extraction techniques of EOs; depending on whether the matrix is immersed in water or suspended above the steam source, there are different procedures, but they can also be obtained by cold pressing or by dry distillation. The common method for extraction of EO from hops is the steam distillation method [193]. However, the choice of each technique depends on the objective to be achieved by research.

3.1.2.2 ESSENTIAL OILS OBTAINED BY STEAM DISTILLATION

The steam distillation technique is based on the physical properties of the EOs to be volatile and easily vaporizable [194]. This extraction technique **(Fig.9)** is usually used for plants not sensitive to heat and uses fresh plants, so that the oils are not altered by storage. The time between the harvesting of the plant and its distillation must be as short as possible in order to avoid the alteration and dispersion of the EO during storage [195]. Before being placed inside the distiller, the plant must be cleaned of insects, a material not suitable for distillation and weeds. The steam, generated by the boiling of the water, makes the cell walls more permeable, until it determines the breakage and vaporization of the essence, which is dragged by the steam and subsequently condensed thanks to a coil cooled by a water recirculation. In the majority of cases, the oil is less dense (lighter) than water,

forming the two player of the distillate and can be separated easily using proper method and instruments [196]. The essence obtained is separated from the aqueous phase and can subsequently undergo further purification processes.

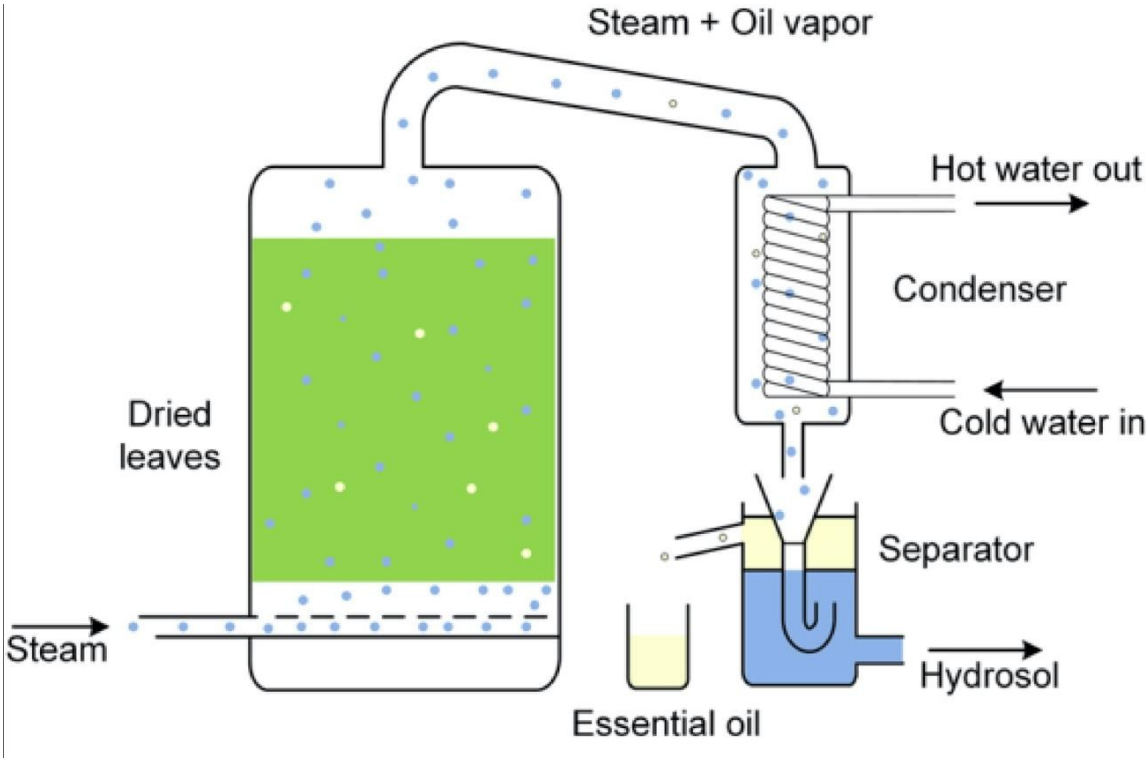


Fig.9 Sci Rep. 2018; 8: 2235. Published online 2018 Feb 2. doi: 10.1038/s41598-017-18141-z

Afterwards, an evaluation was made on the suitability, reliability and applicability of the methods reported in the literature [197]. It was considered useful to study the correlation between the chemical structure of molecules contained in the extracts with their antiseptic, antibacterial, antiviral and anti-infective properties.

3.1.3 CHOICE OF OILS TO BEMIXED

We evaluated the activity of Eos on various strains of pathogenic and non-pathogenic microorganisms. Fourteen EOs were selected and submitted to gas chromatographic analysis, including *Illicium verum, Eucaliptus globulus, Eugenia caryophyllata, Leptospermum scoparium, Mentha arvensis, Mentha piperita, Myrtus communis, Salvia officinalis, Melaleuca alternifolia, Rosmarinus officinalis, Lavandula x intermedia, Thymus capitatus e T.volgare.*

These EOs with antimicrobial activity have been tested on *Streptococcus mutans*e *Lactobacillus spp.*, clinically isolated from subjects undergoing dental surgery. Investigating the relationship between bacterial species and inflammation of the periodontium [198] it has been concluded that some

Lactobacillus strains are able to inhibit the proliferation of pathogens linked to the development of periodontal disease, in particular of some pathogens such as: *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* [199]. This means that favouring the colonization of the oral cavity by some *Lactobacillus* can lead to a general rebalancing of the bacterial flora of the oral cavity by counteracting the inflammation of periodontal tissues [200]. The antibacterial activity was assessed by the Kirby-Bauer test and the minimum inhibitory concentration (MIC). Three EOs, extracted from *Rosmarinus officinalis, Lavandula x intermedia, Thymus capitatus* with antimicrobial activity, were selected for a second screening in combination with each other and with O*puntia ficus indica* and *Olea europaea.*

3.1.4 CHARACTERIZATION OF POWDER OILS

A suitable aliquot of sample was extracted in ethyl-acetate and subjected to sonication in an ultrasound bath for T = 5 min at room temperature. The suspension obtained was centrifuged (T = 5 min, rpm: 14000) and 1 µL of the organic phase analysed by GC-MS analysis

Analysis of essential oils (EO) powder *Lavandino sumian, Origanum vulgare* var. *Hirtum*, Lavanda vera gen, Lavanda mailhette

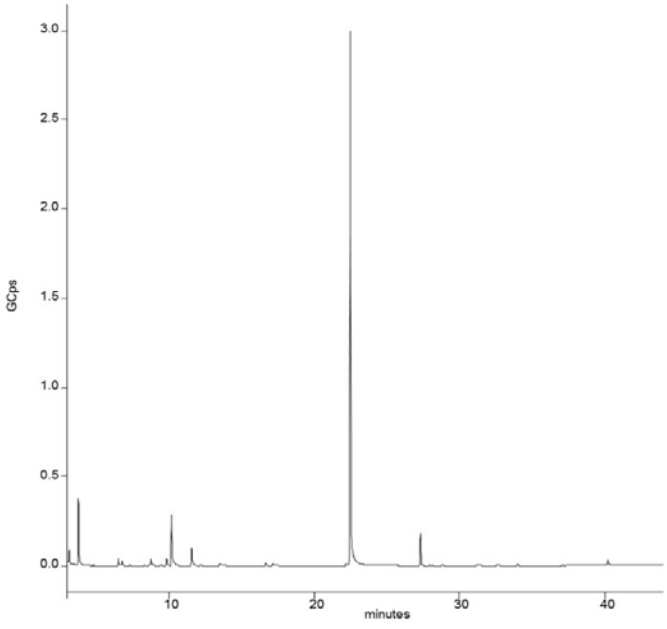


Fig. 1: Gas-chromatographic tracing of OE extracted from the inert support

RT	Peak Name	%
6,526	β-Thujene	0,970
6,772	α-Pinene	0,779
8,75	β-Pinene	1,112
9,842	Terpinolene	1,116
10,164	p-Cymene	7,627
11,557	γ-Terpinene	3,159
16,657	endo-Borneol	0,467
22,176	Thymol	0,360
22,477	Carvacrol	78,805
27,31	β-Caryophyllene	4,612
40,186	β-Bisabolene	0,993

Tab. 1: Gas-chromatographic characterization of powdered OE

3.1.5 GAS-CHROMATOGRAPHIC TRACING OF THE EXTRACTED EOs

3.1.5.1 *Lavandino sumian, Origanum vulgare var. Hirtum, Lavanda vera gen, Lavanda mailhette*

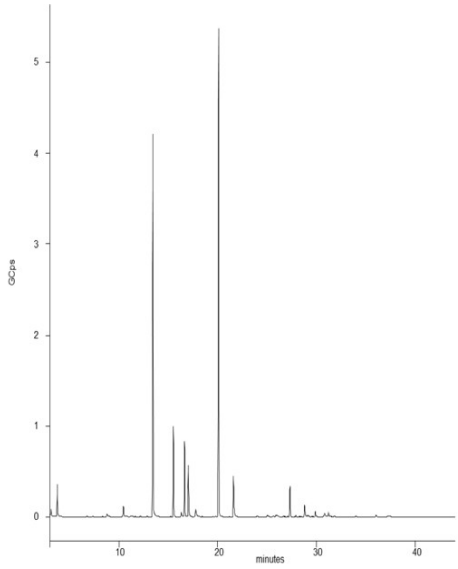


Fig. 2: Gas-chromatographic tracing of OE extracted from *lavandino sumian*

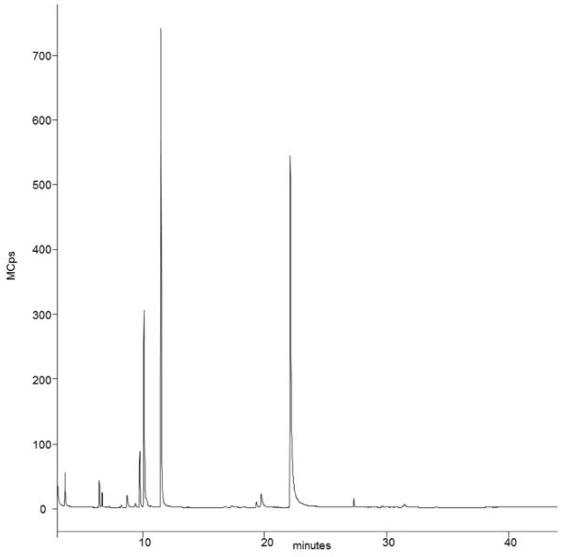


Fig. 3: Gas-chromatographic tracing of OE extracted from *Origanum hirtum*

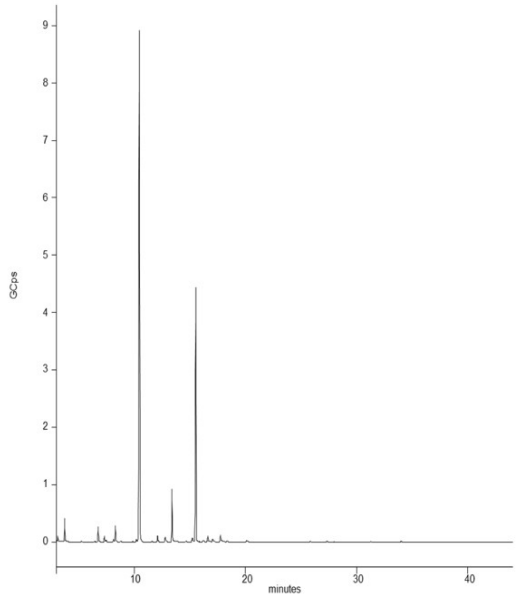


Fig. 4: Gas-chromatographic tracing of OE extracted from lavanda vera gen.

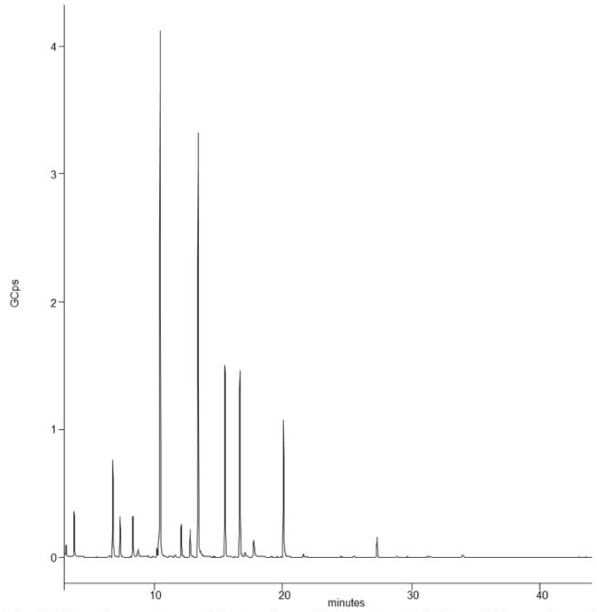
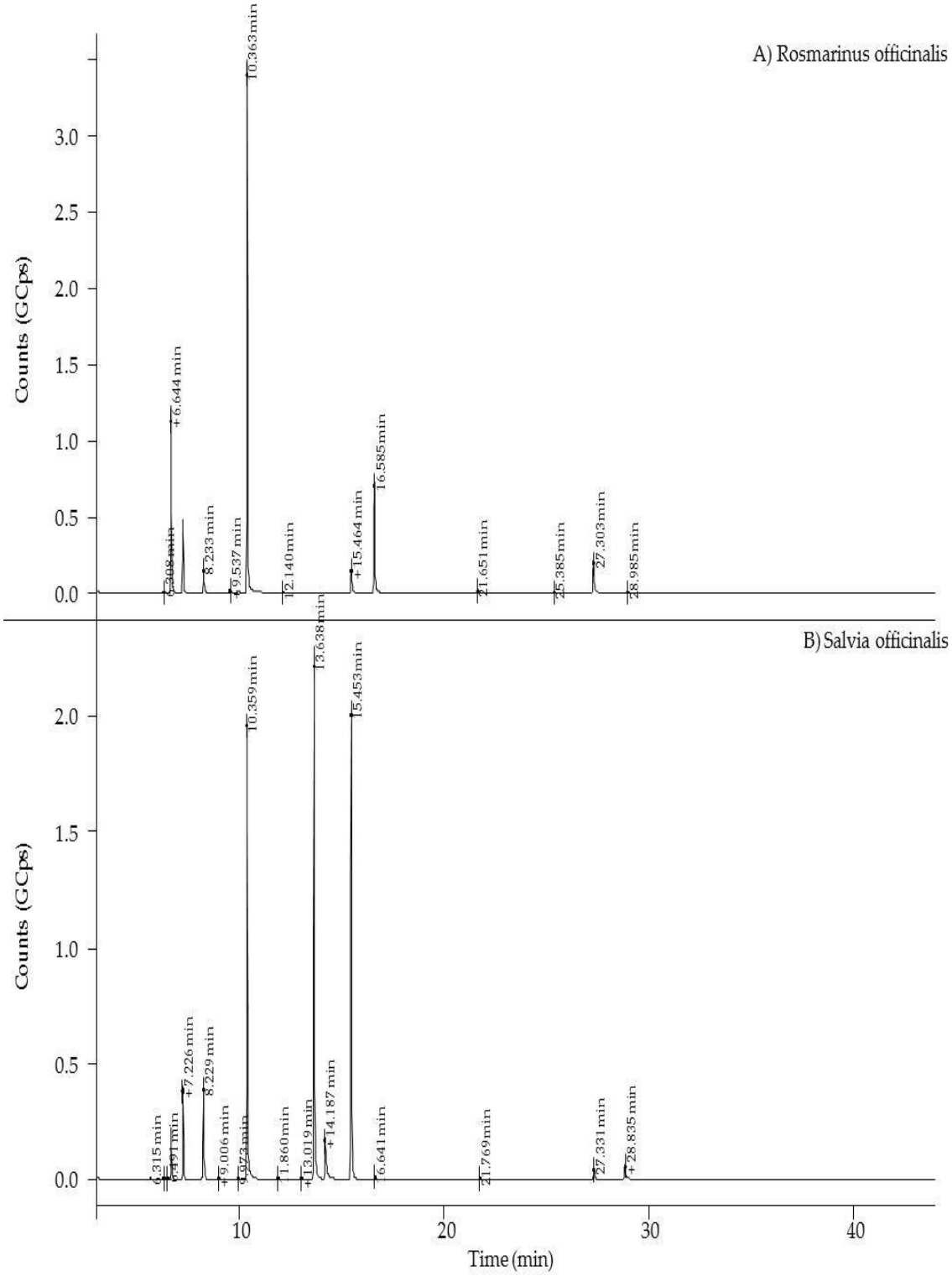
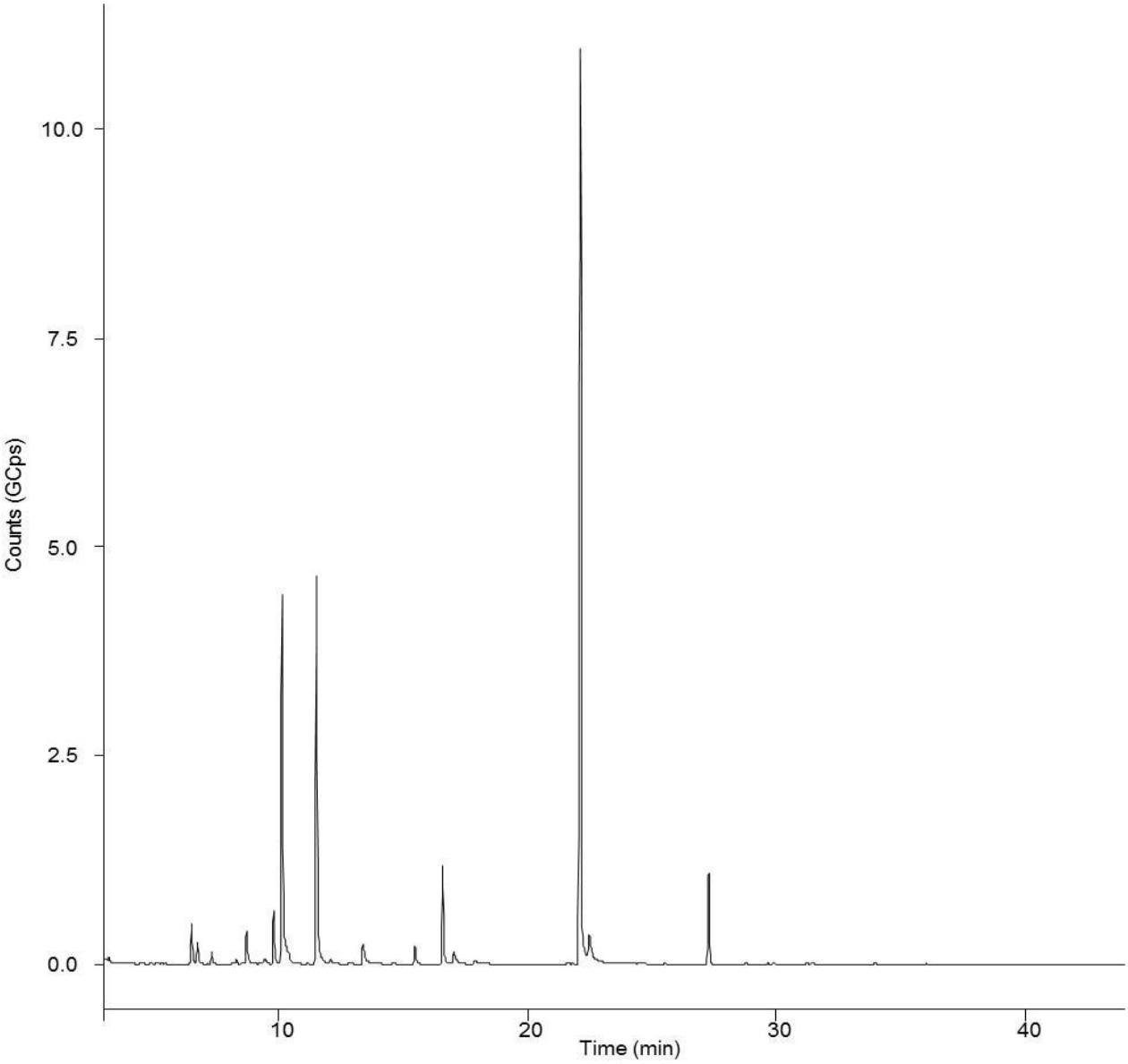


Fig. 5: Gas-chromatographic tracing of OE extracted from di lavanda mailhette

3.1.5.2 *Rosmarinus officinalis* and *Salvia officinalis*



3.1.5.3 *Thymus vulgaris ct. tujanolo L.*



3.1.6 GAS-CHROMATOGRAPHIC CHARACTERIZATION OF EOs POWDER

3.1.6.1 *Lavandino sumian* and *Origanum vulgare var. Hirtum*

RT	Peak Name	%
10,434	Eucalyptol	1,06
13,413	β-Linalool	30,40
15,495	Camphor	6,68
16,292	Lavandulol	0,52
16,632	endo-Borneol	5,66
17,008	L-Terpinen-4-ol	4,38
17,757	α -Terpineol	1,07
20,074	Linalyl acetate	38,06
21,587	Lavandulol acetate	3,85
27,306	β-Caryophyllene	2,31
28,778	β-trans-Farnesene	1,35

RT	PeakName	%
6,437	β-Thujene	1,77
6,681	α-Pinene	0,91
8,716	β-Pinene	1,33
9,389	α-Phellandrene	0,42
9,771	Terpinolene	3,93
10,103	p-Cymene	13,00
11,491	γ-Terpinene	33,68
19,328	Thymol methyl ether	0,54
19,723	Isothymol methyl ether	1,67
22,108	Thymol	42,00
27,321	β-Caryophyllene	0,76

3.1.6.2 *Lavanda vera gen, Lavanda mailhette*

RT	PeakName	%
6,761	α-Pinene	1,52
7,33	Camphene	0,58
8,31	β-Pinene	1,66
10,463	Eucalyptol	57,54
12,085	cis-Linalool oxide	0,73
12,791	trans-Linalool oxide	0,68
13,382	β-Linalool	5,47
15,221	L-Pinocarveol	0,56
15,522	Camphor	26,42
16,618	Isoborneol	0,75
17,748	Myrtenal	1,14

RT	PeakName	%
6,773	α-Pinene	5,21
7,339	Camphene	2,23
8,321	β-Pinene	2,27
10,45	Eucalyptol	27,75
12,085	cis-Linalool oxide	1,91
12,786	trans-Linalool oxide	1,67
13,411	β-Linalool	24,10
15,503	Camphor	10,24
16,643	endo-Borneol	10,17
17,74	α -Terpineol	1,62
20,047	Linalyl acetate	7,61

3.1.6.3 *Rosmarinus officinalis* and *Salvia officinalis*

RT	Peak Name	%
6,308	Tricyclene	0,11
6,644	α-Pinene	15,57
7,22	Camphene	6,80
8,233	β-Pinene	2,55
9,537	3-Carene	0,38
9,94	α-Terpinene	0,28
10,363	Eucalyptol	54,07
15,464	Camphor	2,99
16,585	Borneol	12,28
21,651	Bornyl acetate	0,38
27,303	β-Caryophyllene	4,42

RT	Peak Name	%
6,657	α-Pinene	2,41
7,226	Camphene	4,60
8,229	β-Pinene	5,09
10,359	Eucalyptol	24,28
11,86	γ-Terpinene	0,19
13,638	α-Thujone	29,51
14,187	3-Thujanone	3,88
15,453	Camphor	26,94
16,641	endo-Borneol	0,20
27,331	β-Caryophyllene	0,89
28,835	Humulene	1,41

3.1.6.4 *Thymus vulgaris ct. tujanolo L.*

RT	Peak Name	%
6,51	β-Thujene	1,49
6,756	α-Pinene	0,89
8,719	β-Pinene	1,30
9,813	α-Terpinene	2,13
10,15	p-Cymene	16,62
11,53	γ-Terpinene	14,71
13,396	β-Linalool	1,30
16,612	Borneol	3,98
17,05	L-Terpinen-4-ol	0,82
22,149	Thymol	50,20
27,296	β-Caryophyllene	3,54

3.1.7 CHARACTERIZATION OF ESSENTIAL OILS

Gas chromatography-mass spectrometry (GC-MS) technique has contributed generally to the analysis of EOs [201]. The OE samples were diluted 1: 1000in ethyl acetate and 1 μL of this dilution was injected into GC-MS Bruker Scion SQ. The samples of hydrolates were diluted in ethanol and 1 μL of this dilution was injected into GC-MS.

3.1.7.1 CHARACTERIZATION OF ADDITIONAL COMPONENTS:

3.1.7.1.1 SOLID EXTRACT OF *OPUNTIA FICUS INDICA L. MILL* (CLADODES) AND *OLEA EUROPEAE L.* (LIVES)

- Chemical composition
- Physical-Chemical Properties liquid, amber colour, intense and refreshing flavour, citrus smell
- Microbiological parameters

Analysis	Result	Unit Measure	LQ	Method
Total Plate Count	< LQ	UFC/g	10	ISO 4833 -1, Inclusion.
Yeast and Molds	< LQ	UFC/g	10	ISO 21527 -1-2, Surface.
Total Coliforms	< LQ	UFC/g	10	ISO 4832, Inclusion.
Bacillus Cereus	----	UFC/g	10	ISO 7932: 2004, Surface.
Enterobacteriaceae	Absent 1g	UFC/g		Harmonized USP/EP, Enrichment - Inclusion.
Escherichia Coli	Absent 1g	UFC/g		Harmonized USP/EP, Enrichment - Inclusion.
P. Aeruginosa	Absent 1g	UFC/g		Harmonized USP/EP, Enrichment – Surface.
Staphylococcus Aureus	Absent 1g	UFC/g		Harmonized USP/EP, Enrichment – Surface.
Salmonella spp.	Absent 25g	UFC/25g		ISO 6579, Enrichment – Surface.
Candida A.	Absent 1g	UFC/g		Harmonized USP/EP, Enrichment – Surface.
Clostridia spp.	----	UFC/g		EP, Enrichment – Surface.

< LO: Below the Limit of Quantification. The measurement uncertainty figure is not synonymous with some form of positivity but only with the performance of the method.
LQ: Limit of Quantification: = 10, is the lowest concentration of the parameter sought in the sample that can be detected with acceptable precision (repeatability) and accuracy under well specified conditions.

- Toxicological evaluation of every single component used for the realization of the experimental

product in order to ensure that they were not associated with any adverse events or contraindications in the studies already present in the literature.

Therefore, the purpose of the present research was to create a product suitable for human and marketable administration, and useful in the field of cardiovascular risk prevention in subjects with PD.

After the first selection described above, we moved on the choice and formulation of the experimental product by evaluating a series of factors:

• In-company reproducibility at low costs

• Ease of administration to the subject

• Risks and benefits for public health

The suitability, reliability and applicability of the methods reported in the literature have been evaluated. Furthermore, it was considered useful to study the correlation between the chemical structure of molecules contained in EOs with their antiseptic, antibacterial, antiviral and anti-infective properties. The analysis of the analysed plant extracts led to the identification of a mixture of EOs to be administered (*Rosmarinus officinalis, Lavandula x intermedia, Thymus capitatus*) by irrigation/rinsing and by nebulizing spray.

Following the studies carried out during the previous two months it was possible to carry out a correct research and confirmation of the possible application for human use of EOs of *Rosmarinus officinalis, Lavandula x intermedia* and *T. capitatus* in combination with *Musosave*.

3.1.8 FORMULATION OF THE EXPERIMENTAL PRODUCT

The constant sensation of itching and burning of the gums that warns those affected by PD can be partly alleviated with the use of a disinfectant and antiseptic action mouthwash with each tooth wash, and for this reason we have imagined a product without alcohol, slightly frothy and with a delicate taste, perfect even for those who have a particularly delicate mouth, with a formulation that does not alter the perception of the flavours of food and drink. Although the causes of PD are multiple, a daily and correct cleaning of the oral cavity is essential to avoid excessive formation of tartar for this is thought of a double formulation, which accompanied the use of mouth wash, or exploit the ability to use also a nebulizer spray able to effectively counteract the formation of bacterial plaque, restore the balance of the oral environment, reduce the perception and discomfort of gingival swelling, also favouring the maintenance of the local microcirculation.

3.1.9 PRODUCTION OF THE PRODUCT AND PLACEBO: MOUTHWASH AND NEBULIZER SPRAY, TIMES AND METHODS OF ADMINISTRATION MOUTHWASH/IRRIGATION SOLUTIONS

(**ATTACHMENT 3-4**)

3.1.9.1 MOUTHWASH/IRRIGATION SOLUTIONS

Both product and placebo consist of rinsing about 20 seconds with the mouthwash. The subject should make sure that the mouthwash reaches both the front and the back of the teeth and the gums all under the tongue and on the palate. The subjects also were advised not to rinse at the end of treatment. The operation should be repeated every morning and evening before going to bed for a period of 6 months.

3.1.9.2 NEBULIZER SPRAY

A single dose was sprayed directly into the oral cavity after rinsing it with warm water, 3 times a day. It was recommended to use it after eating a meal or a snack or consuming a drink like tea or coffee.

3.2 EXPERIENCE IN ITALY:

3.2.1 In the following period I improved my Spanish and English language skills, as well as statistics following courses provided by the University of Palermo. The certificates obtained are attached (**Attachment 5-6-7**).

3.2.2. At the same time, the preparation of the study protocol was carried out in order to be submitted to the ethics committee of Granada, and to get the approval at the beginning of the second year when we should start with the collection of saliva samples, sampling plot champions and a crevicular fluid from subjects with PD, including an assessment of metabolic variables of interest, as described in details in the study protocol. The research protocol of the clinical study designed and submitted to the ethics committee of Granada is attached **(Attachment 8)**. Contracting order of experimental products and placebo. Labeling. Inventory and shipping at the University of Granada.

3.3 EXPERIENCE IN SPAIN: CLINICAL STUDY FACILITY AND PATIENT RECRUITMENT

During the 12 months at the University of Granada, Spain, we followed all procedures required for the realization of the clinical trial entitled: "**EFFECT OF ESSENTIAL OILS AS ADJUTANTS ON**

THE TREATMENT OF SUBJECTS WITH PERIODONTITIS: ASSESSMENT OF METABOLIC VARIABLES AS EFFECTMODIFIERS."

3.3.1 CERTIFICATE ARRIVAL

As soon as I made the transfer to Spain I went to the offices to undertake a research work at the Department of Stomatology, School of dentistry, University of Granada, Spain as well as to certified my arrival (**Attachment 9**).

3.3.2 PROBLEM SOLVING

Immediately I took care of the management of problems concerning the logistics of the experimental product, logistics management of the material necessary for laboratory analyses as well as organization and planning of the trial in Spain.

In addition, I contacted the laboratory that deals with the production of the experimental product to provide me with the certification for human use. They certified that RINSE A, RINSE B, NEBULIZER C and NEBULIZER D were authorized for clinical study for use by individuals. These essays are produced and packaged in the laboratories of EGERIA PHARM S.R.L. (Via Carcara, 36 Aci Catena (CT) Italy), a company authorized to produce cosmetic products for human use according to EC No 1223/2009 (**Attachment 10**).

3.4 APPROVAL FROM THE RESEARCH ETHICS COMMITTEE

The study was designed in order to comply with the standards of the last revision of the Helsinki declaration. Approval from the Research Ethics Committee in Human Studies of the University of Granada, Spain, was obtained prior to the beginning of the study (**Attachment 11**).

The clinical trial was also registered in clinicaltrials.gov (NCT04692246). Written informed consent has been obtained from all participants in the study. All obtained data were collected as anonymous and the study was designed following the CONSORT guidelines for reporting clinical trials [202].

3.5 SPANISH LANGUAGE COURSE

During my stay in Spain, I also improved my knowledge of Spanish language attending the Spanish course from June 10thto July 5thprovided by the Escuela Delengua, Granada, Spain. The certificate obtained is attached (**Attachment 12**).

3.6 OBJECTIVES

This study aimed to test the effect as an adjutant to therapy of a nutraceutical composed of several plant extracts in patients with PD and different levels of risk for the MetS. Specifically:

- The response of periodontal clinical variables to non-surgical periodontal treatment in subjects treated with the extract compared to controls;
- The effect on local inflammatory markers in subjects treated with the extract compared to controls;
- The modifier effect of the metabolic syndrome-related variables in the treatment outcomes of the subjects treated with the extract compared to controls.

3.7 STUDY DESIGN

It was a randomized, controlled, double-blinded clinical trial. Subjects with diagnosis of PD according to the joint the European Federation of Periodontology (EFP)/ American Association of Periodontology (AAP) 2018 criteria for the case definition of PD.

- **Inclusion criteria**

Subjects who attended the Department of Stomatology at the University of Granada, School of Dentistry (Granada, Spain) gave written, signed consent to participate in the trial.

- **Exclusion criteria**

Subjects under age of 18 years, as well as those who received periodontal treatment in the last year, anti-microbial therapy in the previous 3 months were excluded from the study. Also, multiple pregnancy and the presence of neoplastic or severe infectious diseases were main exclusion criteria.

3.8 PROJECT MANAGEMENT: PRE-SCREENING E SCREENING

The Faculty of Dentistry of the University of Granada is a teaching, healthcare and research institution. In the Faculty Clinics, clinical practices are developed in order to achieve the objectives defined in the Odontology study plan. The treatments were carried out by students studying the Degree in Dentistry under the direction and supervision of the teachers assigned to each group of clinical practices. For our dental clinic visits, the explorers calibrated with Prof. Francisco Mesa were:

Antonio Magán-Fernández,

Cristina Benavides Reyes,

Maria Torremocha Lopez.

Periodontal examination was performed by a calibrated researcher using PCPUNC15 periodontal probe (Hu-Friedy, Chicago, IL, USA) and dental exploration mirror (**Fig.10**).

Fig.10 PCP-UNC 15 and dental exploration mirror

As a university centre, clinical activities were only carried out during university teaching schedule in collaboration with undergraduate s. For this reason, we used the electronic patient admission database of the centre to screen for potential candidates, based on the inclusion and exclusion criteria that we had established in the study protocol. Subsequently, they were contacted and scheduled visits to start clinical study. We performed a periodontal clinical visit to confirm the eligible patients (screening of subjects with/without PD), illustrated the clinical study and the informed consent were signed by eligible subjects. At screening, subject received an information sheet of the clinical trial and telephone numbers of trial site staff.

3.8.1 SUBJECT RELATED INFORMATION/ASSESSMENT

- **The following sociodemographic variables were collected:**

- Date of birth
- Sex
- Telephone numbers
- Smoking status
- Alcohol consumption

3.8.2 GROUP ALLOCATION OF THE PARTICIPANTS

The researchers performed the clinical examinations in a blinded manner for the group allocation of the participants, since the random allocation and prescription were performed by a different researcher.

- **Test group:** Subjects that received the application of the extract in form of irrigation solutions in the periodontal pockets during regular periodontal treatment. They would continue at home during

the follow-up period, taking the EOs as a rinse twice per day, and as a spray when regular toothbrush could not be performed.

- **Control group**: Subjects that followed the same protocol, but using regular irrigation and a placebo spray at home.

3.8.3 CONCOMITANT ILLNESS AND MEDICAL HISTORY

Information about any illness that was present at the start of the trial and medical event that the subject has experienced in the past were collected and only relevant medical history as judged by investigator was reported. The information included diagnosis, date of inset and date of resolution or continuation, as applicable.

3.8.4 CONCOMITANT MEDICATION

Any medication which was taken during the trial. The information collected for each concomitant includes generic name, indication with start date and total daily dose.

3.8.5 FASTING VISIT

The subjects attended site visits in a fasting state. Fasting was defined as having consumed only water within the last 6 hours prior to the visit.

3.8.6 METABOLIC SYNDROME-RELATED VARIABLES

The following variables were collected during the visits at the cite:

- **Body weight:** measured in kg without shoes and only wearing light clothing.
- **Body height:** measured without shoes in cm.
- **Body mass index:** calculated using the equation: BMI body weight (Kg)/ (height (m) x height(m)).
- **Waist circumference:** was defined as the minimal abdominal circumference located midway between the lower rib margin and iliac crest and has been measured using a non-stretchable measuring tape.
- **Systolic and diastolic blood pressure:** in a sitting position after the subject has been resting for at least 5 minutes. The same equipment, Sphygmomanometer Minimus® III, was been used throughout the trial (**Fig. 11**).
- **Pulse:** pulse (beats per minute) was recorded at site visits after resting for 5minutes in a sitting position.

- **Fasting blood glucose:** The investigator provided with a blood glucose meter use test strip calibrated to plasma values. All measurements performed with capillary blood were automatically calibrated to plasma equivalent glucose values which was been on the display. For this process we used safety lancets because it is important for this process to be safe and easy. Securlancets®unik are sterile single-use safety lancets used to perform on finger pricks in order to collect micro- samples of capillary blood. Study products was been provided previously by the company A. Menarini Diagnostics s.r.l. (**Fig. 12**).

3.8.7 BIOCHEMICAL VARIABLES

Blood samples have been obtained at the beginning of the study for the assessment of:

- Lipid profile
- Panel of inflammatory cytokines.

All samples were centrifuged, within 30 minutes of collection, with Orthodiagnostic system petalfuge III centrifuge unit (**Fig. 13**) for 5 min, and aliquots were made of both serum and plasma and immediately frozen and stored at -80 C. The analyses were planned to be performed at the end of the trial.

Fig. 11 Sphygmomanometer Minimus® III

Fig.12 Glucometer GlucoMen Lx3 and Securlancets unik

Fig.13 Orthodiagnostic system petalfuge III centrifuge unit

3.8.8 PERIODONTAL EXAMINATION

After signing the informed consent, we have agreed on two appointments: one for the extraction of fasting blood sample and collection of clinical and anthropometric parameters and one for the periodontal examination. We explored 62 subjects and randomized to each group, of which 30 on test group and 32 on control group, accordingly to similar studies on the topic available in the literature. All subjects have received standard periodontal treatment with an ultrasonic new generation device (AIRFLOW® Prophylaxis Master, EMS Dental, Nyon, Switzerland) (**Fig.14**). Six surfaces in each implant have been measured: mesiovestibular, vestibular, distovestibular, distolingual, lingual, mesiolingual. All clinical data have been recorded in a periodontal chart. The following periodontal variables were registered: pocket probing depth, clinical attachment loss, gingival recession, gingival bleeding and oral hygiene (**Fig.15**). Pocket probing depth, gingival recession and clinical attachment loss were measured in millimetres. Bleeding on probing (BoP) was registered according to the Ainamo and Bay Index [203]. The presence of plaque was determined by the binary visual index of Tonetti [204]. The number of present teeth was also recorded. The severity of periodontitis was evaluated using a modification of the Periodontal Inflammatory Severity Index (PISIM) [51]. The PISIM score is the sum of the product of the number of sites and the PPD at each site divided by the number of remaining teeth (PISIM=$\sum$ (di ni)/t), where "i" is the site, "d" is the PPD of the site in mm, "n" is the absolute frequency of the sites, and "t" is the number of remaining teeth. Periodontitis extension was measured using the Arbes index, considering the percentage of sites with CAL $\geq$ 3 mm [205]. Samples of gingival crevicular fluid were obtained at the beginning of the study and we collected these by using tips of absorbent paper (perio-paper), that were later diluted in phosphate buffered saline (PBS), after they spun for 10 seconds and centrifuged at 2000 rpm for 15 seconds with VELP Scientifica Infrared Vortex Mixer (**Fig.16**).

Fig.14 AIRFLOW® Prophylaxis Master, EMS Dental, Nyon, Switzerland

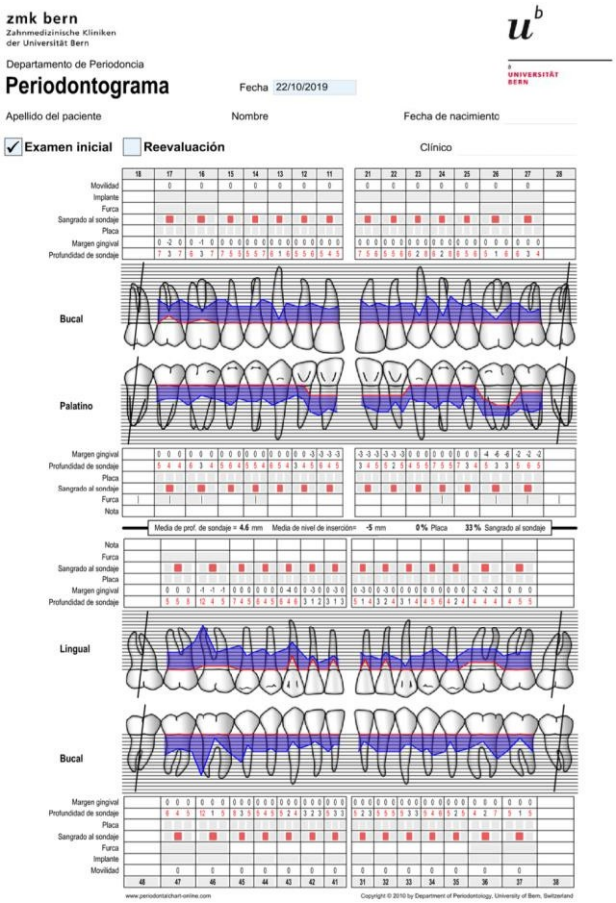


Fig.15 On line Periodontal Chart: Copiright © 2010 by Department of Periodontology, University of Bern, Switzerland

Fig.16 VELP Scientifica Infrared Vortex Mixer

3.8.9 DISPENSING VISIT

After a subject obtained identification code and allocation of group of trial products, we dispensed 6 mouthwash and 3 nebulizer sprays to each subject, while the second delivery was made during the 3-month visit. The investigator instructed the subject to the use of trial product: **mouthwash/Irrigation solutions or nebulizer spray** in a way as it has been described in detail above (section 3.1.9).

3.9 REPORT OF FOLLOW UP OF THE CLINICAL STUDY

A total of 62 subjects (30 on test group and 32 on control group) were included in the present study. All subjects have received standard periodontal treatment with an ultrasonic new generation debridement device. Collection and processing of blood sampling, periodontal examination and medical examination, collection of gingival crevicular fluid, delivery of the experimental product (group A and group B with random assignment). Clinical data were gathered at baseline and at 3 of follow-up. Moreover, they were contacted monthly to improve treatment adherence and to ensure that there were not any changes in concomitant therapy. Yet, all patients underwent a periodontal examination after 3 months in order to verify the adherence to treatment. At the same time, trial product was dispensed for the next 3 months. After that, due to emerging coronavirus pandemic spreading, they were only contacted by phone to reassure them of our constant support and ensure compliance as well as to feel free to contact us for any needs. Every effort was made to follow the study protocol. We were closely monitoring the situation and were continuing to align our operation with guidance from the WHO as well as the national and local government. None of the subjects experienced intolerable adverse effects. Four subjects requested to withdraw from the trial for personal reasons. We recorded a total of 5 dropout: 2 patients due to pregnancy, 2 for being non-compliant and 1 patient for work reasons since he/she had to leave Spain. Five subjects developed mild, transient dental sensitivity, but were able to continue to use the treatment throughout the study. Furthermore, on February 21st, 2020, in relation to the PEC (prot. MIUR 1647 of 05/02/2020), with which the further extension of the research period to be carried out at the foreign office was requested, for my research project scholarship DOT1320519-2, considering the scientific reasons provided and the opinion expressed by the coordinator, and the further extension of the research period has been authorized (from 12 to 18 months) until on 23 October 2020. (**Attachment 1-2**).

As suggested by my coordinator, I had enrolled at the course "*Curso de bioinformática de muestrasbiológicas: bioestadística univariable y multivariable aplicada a resultados con muestras de secuenciaciónmasiva*" (**Fig. 17**), but unfortunately due to the COVID-19 emergency it has been postponed to a later date still to be confirmed. However, on 20thNovember 2019 I attended the training

Course on "*Funcionamiento del microscopio Eclipse 100D y software de analisis de imagen NIS - BR de NIKON*" (**Fig. 18**).

Fig. 17 Course on bioinformatics of biological samples: univariate and multivariable biostatistics applied to results with massive sequencing samples

Fig. 18 Course on "Funcionamiento del microscopio Eclipse 100D y software de analisis de imagen NIS - BR de NIKON".

During the last year of the project (2020), the University of Palermo, Italy (as a leading institution) and the University of Granada, Spain (as a secondary institution) agreed, in accordance with the applicable laws, rules and regulations in force in each respective Countries and Institutions, to jointly organize an international doctoral thesis in cotutelle for the benefit of my research doctoral thesis entitled: “Effect of essential oils as adjutants on the treatment of subjects with PD: assessment of metabolic variables as effect modifiers” (**Attachment 13**).

Moreover, during this year also due to lock down of Coronavirus, I was working on the preparation the following scientific papers and activities:

- Publication of the review article entitled: “Effects of Aging and Diet on Cardioprotection and Cardiometabolic Risk Markers”; DOI: 10.2174/1381612825666191105111232
- Publication of the original article entitled: “Altilix (R) Supplement Containing Chlorogenic Acid and Luteolin Improved Hepatic and Cardiometabolic Parameters in Subjects with Metabolic Syndrome: A 6 Month Randomized, Double-Blind, Placebo-Controlled tudy”; DOI: 10.33 0/ U11112580
- Publication of an editorial comments: The role of periodontal microorganisms in the pathogenesis of myocardial infarction. From PCR techniques to Microbiome Sequencing; DOI: 10.1016/j.tcm.2020.02.006
- I acted as a Reviewer of a review for The Journal of Obstetrics and Gynaecology Research.
- Research and planning work for the drafting of a review relating to my research topic.

3.10 EXPERIENCE IN ITALY: ANALYSIS OF RESULTS

Unfortunately, due to coronavirus pandemic, performing usual trial-related activities are forbidden by the authorities, we were not able to perform the visit at the end of follow-up period (after 6 months). Consequently, we could not collect the MetS-related variables such as body weight, body mass index, waist circumference, blood pressure, but also samples of saliva, subgingival plaque and gingival crevicular fluid, as well as a panel of inflammatory cytokines and biochemical analyses (fasting blood glucose, lipid profile, hepatic enzymes AST, ALT, GGT). Therefore, I had returned to Italy (**Attachment 14)**. Every effort was been made to follow the study protocol. We were closely monitoring the situation and continued our operation in accordance with the guidance from the World Health Organization and the national and regional government policies. During this period, I attended at the course of Bioinformatics at University of Palermo (**Attachment 15)**, improving my English language with a private course, participating to webinars of University of Palermo.

3.10.1 DATA MANAGEMENT AND STATISTICAL ANALYSIS

A database in Excel format was prepared including all recorded variables. The data was analysed by a single researcher. The statistical software SPSS v.21.0 (IBM Inc., Chicago, IL, USA) was used, performing descriptive statistics (means, standard deviations and percentages) and analytical procedures (95% confidence intervals, Mann- Whitney test, Chi-square, Student's t-test for paired samples, and univariate and multivariate regression analysis). A 5% significance level was considered for all tests.

3.10.2 IMPACT OF THE PANDEMIC ON THE THESIS PROJECT

As it is known the current COVID-19 pandemic has affected activities related to clinical research in most parts of the world and its sudden onset and wide-spread impact has been documented [206].
Regarding the present study, when the pandemic broke out, the study protocol was at 5 months of follow-up. Thus, at that moment, the follow-up visit at 3 months have been completed for all participants and in the following months we should do activities related to the final visit after 6 months of the treatment. Especially during the first months of the pandemic one enormous effort has been made in order to keep the contact with the enrolled patients and make them to feel safe and secure. Likely, they have been covered with the trial product and continued to receive the treatment as at the previous visit, performed at the site, they were provided with a medication for a longer period of time. However, the following in-person scheduled study visits at the end of follow-up period could not be performed due to quarantines that resulted in study participants' inaccessibility (as well as trial personnel) to the site (clinics were closed). In order to continue giving support and care to the patients, call visits were made every month. Of course, in a dealing with the reality of COVID-19, the most important thing was to ensure the safety of patients as well as clinic staff and researchers. Consequently, such situation influenced research activities. As subjects could not come in to a clinic, the final both blood and dental sampling could not be made. Thus, we were experiencing a delay in timelines and operational gaps that later resulted in a complete halt of operations in lieu of this pandemic. On the other hand, such operational gaps negatively impacted the trial program and data integrity, affecting also clinical research outcomes. The difference between the objectives set at the beginning of the project and those actually achieved are the following: preliminary data has been obtained after 3 months, the final study visit after 6 months could not be completed including the measurement of clinical and biochemical variables. However, it should be highlighted that we have some encouraging preliminary results, and we hope that in the future someone else will be able to

carry out the whole project in full. It is important to mention that the project was not closed, and we are expecting that it can be continued in the following months/years, once pre-pandemic conditions are restored. In addition, our findings together with the data available in the literature may indicate further directions in *in vivo* and clinical studies in order to select efficacious formulations of nutraceutical and to support their application in dentistry clinical practice and CVD prevention.

4. RESULTS

Baseline measurements of demographic variables and risk factors in both groups are presented in **Table 1**.

Table 1. Demographic variables and risk factors.

Variable	Placebo (n=24)	Test (n=26)	p-value
Sex, n (%)			0.913[b]
Male	8 (33.3)	8 (30.8)	
Female	16 (66.7)	18 (69.2)	
Age (yrs.), range[a]	28-66	25-72	
Age (yrs.), mean±sd[a]	48±11	50±12	0.509[c]
Tobacco, n (%)			0.257[d]
No	11 (45.8)	14 (56.0)	
Ex-smoker	1 (4.2)	4 (16.0)	
Smoker	12 (50.0)	7 (28.0)	
No answer	-	1	
BMI, mean±sd	27.7±5.2	28.3±6.1	0.700[c]
Glucose, mean±sd	110±36	110±13	0.952[c]
Diabetes, n (%)			1[e]
No	21 (91.3)	21 (91.3)	
Yes	2 (8.7)	2 (8.7)	
No answer	1	3	
Hypertension, n (%)			0.721[b]
No	17 (73.9)	19 (82.6)	
Yes	6 (26.1)	4 (17.4)	
No answer	1	3	
Dislipemia, n (%)			1[e]
No	22 (95.7)	22 (95.7)	
Yes	1 (4.3)	1 (4.3)	
No answer	1	3	

a: 1 missing.
b: Chi cuadrado con corrección de Yates
c: T-student.
d: Test de Mann-Whitney
e: Test exacto de Fisher bilateral

The baseline characteristics of the subjects were observed (sex, age, body mass index, glycemia, tobacco and alcohol consumption, diabetes, hypertension, and dyslipidaemia) and no differences were found between groups.

Periodontal clinical variables are presented in **Table 2 and Table 3**.

Table 2. Periodontal Variables

Baseline	Placebo (n=24)	Test (n=26)	p-value[a]
Pockets of 4 mm	23.4±11.4	20.3±14.2	0.207
Pockets of 5 mm	12.1±12.8	5.6±6.8	**0.007**
Pockets of 6 mm	4.6±5.7	1.5±3.4	**0.020**
Pockets of 7 mm	1.6±2.7	0.5±1.4	0.069
Pockets of 8 mm	0.3±0.7	0.1±0.3	0.175
Pockets of 9 mm	0.1±0.3	0.3±1.0	0.681
Pockets of 10 mm	0	0	-
Pockets of 11 mm	0	0	-
Pockets of 12 mm	0.1±0.4	0.0±0.0	0.298
Nº Teeth	22.6±6.2	22.8±5.5	0.852
Nº Pockets	42.1±24.5	28.4±22.0	**0.006**
PISIM index	9.3±5.8	5.7±4.2	**0.018**
Sites with CAL > 3 mm	49.5±25.7	37.2±24.6	**0.019**
ARBES index	40.2±22.2	29.6±21.1	0.052
Bleeding on probing	67.1±23.8	59.4±26.6	0.472
3 months			
Pockets of 4 mm	11.7±12.6	6.1±5.9	0.114
Pockets of 5 mm	5.2±8.8	1.6±2.0	0.223
Pockets of 6 mm	2.1±4.9	0.3±1.2	**0.014**
Pockets of 7 mm	0.5±1.6	0.0±0.0	0.066
Pockets of 8 mm	0.04±0.2	0.0±0.0	0.298
Pockets of 9 mm	0.1±0.4	0.0±0.0	0.298
Pockets of 10 mm	0	0	-
Pockets of 11 mm	0.00±0.0	0.04±0.2	0.337
Pockets of 12 mm	0	0	-
Nº Teeth	22.5±6.2	22.6±5.7	0.867
Nº Pockets	19.9±25.4	8.0±7.9	0.073
PISIM index	4.2±5.1	1.7±1.7	0.050
Sites with CAL > 3 mm	29.3±29.6	19.8±22.3	0.130
ARBES index	24.3±22.4	18.2±25.2	0.125
Bleeding on probing	33.3±26.2	27.7±22.7	0.472

a: Mann-Whitney's U Test

Table 3. Relative change in periodontal variables during the study

Difference % Pre-Post	Placebo (n=24)	Test (n=26)	p-value[a]	Difference % Placebo-Test[b]	
	mean±sd	mean±sd		mean±SE	95% CI
Nº Pockets	-64±27	-71±24	0.387	10.9±12.1	-12.7 a 34.6
PISIM Index	-65±27	-72±24	0.466	10.8±11.9	-12.5 a 34.0
ARBES Index	-50±28	-51±42	0.560	2.0±20.2	-37.6 a 41.6
Bleeding on Probing	-34±33	-32±28	0.690	-5.9±24.7	-54.2 a 42.5

a: Mann-Whitney's U Test
b Pre-post test According to Clark et al. 1985 [207]

Regarding periodontal variables assessed in the present study the statistically significant differences between 2 groups were found in the following parameters: BP5T0, BP6T0, NBOLT0, BOLTOTT0, PISIMT0, SPIM3MT0 and BP6T1.

Cardiometabolic variables related to the metabolic syndrome are presented in **Table 4**.

Table 4. Within-group analysis of cardiometabolic variables after periodontal treatment in each study group (n=50)

	Test group (n=26)			**Placebo group (n=24)**		
	Baseline	**3 months**	**p-value***	**Baseline**	**3 months**	**p-value***
Weight	79.5 ± 19.6	78.9 ± 19.1	0.250	78.6 ± 19.1	79.6 ± 19.0	0.364
BMI	28.3 ± 6.1	28.0 ± 5.9	0.246	27.7 ± 5.2	28.0 ± 4.9	0.299
Waist circumference	99.1 ± 15.7	96.3 ± 16.2	0.336	96.4 ± 12.8	97.3 ± 12.1	0.635
Systolic BP	113.3 ± 17.9	124.8 ± 19.8	0.117	116.7 ± 16.5	120.3 ± 9.9	0.755
Diastolic BP	72.1 ± 11.3	76.7 ± 6.7	0.086	75.0 ± 10.6	73.9 ± 11.9	0.296
Pulse	69.7 ± 10.9	72.6 ± 9.5	0.395	72.6 ± 7.6	73.4 ± 12.1	0.784
Glycemia	109.9 ± 13.3	98.2 ± 12.7	**<0.001**	110.3 ± 36.1	106.7 ± 31.6	**0.029**

All values expressed in mean ± standard deviation. *Student's T-test for paired samples.

There were no statistically significant differences in assessed cardiometabolic variables between 2 groups, except glycemia level that decreased significantly in both groups. In the test group a slight increase in systolic, diastolic blood pressure and pulse were observed, as well as a slight decrease in body weight, body mass index and waist circumference.

In the control group there is an increase in body weight, body mass index, systolic blood pressure, pulse and waist circumference, while diastolic blood pressure decreased.

5. DISCUSSION

Preliminary data of the present study indicates that several periodontal clinical variables improved significantly in the test group compared to the control group after 3 months of trial period. Improvement in some cardiovascular variables have been also observed, although there was not any statistically significant difference between 2 groups, except fasting glycemia that improved in both groups. Our results may suggest, in line with findings already present in the literature, the use of nutraceutical as a prevention or adjunct to conventional periodontal treatment. It is known that chlorhexidine, one of the most effective drugs used for periodontal treatment, has side effects, including toxicity, especially when used for prolonged periods, so the addition of nutraceutical could be an effective alternative option characterized by the absence of such side effects, containing herbal agents which have antimicrobial and anti-inflammatory properties and may further improve patient compliance too [208, 209].

Regarding the efficacy of the nutraceuticals, numerous preclinical studies have demonstrated the antimicrobial, antioxidant, anti-inflammatory as well as anticancer activities *in vitro* and *in vivo*. However, the human studies are still scarce and limited data does not allow nutraceuticals' use in daily dentistry clinical practice. A very recent systematic review indicates nutritional intervention as beneficial in PD treatment, highlighting that the evidence in general, is inconsistent and imprecise [210]. Three clinical studies using nutritional supplements reported improvements in at least one clinical parameter of PD: reduction of probing depths, attachment gain, reduced bleeding on probing after only 2 or 6 months, respectively [211-213]. We should highlight that our patients kept their dietary habits unchanged trough the trial and that they used spray or mouthwash on daily basis, so the beneficial effect in the present study were seen after only 2 months of using of nutritional supplements, as previously documented in the literature.

It is widely known that EOs are recognized for their antimicrobial, antiviral and antifungal activity, but also recent studies have demonstrated potent anti-oxidant, anti-inflammatory, antidiabetic properties as well as cancer suppressor activity [214]. Thus, the potential of EOs as effective and safe phytotherapeutic agents should not be underestimated, although their efficacy in oral health is well documented [215]. The antibacterial activity of EOs as well as their isolated constituents and their potential applicability in novel dental formulations have been summarized in a systematic review [209], emphasizing the need for further non-clinical and clinical studies, as the evidence on the anti-caries potential of EOs is mainly based on *in vitro* studies, while clinical trials are limited. Yet, the available data from the literature supports the use of *Rosmarinus officinalis*, *Lavandula x intermedia*, and *Thymus capitatus*, the main components of the extract used in the present study, in the treatment

of acute inflammatory conditions. As mentioned above, the measurement of inflammatory markers was planned, but unluckily not realized due to the pandemic restrictions. The mechanisms which could explain anti-inflammatory effect have been described in detail in the introduction section. Immunomodulatory and anti-inflammatory properties of compounds found in the lavender EOs have been investigated [216]. Similarly, plant species *Rosmarinus officinalis L.* are also known by its anti-inflammatory activity that are attributed to its EOs (frequently reported molecules are 1,8-cineole, α-pinene, and camphor) and are explained through inhibition of NF-κB transcription and suppression of arachidonic acid cascade [217]. Yet, this plant exerts antioxidant activity preventing injury caused by the ROS of inflammation, it also has smooth muscle relaxant activity and low toxicity, supporting its ethnopharmacological uses in inflammatory-related diseases, and potential future applications. It has been supposed that the high doses of EOs are needed in order to exert pharmacological effects, but recent studies indicate that this matter can be avoided using the oil formulated as nano-emulsions to improve its bioavailability [217]; thus, more investigations are needed in the future to confirm this finding. Interestingly, in addition to antioxidant, anti-inflammatory, antimicrobial, spasmolytic, antinociceptive, antitumor activity, some EOs, such as those from *Thymus vulgaris L.* may enhances cognitive function as shown in animal models [218]. All these beneficial actions increase an interest in future investigation of these plants, including the field of PD. Recent research suggests that oxygenated terpenoids found in the EOs diffuse within the bacterial cell membrane, irreversibly damaging it, and cause cell death. In this regard, recently Anusha D. *et al*. assessed the efficacy of mouthwash containing EOs and curcumin (MEC) as an adjunct to nonsurgical periodontal therapy (scaling and root planning, SRP) on the disease activity of rheumatoid arthritis (RA) among RA subjects with chronic periodontitis (CP). Also, they investigated epigenetic modifications including chemical alterations of DNA and associated proteins influencing the remodelling of the chromatin and gene malfunctions which may be related to both PD and RA. The results revealed that MEC as an adjunct to SRP, as an effective approach in reducing the disease activity of both RA and CP, thereby warranting its use [219]. Finally, the utilization of such agents may reduce the intake of the drugs and consequently minimize the risk of a possible appearance of drug resistance as well as other risk factors.

Regarding the baseline values of the periodontal clinical variables, it has been observed in both groups that periodontal health improved through the follow up period. Such changes in both groups might be due to the fact that the subjects have been closely monitored with a greater knowledge about the disease, but also a greater motivation towards oral hygiene. This is related to the reduction in the number of periodontal pockets, PISIM index, ARBES index (The mean ± S.D. of percentage of sites with a loss of attachment ≥3 mm) and bleeding on probing. Clearly, it is necessary to carry out disease

maintenance and support therapy in order to maintain these values. However, several PD variables improved significantly in test group compared to the control group. Carvacrol has been described to contribute significantly to the antimicrobial activity [220], but also, we cannot exclude the possibility that other compounds may also have antimicrobial effects. For instance, carvacrol in combination with 4 EOs: eugenol, thymol, p-cymene and γ-terpinene synergistically increased the antibiotic effect of tetracycline against oral strains and references strains, having an effect on biofilm inhibition on both polystyrene and tooth surface [221]. Other similar combinations of EOs (especially menthol, thymol and carvacrol) have been suggested to be helpful in the field of dentistry including production of mouthwashes, toothpastes and other products for oral hygiene [222], that further support our findings. Over years, the fundamental treatment of PD has been the removal of plaque/biofilm and calculus through SRP. However, it has been noted that some patients might not respond to such mechanical debridement alone as this treatment does not target specific periodontopathogens, but aims to remove the whole dental plaque. Certain periodontopathogens such as *P. gingivalis* may infect the gingival tissues and are thus spared from such mechanical treatment providing the source for recolonization of periodontal pockets [223]. Consequently, chemical plaque control is suggested as an adjuvant treatment for such periodontopathogens, that further support use of nutraceuticals and our findings. In addition, it has been shown that the use of aloe vera and tea tree oil mouthwashes can decrease plaque, gingivitis and *S. mutans* in the oral cavity in children and such activity was even comparable to that seen after chlorhexidine treatment [208], as also seen with the mixture of EOs (clove, bergamote , and orange or binary - clove, bergamote), and such an inhibitory effect was maximized when they were used with chlorhexidine [224], that previously has been shown *in vitro* [225]. Based on a promising antimicrobial effects, it has been suggested that different EOs formulations may be used as toothbrush sanitizers to help prevent the establishment of bacterial biofilm [226], and may substitute the standard mouth rinses, without any side effects on cell viability [227]. The decrease of the virulence of *C. albicans* were documented after use of the EOs of *Origanum vulgare* [228, 229], present also in our mixture. Similarly, the antibacterial and antioxidant capacity of the EO of *T. capitatus* (also present in our plant mixture) was demonstrated against several cariogenic bacteria, suggesting it to be used as a mouthwash [230]. Recent findings indicate that flavonoids are highly promising for both prevention and reduction of PD, and they can be delivered easily to subjects via mouthwash, toothpaste, and food products. These nutraceutical compounds may also help to alleviate the symptoms and even the progression of PD [231]. Precisely, it has been shown that flavonoids affect a diverse array of periodontally cells, including epithelial gingival fibroblasts, PDLCs, and osteoclasts. They also may be useful in dealing with bacterial infection and plaque formation, helpful in postoperative healing of dental sockets and other traumatized tissues, as

well as to contribute to the preservation and regeneration of damaged periodontal tissues [231]. Such products may also prevent and/or slow the development and progression of periodontal disease.

A clinical study using freshly-squeezed pomegranate juice as a mouth rinse in subjects without periodontal disease, where significant reductions in the colony-forming units (CFUs) of *Lactobacillus* and *Streptococcus species* were demonstrated [232]. It is known that pomegranates are rich in polyphenols, tannins, anthocyanins, and ellagic acid, which all may contribute to the antimicrobial properties of this mouth rinse. In another clinical study where patients with CP were included, the use of a gel containing 1% curcumin, a bioactive substance found in turmeric, resulted in significant bactericidal effects on *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *Capnocytophaga* [233]. Also, turmeric's curcumin convincingly showed both RA-related anti-bacterial activity and immunosuppressant/regulatory action [234] and have been suggested as ideal candidates to focus on in the years to come, including the possible turmeric role for efficient whitening of teeth (alone or in combination with coconut oil), but the data are still missing.

Similarly, other clinical studies have showed beneficial effects of dietary polyphenols on PD including indicators of PD severity such as probing depth (PD), gingival index (GI), and clinical attachment level (CAL). In details, supplementation with a capsule containing selected dehydrated fruits and vegetables significantly reduced PD compared with placebo [211]. Sub-gingival application of a gel containing *Emblica officinalis* or gooseberry extract (10%) reduced PD, increased CAL, and improvements in the modified sulcus bleeding index were seen too [235]. Similarly, intra-pocket application of a green tea extract gel led to decrease in PD, GI, and relative CAL (rCAL) in patients with CP [236]. In addition, it is known that dietary polyphenols possess anti-inflammatory and antioxidant properties. When patients with CP were treated with either a green tea dentifrice containing 60% to 90% epigallocatechin or a standard fluoride/triclosan dentifrice, the green tea treatment significantly increased glutathione-S-transferase activity (an endogenous antioxidant), and consequently decreased gingival inflammation [237]. Dietary polyphenols also have been shown to ameliorate gingival bleeding as well as alveolar bone loss in animals and human studies by suppressing osteoclastogenesis and inhibiting inflammatory cytokines. Although such data is promising, further research is needed to confirm the effects of polyphenols for both prevention and treatment of PD [131]. Yet, the selection of polyphenols at each meal, including a concomitant decrease in sugar intake, and in combination with standard oral hygiene care may play an important role in the prevention and in managing of PD. This, at least in part, was achieved in the present trial by carefully selected contain of the extract that was used. In this context, it is important to mention the lack of characterization and standardization of polyphenol content in foods and beverages that may be associated different outcomes in clinical trials. The latest finding from the literature indicates

that he plant natural products curcumin, baicalin and platycodin D should be further evaluated as PD-1/PD-L1 checkpoint modulators active against PD [238].

The gel of *Myracrodruon urundeuva* (5%) and *Lippia sidoides* (0.5%), and *Ginkgo biloba* extract (28-56 mg/kg) and propolis (100-200 mg/kg) extracts showed strong alveolar bone protective effectiveness in PD induced in rats when compared to the treatment with doxycycline [239].

The fact of using an ultrasonic method in the present study has a series of advantages compared to the manual (curettes) such as: better access to deep periodontal pockets and furcation lesions, faster treatment and therefore more subject comfort and less residual stone. However, it should be mentioned that the roughness parameters that are achieved with ultrasound are similar to those obtained with curettes [240, 241].

Regarding the cardiometabolic variables that have been studied, the fact that there were not significant differences between the two groups guarantees homogeneity in the experimental protocol of the treatment. This implies that the nutraceutical has no effects at the cardiometabolic level. However, we should emphasize that a very short follow up period may influence missing of significant changes in such parameters as some of them improved in the test group compared to the controls and that increasing scientific evidence support the relationship between periodontists and CVD, as well as nutraceutical use in different metabolic disorders. For instance, Iauk *et al.* have investigated the hypoglycaemic activity of *T. capitatus* via the inhibition of α-amylase and α-glucosidase, offering an additional strategy to control postprandial hyperglycaemia in type 2 diabetes management [242]. A continuous inflammatory mediator activation is a common factor for PD and diabetes. Yet, defect of bacteria elimination ability and hyper-responsiveness of monocytes in diabetic patients is associated with persistent elevation of systemic inflammatory mediators and, consequently, to prolonged exposure to inflammatory cytokines which interact with traditional CV risk factor that further may lead to endothelial dysfunction, the first phase of atherogenesis [243]. Interestingly, a very recent systematic review and meta-analysis examined the risk of incident CVD in people with PD considering the longitudinal cohort studies [244]. The findings show a higher risk of all incident CVD outcomes in PD populations compared to non-PD, and, importantly, this risk was consistent across the PD diagnosis method (there was not difference between clinical and self-reported diagnosis), PD severity (associated with PD severity), gender and study regions (no differences between man, women, and regions). The results also indicate that PD precedes CVD and, therefore, early diagnosis of PD together with prompt management may prevent morbidity and mortality from CVD. The management of PD also includes the management of systemic risk factors, such as smoking and stress, which are also risk factors for CVD. Thus, early interventions to target

these risk factors along with PD treatment to remove causative bacterial agents might have an important role in preventing CVD morbidity and mortality [244].

The most recent consensus document regarding associations between PD and CVD derives from the workshop organized by the European Federation of Periodontology (EFP) and the World Heart Federation (WHF) in February 2019 [245]. According to its recommendations, PD is an established, novel CV risk factor that influences the management of subjects suffering from CVD or at increased CVD. In other words, the management of traditional CV risk factors, such as hypertension, is required in the presence of PD, and that a good periodontal health is of pivotal for achieving overall health [246]. However, it remains to be determined if periodontal treatment in subjects with hypertension would translate into a reduced CV risk. Again, inflammation appears to be one of the main connections between CVD and PD. It should be mentioned that the available data mainly comes from observational studies, assessing major outcomes such as myocardial infarction, stroke, heart failure, or death from CVD, but only a few studies investigated preclinical markers of CVD in subjects with PD. Very interestingly, some studies suggest that healthy subjects with PD may have signs of atherosclerosis, thus PD may be considered in case of CV events that cannot be fully explained by the presence of common CV risk factors [117, 245]. Future studies are needed to better understand the relationship between both diseases and to detect early stages of CVD or alterations in CV structure and function linked to PD. The last published randomized controlled trials confirm a positive effect of periodontal treatment on surrogate CV measures, while its effect on the incidence of CVD events (myocardial infarction and stroke) have not been investigated in powered randomized controlled studies with adequate control of traditional CV risk factors [247, 248]. Interestingly, it has been speculated that botanical products may become a new perspective in stem cell-based periodontal regeneration thanks to their angiogenic properties that may be beneficial for bone formation and periodontal regeneration [249].

Based on the literature available up to date, there is a tendency to use the nutraceutical as a mouthwash since its usefulness has been demonstrated reducing a large number of microorganisms that affect the oral cavity [250, 251]. As mentioned above, the oral hygiene of the subject improved considerably in the present study since there was a greater involvement in it. This fact not only influences at the oral level but also at the systemic level given that the consequences of periodontal disease at the oral level are bleeding, halitosis, tooth loss, etc., while at the systemic level they include bacteraemia, systemic inflammation, diabetes, CVD, etc. Thus, improving oral hygiene implies reducing the appearance of PD and consequently its complications and related disorders. It is clear that available data reported in the literature are still not enough to draw final conclusions or indicate on therapeutic supplements use as an adjunct to periodontal therapy. Therefore, long-term studies are needed to ascertain the direct

effects of nutraceuticals on the outcomes in periodontal diseases. We believe that our data can contribute to further support of the use of plant extracts in prevention and treatment of PD. It should be mentioned that the present project is delayed and not closed, so it can be carried out in the coming months/years, as soon as the pandemic situation is resolved and the clinics will be able to resume functioning without interruption (at the moment, it would make no sense to resume recruiting patients, as we do not know if the third wave will lead to a new closure of the clinics; it is necessary to have the clinics open continuously to obtain biological samples (blood, etc.) of the same patients at the right time points as scheduled by the project.

Due to ongoing COVID-19 pandemic, we could not confirm our hypothesis and value the outcome after 6 months, although we get encouraging preliminary data, after 3 months, that should be confirmed by future studies. However, in my opinion the project remains a valid project and with the work that has been done and with the obtained preliminary data, the experimental protocol has been validated. Nevertheless, the baseline periodontal conditions of the placebo group were significantly worse compared to the Test group, despite the randomization procedure was followed in a strict way, that also has been seen in other randomized controlled trial showing that randomisation by itself does not ensure a balanced distribution [252]. The impact of such asymmetrical patient allocation due to randomness on the observed treatment effects may have had an impact on our results and only a longer follow-up as well as larger samples could have provided more evidence on these results, since periodontal conditions may have been more equal between patients after the impact of non-surgical treatment. Also, we do believe that the current project can be further carried out and may strength these preliminary findings reducing the degree of these imbalances at baseline which limit the conclusions and does not allow us to interpret the different outcomes between the test and control group as simply reflecting the supplement's effectiveness. However, it can be noticed that, after only 3 months, improvements in periodontal conditions were somewhat higher in the test group compared to the controls (about 70-80% vs about 50%). We assume that this percentage would be higher after 6 months and that this difference did not occur by chance, and future investigations will hopefully conform this assumption. From this reason, together with other previously mentioned limitations, we consider our results as preliminary. On the other hand, we highlighted the strengths of the present study that have been discussed in details. It should be also emphasized that, in addition to randomisation, and the fact that person who generated the allocation sequence has not taken decisions about the eligibility and entry of patients, trial involved other complex processes such as blinding and close monitoring of participants in order to avoid bias to results. During the time when our study was ongoing, to the best of our knowledge, only one similar randomized controlled clinical study has been published [219], while a few recent systematic as well as meta-analysis review articles indicate on a

need for the further research in the field, highlighting beneficial effects seen mostly in preclinical research and a need for clinical research.

Finally, despite the fact that there were no significant differences in the metabolic parameters, clinical experience supported by significant changes in several PD variables, suggests that the organoleptic properties of the nutraceutical exert favourable effects in subjects with PD. This is also related to the fact that none subject reported an adverse reaction and also, there was a good compliance/tolerance rate within the sample. It should be emphasized that the use of this nutraceutical has motivated several subjects to quit smoking, which represents one of the important risk factors for the development of PD, but also an important CVD risk factor. Also, we wish to highlight that the benefits of nutraceuticals may differ between individuals and age, gender, genetics, BMI and, overall health status have been suggested to be important predictors in the response, especially regarding the changes in the metabolic parameters where also lifestyle and diet are important factors. In this context, a personalized therapeutic approach should be used by clinicians considering also possible drug interactions. Yet, with a broader understanding of how prevention of PD can impact CVD and diabetes would not only provide additional preventative measures for CVDs, but also decrease the economic burden on the health system [253]. We believe that use of nutraceutical in combination with healthy lifestyle, including smoking control, could have an important role in preventing PD (as it is well known that PD is preventable [254]), and consequently CVD. In addition, gaining a broader understanding of the link between oral microbiota and our organism can lead to the development of a novel therapeutic approaches to prevent and treat metabolic diseases targeting the oral microbiota [255]. On the other hand, the development of metabolic disorders in subjects at risk, may be prevented by the identification of new bacterial biomarkers from oral cavity.

6. CONCLUSION

The response of some periodontal clinical variables was more effective in subjects treated with the extract compared to controls, while more favourable effects on MetS-related variables have not been seen in subjects treated with the extract compared to controls, excepting glycemia level that significantly improved in both groups. Although the final conclusion cannot be driven, our preliminary results suggest that nutraceutical based on EOs as an adjuvant therapeutic agent for periodontal treatment might have some beneficial additional effect on PD variables, preventing progression of disease, when used as an irrigation solution and/or mouthwash. However, more clinical trials with a longer follow-up period are needed, in order to confirm such benefits of the studied product with the particular focus on assessing changes in cardiovascular risk markers. There is significant research potential in this area, and we hope that in the future someone else will be able to carry out the whole project in full because the answers obtained may be useful for CV prevention too.

	Componenti	%	Cod.	INCI
1.	AQUA	80,8300	EGERIA	Aqua
2.	GLICEROLO VEGETALE FU-Ph.Eur.	15,0000	004742	Glycerin
3.	OLIO RICINO IDROGENATO ETOSS. 40 MOLI	1,0000	002903	PEG-40 hydrogenated castor oil
4.	XILITOLO POLVERE Ph.Eur.-USP E 967	1,0000		Xylitol
5.	ACNIBIO PE 9010	0,5000	010062	Phenoxyethanol, Ethylhexylglycerin
6.	MUCOSAVE CG	0,5000		Opuntia ficus-indica stem extract, Olea europaea leaf extract, Maltodextrin
7.	PROTELAN LS-9011/SL	0,5000	003810	Aqua, Sodium lauroyl sarcosinate
8.	FENOSSIETANOLO	0,3000	004306	Phenoxyethanol
9.	CITRUS SINENSIS ESSENTIAL OIL	0,2000		Citrus sinensis peel oil expressed
10.	LAVANDULA INTERMEDIA ESSENTIAL OIL	0,1000		Lavandula intermedia flower/leaf/stem oil
11.	ROSMARINUS OFFICINALIS ESSENTIAL OIL	0,0300		Rosmarinus officinalis flower oil
12.	THYMUS VULGARIS ESSENTIAL OIL	0,0300		Thymus vulgaris oil
13.	TRIETANOLAMMINA 99% COSMETIC GRADE	0,0100	003885	Triethanolamine
	Totale	100,0000		

Mouthwash / Irrigation solutions, scalar qualitative and quantitative composition of the raw materials and related ingredient lists

	Componenti	%	Cod.	INCI
1.	AQUA	81,3300	EGERIA	Aqua
2.	GLICEROLO VEGETALE FU-Ph.Eur.	12,0000	004742	Glycerin
3.	POLIVINILPIRROLIDONE K 30 (LUVISKOL K30)	3,0000		PVP
4.	OLIO RICINO IDROGENATO ETOSS. 40 MOLI	1,0000	002903	PEG-40 hydrogenated castor oil
5.	XILITOLO POLVERE Ph.Eur.-USP E 967	1,0000		Xylitol
6.	ACNIBIO PE 9010	0,5000	010062	Phenoxyethanol, Ethylhexylglycerin
7.	MUCOSAVE CG	0,5000		Opuntia ficus-indica stem extract, Olea europaea leaf extract, Maltodextrin
8.	FENOSSIETANOLO	0,3000	004306	Phenoxyethanol
9.	CITRUS SINENSIS ESSENTIAL OIL	0,2000		Citrus sinensis peel oil expressed
10.	LAVANDULA INTERMEDIA ESSENTIAL OIL	0,1000		Lavandula intermedia flower/leaf/stem oil
11.	ROSMARINUS OFFICINALIS ESSENTIAL OIL	0,0300		Rosmarinus officinalis flower oil
12.	THYMUS VULGARIS ESSENTIAL OIL	0,0300		Thymus vulgaris oil
13.	TRIETANOLAMMINA 99% COSMETIC GRADE	0,0100	003885	Triethanolamine
	Totale	100,0000		

Nebulizer spray, scalar qualitative and quantitative composition of the raw materials and related ingredient lists

(Attachment 3-4)

RESEARCH PROTOCOL

EFFECT OF ESSENTIAL OILS AS ADJUTANTS ON THE TREATMENT OF SUBJECTS WITH PERIODONTITIS: ASSESSMENT OF METABOLIC VARIABLES AS EFFECT MODIFIERS.

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Research project

reference:

ClinicalTrials.gov

Identifier:

Keywords: Anti-inflammation; Nutraceuticals; Periodontal therapy; Periodontitis.

(Attachment 8)

7. REFERENCES

1. Mealey, B.L., T.W. Oates, and P. American Academy of, *Diabetes mellitus and periodontal diseases.* J Periodontol, 2006. **77**(8): p. 1289-303.
2. Kebschull, M., R.T. Demmer, and P.N. Papapanou, *"Gum bug, leave my heart alone!"--epidemiologic and mechanistic evidence linking periodontal infections and atherosclerosis.* J Dent Res, 2010. **89**(9): p. 879-902.
3. Forner, L., et al., *Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation.* J Clin Periodontol, 2006. **33**(6): p. 401-7.
4. Leon, R., et al., *Detection of Porphyromonas gingivalis in the amniotic fluid in pregnant women with a diagnosis of threatened premature labor.* J Periodontol, 2007. **78**(7): p. 1249-55.
5. D'Aiuto, F., et al., *Association of the metabolic syndrome with severe periodontitis in a large U.S. population-based survey.* J Clin Endocrinol Metab, 2008. **93**(10): p. 3989-94.
6. Han, W., et al., *Abnormalities in periodontal and salivary tissues in conditional presenilin 1 and presenilin 2 double knockout mice.* Mol Cell Biochem, 2011. **347**(1-2): p. 13-20.
7. Ohnishi, T., et al., *Oxidative stress causes alveolar bone loss in metabolic syndrome model mice with type 2 diabetes.* J Periodontal Res, 2009. **44**(1): p. 43-51.
8. Andriankaja, O.M., et al., *Association between metabolic syndrome and periodontal disease.* Aust Dent J, 2010. **55**(3): p. 252-9.
9. Rizzo, M., et al., *Small, dense low-density lipoproteins (LDL) are predictors of cardio- and cerebro-vascular events in subjects with the metabolic syndrome.* Clin Endocrinol (Oxf), 2009. **70**(6): p. 870-5.
10. Rizzo, M., et al., *Heat-shock protein 60 kDa and atherogenic dyslipidemia in patients with untreated mild periodontitis: a pilot study.* Cell Stress Chaperones, 2012. **17**(3): p. 399-407.
11. Shamaei-Tousi, A., et al., *Differential regulation of circulating levels of molecular chaperones in patients undergoing treatment for periodontal disease.* PLoS One, 2007. **2**(11): p. e1198.
12. Choi, J., et al., *Identification of immunoreactive epitopes of the Porphyromonas gingivalis heat shock protein in periodontitis and atherosclerosis.* J Periodontal Res, 2011. **46**(2): p. 240-5.
13. Santini, A., et al., *Nutraceuticals: opening the debate for a regulatory framework.* Br J Clin Pharmacol, 2018. **84**(4): p. 659-672.
14. Aronson, J.K., *Defining 'nutraceuticals': neither nutritious nor pharmaceutical.* Br J Clin Pharmacol, 2017. **83**(1): p. 8-19.

15. *The Foundation for Innovation in Medicine*. Available from: http:// www.fimdefelice.org.
16. Castellino, G., et al., *Effects of Essential Oils and Selected Compounds from Lamiaceae Family as Adjutants on the Treatment of Subjects with Periodontitis and Cardiovascular Risk.* Applied Sciences, 2021. **11**(20): p. 9563.
17. Barnes, J., *Adverse drug reactions and pharmacovigilance of herbal medicines. In: Stephens' Detection and Evaluation of Adverse Drug Reactions: Principles and Practice, 6th edn, eds Talbot J, Aronson JK. Oxford: Wiley-Blackwell, 2011*.
18. Vlietinck, A., L. Pieters, and S. Apers, *Legal requirements for the quality of herbal substances and herbal preparations for the manufacturing of herbal medicinal products in the European union.* Planta Med, 2009. **75**(7): p. 683-8.
19. *The European Parliament and the Council of the European Union. Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004, amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use.* Off J Eur Union, 2004. **L136**: p. 85-90.
20. *World Health Organization. WHO Traditional Medicine Strategy 2002–2005. Geneva: World Health Organization, 2002.*
21. Balic, A., *Biology Explaining Tooth Repair and Regeneration: A Mini-Review.* Gerontology, 2018. **64**(4): p. 382-388.
22. Yuan, Y. and Y. Chai, *Regulatory mechanisms of jaw bone and tooth development.* Curr Top Dev Biol, 2019. **133**: p. 91-118.
23. Madani, M., T. Berardi, and E.T. Stoopler, *Anatomic and examination considerations of the oral cavity.* Med Clin North Am, 2014. **98**(6): p. 1225-38.
24. Havale, R., et al., *Dental notation for primary teeth: a review and suggestion of a novel system.* Eur J Paediatr Dent, 2015. **16**(2): p. 163-6.
25. Al-Johany, S.S., *Tooth Numbering System in Saudi Arabia: Survey.* Saudi Dent J, 2016. **28**(4): p. 183-188.
26. Lindhe, J. and L. P.N., *Clinical periodontology and implant dentistry.* . 2015. 1480.
27. Pugsley, M.K. and R. Tabrizchi, *The vascular system. An overview of structure and function.* J Pharmacol Toxicol Methods, 2000. **44**(2): p. 333-40.
28. Borysenko, M. and T. Beringer, *Functional Histology (pp. 195-208). Boston: Little Brown and Company; 2nd Revised edition*. 1984.
29. Nakashima, Y., et al., *Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration.* Arterioscler Thromb Vasc Biol, 2007. **27**(5): p. 1159-65.

30. Wang, D., et al., *Roles of Cells from the Arterial Vessel Wall in Atherosclerosis.* Mediators Inflamm, 2017. **2017**: p. 8135934.
31. Waller, B.F., et al., *Anatomy, histology, and pathology of coronary arteries: a review relevant to new interventional and imaging techniques--Part I.* Clin Cardiol, 1992. **15**(6): p. 451-7.
32. Nakashima, Y., T.N. Wight, and K. Sueishi, *Early atherosclerosis in humans: role of diffuse intimal thickening and extracellular matrix proteoglycans.* Cardiovasc Res, 2008. **79**(1): p. 14-23.
33. Ait-Oufella, H., et al., *Adaptive (T and B cells) immunity and control by dendritic cells in atherosclerosis.* Circ Res, 2014. **114**(10): p. 1640-60.
34. Subbotin, V.M., *Excessive intimal hyperplasia in human coronary arteries before intimal lipid depositions is the initiation of coronary atherosclerosis and constitutes a therapeutic target.* Drug Discov Today, 2016. **21**(10): p. 1578-1595.
35. Haverich, A., *A Surgeon's View on the Pathogenesis of Atherosclerosis.* Circulation, 2017. **135**(3): p. 205-207.
36. Houtkamp, M.A., et al., *Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses.* J Pathol, 2001. **193**(2): p. 263-9.
37. Campbell, K.A., et al., *Lymphocytes and the adventitial immune response in atherosclerosis.* Circ Res, 2012. **110**(6): p. 889-900.
38. Xu, F., et al., *Activation of adventitial fibroblasts contributes to the early development of atherosclerosis: a novel hypothesis that complements the "Response-to-Injury Hypothesis" and the "Inflammation Hypothesis".* Med Hypotheses, 2007. **69**(4): p. 908-12.
39. Xu, F., et al., *NADPH oxidase p47phox siRNA attenuates adventitial fibroblasts proliferation and migration in apoE(-/-) mouse.* J Transl Med, 2015. **13**: p. 38.
40. Cooper, L.L., et al., *Microvascular Function Contributes to the Relation Between Aortic Stiffness and Cardiovascular Events: The Framingham Heart Study.* Circ Cardiovasc Imaging, 2016. **9**(12).
41. Stefanadis, C., et al., *Coronary Atherosclerotic Vulnerable Plaque: Current Perspectives.* J Am Heart Assoc, 2017. **6**(3).
42. Chen, C. and D.B. Khismatullin, *Oxidized low-density lipoprotein contributes to atherogenesis via co-activation of macrophages and mast cells.* PLoS One, 2015. **10**(3): p. e0123088.
43. Pietiainen, M., et al., *Mediators between oral dysbiosis and cardiovascular diseases.* Eur J Oral Sci, 2018. **126 Suppl 1**: p. 26-36.

44. Gonzalez Navarro, B., X. Pinto Sala, and E. Jane Salas, *Relationship between cardiovascular disease and dental pathology. Systematic review.* Med Clin (Barc), 2017. **149**(5): p. 211-216.
45. Tonetti, M.S., T.E. Van Dyke, and E.F.P.A.A.P.w. Working group 1 of the joint, *Periodontitis and atherosclerotic cardiovascular disease: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases.* J Clin Periodontol, 2013. **40 Suppl 14**: p. S24-9.
46. Perk, J., et al., *European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts).* Eur Heart J, 2012. **33**(13): p. 1635-701.
47. Lockhart, P.B., et al., *Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association?: a scientific statement from the American Heart Association.* Circulation, 2012. **125**(20): p. 2520-44.
48. Ryden, L., et al., *Periodontitis Increases the Risk of a First Myocardial Infarction: A Report From the PAROKRANK Study.* Circulation, 2016. **133**(6): p. 576-83.
49. Bartova, J., et al., *Periodontitis as a risk factor of atherosclerosis.* J Immunol Res, 2014. **2014**: p. 636893.
50. Beukers, N.G., et al., *Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands.* J Epidemiol Community Health, 2017. **71**(1): p. 37-42.
51. Marfil-Alvarez, R., et al., *Acute myocardial infarct size is related to periodontitis extent and severity.* J Dent Res, 2014. **93**(10): p. 993-8.
52. Amar, S., et al., *Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation.* Arterioscler Thromb Vasc Biol, 2003. **23**(7): p. 1245-9.
53. Holtfreter, B., et al., *Periodontitis is associated with endothelial dysfunction in a general population: a cross-sectional study.* PLoS One, 2013. **8**(12): p. e84603.
54. Moura, M.F., et al., *Periodontitis and Endothelial Dysfunction: Periodontal Clinical Parameters and Levels of Salivary Markers Interleukin-1beta, Tumor Necrosis Factor-alpha, Matrix Metalloproteinase-2, Tissue Inhibitor of Metalloproteinases-2 Complex, and Nitric Oxide.* J Periodontol, 2017. **88**(8): p. 778-787.
55. Punj, A., S.B. Shenoy, and K. Subramanyam, *Comparison of Endothelial Function in Healthy Patients and Patients With Chronic Periodontitis and Myocardial Infarction.* J Periodontol, 2017. **88**(12): p. 1234-1243.

56. D'Aiuto, F., M. Orlandi, and J.C. Gunsolley, *Evidence that periodontal treatment improves biomarkers and CVD outcomes.* J Clin Periodontol, 2013. **40 Suppl 14**: p. S85-105.
57. Mercanoglu, F., et al., *Endothelial dysfunction in patients with chronic periodontitis and its improvement after initial periodontal therapy.* J Periodontol, 2004. **75**(12): p. 1694-700.
58. Seinost, G., et al., *Periodontal treatment improves endothelial dysfunction in patients with severe periodontitis.* Am Heart J, 2005. **149**(6): p. 1050-4.
59. Elter, J.R., et al., *The effects of periodontal therapy on vascular endothelial function: a pilot trial.* Am Heart J, 2006. **151**(1): p. 47.
60. Blum, A., et al., *Periodontal care may improve endothelial function.* Eur J Intern Med, 2007. **18**(4): p. 295-8.
61. Desvarieux, M., et al., *Changes in clinical and microbiological periodontal profiles relate to progression of carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology study.* J Am Heart Assoc, 2013. **2**(6): p. e000254.
62. Kiechl, S., et al., *Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study.* Circulation, 2001. **103**(8): p. 1064-70.
63. Prasad, A., et al., *Predisposition to atherosclerosis by infections: role of endothelial dysfunction.* Circulation, 2002. **106**(2): p. 184-90.
64. Spahr, A., et al., *Periodontal infections and coronary heart disease: role of periodontal bacteria and importance of total pathogen burden in the Coronary Event and Periodontal Disease (CORODONT) study.* Arch Intern Med, 2006. **166**(5): p. 554-9.
65. Elkaim, R., et al., *Prevalence of periodontal pathogens in subgingival lesions, atherosclerotic plaques and healthy blood vessels: a preliminary study.* J Periodontal Res, 2008. **43**(2): p. 224-31.
66. Chen, Y.W., et al., *Periodontitis may increase the risk of peripheral arterial disease.* Eur J Vasc Endovasc Surg, 2008. **35**(2): p. 153-8.
67. Gaetti-Jardim, E., et al., *Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries.* J Med Microbiol, 2009. **58**(Pt 12): p. 1568-1575.
68. Figuero, E., et al., *Detection of periodontal bacteria in atheromatous plaque by nested polymerase chain reaction.* J Periodontol, 2011. **82**(10): p. 1469-77.
69. Chiu, B., *Multiple infections in carotid atherosclerotic plaques.* Am Heart J, 1999. **138**(5 Pt 2): p. S534-6.
70. Kinane, D.F., et al., *Bacteraemia following periodontal procedures.* J Clin Periodontol, 2005. **32**(7): p. 708-13.

71. Takeuchi, H., et al., *Exit of intracellular Porphyromonas gingivalis from gingival epithelial cells is mediated by endocytic recycling pathway.* Cell Microbiol, 2011. **13**(5): p. 677-91.
72. Carrion, J., et al., *Microbial carriage state of peripheral blood dendritic cells (DCs) in chronic periodontitis influences DC differentiation, atherogenic potential.* J Immunol, 2012. **189**(6): p. 3178-87.
73. Cekici, A., et al., *Inflammatory and immune pathways in the pathogenesis of periodontal disease.* Periodontol 2000, 2014. **64**(1): p. 57-80.
74. Pussinen, P.J., et al., *Periodontitis decreases the antiatherogenic potency of high density lipoprotein.* J Lipid Res, 2004. **45**(1): p. 139-47.
75. Bengtsson, T., et al., *The periodontal pathogen Porphyromonas gingivalis cleaves apoB-100 and increases the expression of apoM in LDL in whole blood leading to cell proliferation.* J Intern Med, 2008. **263**(5): p. 558-71.
76. Morishita, M., et al., *A. actinomycetemcomitans LPS enhances foam cell formation induced by LDL.* J Dent Res, 2013. **92**(3): p. 241-6.
77. Takahashi, Y., et al., *Fimbria-dependent activation of pro-inflammatory molecules in Porphyromonas gingivalis infected human aortic endothelial cells.* Cell Microbiol, 2006. **8**(5): p. 738-57.
78. Gibson, F.C., 3rd, et al., *Innate immune recognition of invasive bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2004. **109**(22): p. 2801-6.
79. Mesa, F., et al., *Periodontitis, blood lipids and lipoproteins.* Clinical Lipidology, 2014. **9**(2): p. 261-276.
80. Hashimoto, M., et al., *Selective proteolysis of apolipoprotein B-100 by Arg-gingipain mediates atherosclerosis progression accelerated by bacterial exposure.* J Biochem, 2006. **140**(5): p. 713-23.
81. Tamaki, N., et al., *Periodontal treatment decreases plasma oxidized LDL level and oxidative stress.* Clin Oral Investig, 2011. **15**(6): p. 953-8.
82. Raetz, C.R. and C. Whitfield, *Lipopolysaccharide endotoxins.* Annu Rev Biochem, 2002. **71**: p. 635-700.
83. Liljestrand, J.M., et al., *Lipopolysaccharide, a possible molecular mediator between periodontitis and coronary artery disease.* J Clin Periodontol, 2017. **44**(8): p. 784-792.
84. Tada, H., et al., *Proteolysis of CD14 on human gingival fibroblasts by arginine-specific cysteine proteinases from Porphyromonas gingivalis leading to down-regulation of lipopolysaccharide-induced interleukin-8 production.* Infect Immun, 2002. **70**(6): p. 3304-7.

85. Tada, H., et al., *Proteolysis of ICAM-1 on human oral epithelial cells by gingipains.* J Dent Res, 2003. **82**(10): p. 796-801.
86. Romandini, M., et al., *Periodontitis and platelet count: A new potential link with cardiovascular and other systemic inflammatory diseases.* J Clin Periodontol, 2018. **45**(11): p. 1299-1310.
87. Li, X., et al., *An ultrastructural study of Porphyromonas gingivalis-induced platelet aggregation.* Thromb Res, 2008. **122**(6): p. 810-9.
88. Choi, J.I., et al., *Epitope mapping of Porphyromonas gingivalis heat-shock protein and human heat-shock protein in human atherosclerosis.* J Dent Res, 2004. **83**(12): p. 936-40.
89. Ford, P.J., et al., *Anti-P. gingivalis response correlates with atherosclerosis.* J Dent Res, 2007. **86**(1): p. 35-40.
90. Aarabi, G., G. Heydecke, and U. Seedorf, *Roles of Oral Infections in the Pathomechanism of Atherosclerosis.* Int J Mol Sci, 2018. **19**(7).
91. Yamazaki, K., et al., *T-cell clonality to Porphyromonas gingivalis and human heat shock protein 60s in patients with atherosclerosis and periodontitis.* Oral Microbiol Immunol, 2004. **19**(3): p. 160-7.
92. Gerber, P.A., D. Nikolic, and M. Rizzo, *Small, dense LDL: an update.* Curr Opin Cardiol, 2017. **32**(4): p. 454-459.
93. Nikolic, D., et al., *Lipoprotein subfractions in metabolic syndrome and obesity: clinical significance and therapeutic approaches.* Nutrients, 2013. **5**(3): p. 928-48.
94. Nepomuceno, R., et al., *Serum lipid levels in patients with periodontal disease: A meta-analysis and meta-regression.* J Clin Periodontol, 2017. **44**(12): p. 1192-1207.
95. Magan-Fernandez, A., et al., *Association of simvastatin and hyperlipidemia with periodontal status and bone metabolism markers.* J Periodontol, 2014. **85**(10): p. 1408-15.
96. Hasturk, H. and A. Kantarci, *Activation and resolution of periodontal inflammation and its systemic impact.* Periodontol 2000, 2015. **69**(1): p. 255-73.
97. Teng, Y.T., *The role of acquired immunity and periodontal disease progression.* Crit Rev Oral Biol Med, 2003. **14**(4): p. 237-52.
98. Kinane, D.F. and D.F. Lappin, *Clinical, pathological and immunological aspects of periodontal disease.* Acta Odontol Scand, 2001. **59**(3): p. 154-60.
99. Barros, S.P., et al., *Gingival crevicular fluid as a source of biomarkers for periodontitis.* Periodontol 2000, 2016. **70**(1): p. 53-64.
100. Haffajee, A.D. and S.S. Socransky, *Microbial etiological agents of destructive periodontal diseases.* Periodontol 2000, 1994. **5**: p. 78-111.

101. Caton, J.G., et al., *A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification.* J Periodontol, 2018. **89 Suppl 1**: p. S1-S8.
102. Tonetti, M.S. and M. Sanz, *Implementation of the new classification of periodontal diseases: Decision-making algorithms for clinical practice and education.* J Clin Periodontol, 2019. **46**(4): p. 398-405.
103. Hammer, K.A., C.F. Carson, and T.V. Riley, *Antifungal effects of Melaleuca alternifolia (tea tree) oil and its components on Candida albicans, Candida glabrata and Saccharomyces cerevisiae.* J Antimicrob Chemother, 2004. **53**(6): p. 1081-5.
104. Weber, C. and H. Noels, *Atherosclerosis: current pathogenesis and therapeutic options.* Nat Med, 2011. **17**(11): p. 1410-22.
105. *World Health Organization: Cardiovascular diseases (CVDs).* 2019; Available from: https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
106. Wong, B.W., et al., *The biological role of inflammation in atherosclerosis.* Can J Cardiol, 2012. **28**(6): p. 631-41.
107. Rizzo, M., et al., *Should we measure routinely oxidised and atherogenic dense low-density lipoproteins in subjects with type 2 diabetes?* Int J Clin Pract, 2010. **64**(12): p. 1632-42.
108. Raggi, P., et al., *Role of inflammation in the pathogenesis of atherosclerosis and therapeutic interventions.* Atherosclerosis, 2018. **276**: p. 98-108.
109. Viola, J. and O. Soehnlein, *Atherosclerosis - A matter of unresolved inflammation.* Semin Immunol, 2015. **27**(3): p. 184-93.
110. Tabas, I. and K.E. Bornfeldt, *Macrophage Phenotype and Function in Different Stages of Atherosclerosis.* Circ Res, 2016. **118**(4): p. 653-67.
111. Bories, G.F.P. and N. Leitinger, *Macrophage metabolism in atherosclerosis.* FEBS Lett, 2017. **591**(19): p. 3042-3060.
112. Asadullah, K., W. Sterry, and H.D. Volk, *Interleukin-10 therapy--review of a new approach.* Pharmacol Rev, 2003. **55**(2): p. 241-69.
113. Murray, P.J., *The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription.* Proc Natl Acad Sci U S A, 2005. **102**(24): p. 8686-91.
114. Mallat, Z., et al., *Protective role of interleukin-10 in atherosclerosis.* Circ Res, 1999. **85**(8): p. e17-24.
115. Kim, M., et al., *Targeted delivery of anti-inflammatory cytokine by nanocarrier reduces atherosclerosis in Apo E(-/-) mice.* Biomaterials, 2020. **226**: p. 119550.

116. Libby, P., et al., *Inflammation in atherosclerosis: from pathophysiology to practice.* J Am Coll Cardiol, 2009. **54**(23): p. 2129-38.
117. Carrizales-Sepulveda, E.F., et al., *Periodontal Disease, Systemic Inflammation and the Risk of Cardiovascular Disease.* Heart Lung Circ, 2018. **27**(11): p. 1327-1334.
118. Golia, E., et al., *Inflammation and cardiovascular disease: from pathogenesis to therapeutic target.* Curr Atheroscler Rep, 2014. **16**(9): p. 435.
119. Patti, A.M., et al., *Natural approaches in metabolic syndrome management.* Arch Med Sci, 2018. **14**(2): p. 422-441.
120. Toth, P.P., et al., *Bergamot Reduces Plasma Lipids, Atherogenic Small Dense LDL, and Subclinical Atherosclerosis in Subjects with Moderate Hypercholesterolemia: A 6 Months Prospective Study.* Front Pharmacol, 2015. **6**: p. 299.
121. Castellino, G., et al., *Altilix((R)) Supplement Containing Chlorogenic Acid and Luteolin Improved Hepatic and Cardiometabolic Parameters in Subjects with Metabolic Syndrome: A 6 Month Randomized, Double-Blind, Placebo-Controlled Study.* Nutrients, 2019. **11**(11).
122. Cicero, A.F.G., A. Colletti, and S. Bellentani, *Nutraceutical Approach to Non-Alcoholic Fatty Liver Disease (NAFLD): The Available Clinical Evidence.* Nutrients, 2018. **10**(9).
123. Kononen, E., M. Gursoy, and U.K. Gursoy, *Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues.* J Clin Med, 2019. **8**(8).
124. Eke, P.I., et al., *Periodontitis prevalence in adults >/= 65 years of age, in the USA.* Periodontol 2000, 2016. **72**(1): p. 76-95.
125. Susin, C., A.N. Haas, and J.M. Albandar, *Epidemiology and demographics of aggressive periodontitis.* Periodontol 2000, 2014. **65**(1): p. 27-45.
126. Hajishengallis, G. and R.J. Lamont, *Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology.* Mol Oral Microbiol, 2012. **27**(6): p. 409-19.
127. Ramanauskaite, E. and V. Machiulskiene, *Antiseptics as adjuncts to scaling and root planing in the treatment of periodontitis: a systematic literature review.* BMC Oral Health, 2020. **20**(1): p. 143.
128. Yilmaz, H.G. and H. Bayindir, *Clinical evaluation of chlorhexidine and essential oils for adjunctive effects in ultrasonic instrumentation of furcation involvements: a randomized controlled clinical trial.* Int J Dent Hyg, 2012. **10**(2): p. 113-7.
129. Varela-Lopez, A., et al., *Nutraceuticals in Periodontal Health: A Systematic Review on the Role of Vitamins in Periodontal Health Maintenance.* Molecules, 2018. **23**(5).

130. Nittayananta, W., et al., *Oral spray containing plant-derived compounds is effective against common oral pathogens.* Arch Oral Biol, 2018. **90**: p. 80-85.
131. Basu, A., E. Masek, and J.L. Ebersole, *Dietary Polyphenols and Periodontitis-A Mini-Review of Literature.* Molecules, 2018. **23**(7).
132. Zhu, F., B. Du, and B. Xu, *Anti-inflammatory effects of phytochemicals from fruits, vegetables, and food legumes: A review.* Crit Rev Food Sci Nutr, 2018. **58**(8): p. 1260-1270.
133. Blaizot, A., et al., *Periodontal diseases and cardiovascular events: meta-analysis of observational studies.* Int Dent J, 2009. **59**(4): p. 197-209.
134. Zeng, X.T., et al., *Periodontal disease and carotid atherosclerosis: A meta-analysis of 17,330 participants.* Int J Cardiol, 2016. **203**: p. 1044-51.
135. Humphrey, L.L., et al., *Periodontal disease and coronary heart disease incidence: a systematic review and meta-analysis.* J Gen Intern Med, 2008. **23**(12): p. 2079-86.
136. Mustapha, I.Z., et al., *Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis.* J Periodontol, 2007. **78**(12): p. 2289-302.
137. Bahekar, A.A., et al., *The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: a meta-analysis.* Am Heart J, 2007. **154**(5): p. 830-7.
138. Loos, B.G., *Systemic markers of inflammation in periodontitis.* J Periodontol, 2005. **76**(11 Suppl): p. 2106-15.
139. Furuhashi, M., et al., *Lipid chaperones and metabolic inflammation.* Int J Inflam, 2011. **2011**: p. 642612.
140. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-7.
141. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory mechanisms in obesity.* Annu Rev Immunol, 2011. **29**: p. 415-45.
142. Khansari, N., Y. Shakiba, and M. Mahmoudi, *Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer.* Recent Pat Inflamm Allergy Drug Discov, 2009. **3**(1): p. 73-80.
143. Dandekar, A., R. Mendez, and K. Zhang, *Cross talk between ER stress, oxidative stress, and inflammation in health and disease.* Methods Mol Biol, 2015. **1292**: p. 205-14.
144. Arulselvan, P., et al., *Role of Antioxidants and Natural Products in Inflammation.* Oxid Med Cell Longev, 2016. **2016**: p. 5276130.

145. Bonesi, M., et al., *Anti-inflammatory and Antioxidant Agents from Salvia Genus (Lamiaceae): An Assessment of the Current State of Knowledge.* Antiinflamm Antiallergy Agents Med Chem, 2017. **16**(2): p. 70-86.
146. Aziz, Z.A.A., et al., *Essential Oils: Extraction Techniques, Pharmaceutical And Therapeutic Potential - A Review.* Curr Drug Metab, 2018. **19**(13): p. 1100-1110.
147. Dajic Stevanovic, Z., et al., *Natural Macromolecules as Carriers for Essential Oils: From Extraction to Biomedical Application.* Front Bioeng Biotechnol, 2020. **8**: p. 563.
148. Uritu, C.M., et al., *Medicinal Plants of the Family Lamiaceae in Pain Therapy: A Review.* Pain Res Manag, 2018. **2018**: p. 7801543.
149. Huo, M., et al., *Suppression of LPS-induced inflammatory responses by gossypol in RAW 264.7 cells and mouse models.* Int Immunopharmacol, 2013. **15**(2): p. 442-9.
150. Ma, J., et al., *Linalool inhibits cigarette smoke-induced lung inflammation by inhibiting NF-kappaB activation.* Int Immunopharmacol, 2015. **29**(2): p. 708-713.
151. Khaleel, C., N. Tabanca, and G.J.O.C. Buchbauer, *α-Terpineol, a natural monoterpene: A review of its biological properties.* 2018. **16**: p. 349 - 361.
152. Hart, P.H., et al., *Terpinen-4-ol, the main component of the essential oil of Melaleuca alternifolia (tea tree oil), suppresses inflammatory mediator production by activated human monocytes.* Inflamm Res, 2000. **49**(11): p. 619-26.
153. Nogueira, M.N., et al., *Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1beta, IL-6 and IL-10 on human macrophages.* Inflamm Res, 2014. **63**(9): p. 769-78.
154. Zhang, H., et al., *Serine Alleviates Dextran Sulfate Sodium-Induced Colitis and Regulates the Gut Microbiota in Mice.* Front Microbiol, 2018. **9**: p. 3062.
155. Peng, L.Y., et al., *Madecassoside Protects Against LPS-Induced Acute Lung Injury via Inhibiting TLR4/NF-kappaB Activation and Blood-Air Barrier Permeability.* Front Pharmacol, 2020. **11**: p. 807.
156. Kummer, R., et al., *Evaluation of Anti-Inflammatory Activity of Citrus latifolia Tanaka Essential Oil and Limonene in Experimental Mouse Models.* Evid Based Complement Alternat Med, 2013. **2013**: p. 859083.
157. Yoon, W.J., N.H. Lee, and C.G. Hyun, *Limonene suppresses lipopolysaccharide-induced production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines in RAW 264.7 macrophages.* J Oleo Sci, 2010. **59**(8): p. 415-21.

158. Rufino, A.T., et al., *Evaluation of the anti-inflammatory, anti-catabolic and pro-anabolic effects of E-caryophyllene, myrcene and limonene in a cell model of osteoarthritis.* Eur J Pharmacol, 2015. **750**: p. 141-50.
159. Botelho, M.A., et al., *Effects of a herbal gel containing carvacrol and chalcones on alveolar bone resorption in rats on experimental periodontitis.* Phytother Res, 2008. **22**(4): p. 442-9.
160. Guimaraes, A.G., et al., *Carvacrol attenuates mechanical hypernociception and inflammatory response.* Naunyn Schmiedebergs Arch Pharmacol, 2012. **385**(3): p. 253-63.
161. Hotta, M., et al., *Carvacrol, a component of thyme oil, activates PPARalpha and gamma and suppresses COX-2 expression.* J Lipid Res, 2010. **51**(1): p. 132-9.
162. Landa, P., et al., *In vitro anti-inflammatory activity of carvacrol: Inhibitory effect on COX-2 catalyzed prostaglandin E(2) biosynthesis.* Arch Pharm Res, 2009. **32**(1): p. 75-8.
163. Joca, H.C., et al., *Carvacrol modulates voltage-gated sodium channels kinetics in dorsal root ganglia.* Eur J Pharmacol, 2015. **756**: p. 22-9.
164. Joca, H.C., et al., *Carvacrol decreases neuronal excitability by inhibition of voltage-gated sodium channels.* J Nat Prod, 2012. **75**(9): p. 1511-7.
165. Goncalves, J.C., et al., *Essential oil composition and antinociceptive activity of Thymus capitatus.* Pharm Biol, 2017. **55**(1): p. 782-786.
166. Cosentino, S., et al., *In-vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils.* Lett Appl Microbiol, 1999. **29**(2): p. 130-5.
167. Chauhan, A.K., et al., *Potentiation of macrophage activity by thymol through augmenting phagocytosis.* Int Immunopharmacol, 2014. **18**(2): p. 340-6.
168. Vigo, E., et al., *In-vitro anti-inflammatory effect of Eucalyptus globulus and Thymus vulgaris: nitric oxide inhibition in J774A.1 murine macrophages.* J Pharm Pharmacol, 2004. **56**(2): p. 257-63.
169. Marsik, P., et al., *In vitro inhibitory effects of thymol and quinones of Nigella sativa seeds on cyclooxygenase-1- and -2-catalyzed prostaglandin E2 biosyntheses.* Planta Med, 2005. **71**(8): p. 739-42.
170. Liang, D., et al., *Thymol inhibits LPS-stimulated inflammatory response via down-regulation of NF-kappaB and MAPK signaling pathways in mouse mammary epithelial cells.* Inflammation, 2014. **37**(1): p. 214-22.
171. Yalcin, H., et al., *Gas chromatography/mass spectrometry analysis of Laurus nobilis essential oil composition of northern Cyprus.* J Med Food, 2007. **10**(4): p. 715-9.
172. Bastos, V.P., et al., *Inhaled 1,8-cineole reduces inflammatory parameters in airways of ovalbumin-challenged Guinea pigs.* Basic Clin Pharmacol Toxicol, 2011. **108**(1): p. 34-9.

173. Kennedy-Feitosa, E., et al., *Eucalyptol attenuates cigarette smoke-induced acute lung inflammation and oxidative stress in the mouse.* Pulm Pharmacol Ther, 2016. **41**: p. 11-18.
174. Juergens, U.R., et al., *Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial.* Respir Med, 2003. **97**(3): p. 250-6.
175. Kim, K.Y., H.S. Lee, and G.H. Seol, *Eucalyptol suppresses matrix metalloproteinase-9 expression through an extracellular signal-regulated kinase-dependent nuclear factor-kappa B pathway to exert anti-inflammatory effects in an acute lung inflammation model.* J Pharm Pharmacol, 2015. **67**(8): p. 1066-74.
176. Juergens, U.R., et al., *Inhibitory activity of 1,8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes.* Pulm Pharmacol Ther, 2004. **17**(5): p. 281-7.
177. Vazquez-Ramirez, R., et al., *Reversing gastric mucosal alterations during ethanol-induced chronic gastritis in rats by oral administration of Opuntia ficus-indica mucilage.* World J Gastroenterol, 2006. **12**(27): p. 4318-24.
178. Galati, E.M., et al., *Study on the increment of the production of gastric mucus in rats treated with Opuntia ficus indica (L.) Mill. cladodes.* J Ethnopharmacol, 2002. **83**(3): p. 229-33.
179. Wittschier, N., G. Faller, and A. Hensel, *Aqueous extracts and polysaccharides from liquorice roots (Glycyrrhiza glabra L.) inhibit adhesion of Helicobacter pylori to human gastric mucosa.* J Ethnopharmacol, 2009. **125**(2): p. 218-23.
180. Fentoglu, O. and F.Y. Bozkurt, *The Bi-Directional Relationship between Periodontal Disease and Hyperlipidemia.* Eur J Dent, 2008. **2**(2): p. 142-6.
181. Fentoglu, O., et al., *Short-term effects of periodontal therapy as an adjunct to anti-lipemic treatment.* Oral Dis, 2010. **16**(7): p. 648-54.
182. Losche, W., et al., *Lipoprotein-associated phospholipase A2 and plasma lipids in patients with destructive periodontal disease.* J Clin Periodontol, 2005. **32**(6): p. 640-4.
183. Armitage, G.C., *Periodontal diagnoses and classification of periodontal diseases.* Periodontol 2000, 2004. **34**: p. 9-21.
184. Reichling, J., et al., *Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties--an overview.* Forsch Komplementmed, 2009. **16**(2): p. 79-90.
185. Burt, S., *Essential oils: their antibacterial properties and potential applications in foods--a review.* Int J Food Microbiol, 2004. **94**(3): p. 223-53.
186. Ansari, M.N., et al., *Evaluation of bronchodialatory and antimicrobial activities of Otostegia fruticosa: A multi-mechanistic approach.* Saudi Pharm J, 2020. **28**(3): p. 281-289.
187. Gonzalez-Mas, M.C., et al., *Volatile Compounds in Citrus Essential Oils: A Comprehensive Review.* Front Plant Sci, 2019. **10**: p. 12.

188. Moghaddam, M. and L. Mehdizadeh, *Chemistry of Essential Oils and Factors Influencing Their Constituents.* Soft Chemistry and Food Fermentation, 2017. **3**: p. 379-419.
189. Swamy, M.K., M.S. Akhtar, and U.R. Sinniah, *Antimicrobial Properties of Plant Essential Oils against Human Pathogens and Their Mode of Action: An Updated Review.* Evid Based Complement Alternat Med, 2016. **2016**: p. 3012462.
190. Scorzoni, L., et al., *The use of standard methodology for determination of antifungal activity of natural products against medical yeasts Candida sp and Cryptococcus sp.* Brazilian Journal of Microbiology, 2007. **38**(3): p. 391-397.
191. Angioni, A., et al., *Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and flowers.* J Agric Food Chem, 2006. **54**(12): p. 4364-70.
192. Rizza, L., et al., *Caco-2 cell line as a model to evaluate mucoprotective proprieties.* Int J Pharm, 2012. **422**(1-2): p. 318-22.
193. Ligor, M., et al., *Comparative Gas Chromatographic–Mass Spectrometric Evaluation of Hop (Humulus lupulus L.) Essential Oils and Extracts Obtained Using Different Sample Preparation Methods.* Food Analytical Methods, 2013. **7**(7): p. 1433-1442.
194. Gavahian, M., et al., *Comparison of extraction parameters and extracted essential oils from Mentha piperita L. using hydrodistillation and steamdistillation.* International Food Research Journal, 2015. **22**(1): p. 283-288.
195. Babu, K.G.D. and V.K. Kaul, *Variation in essential oil composition of rose-scented geranium (Pelargonium sp.) distilled by different distillation techniques.* Flavour and Fragrance Journal, 2005. **20**(2): p. 222-231.
196. Rao, V. and D. Pandey, *Extraction of essential oil and its applications. Rourkela, Orissa (India): National Institute of Technology.* 2007.
197. Kabuba, J. and R. Huberts, *Steam Extraction of Essential Oils: Investigation of Process Parameters.* Canadian Journal of Chemical Engineering, 2009. **87**(6): p. 915-920.
198. Kononen, E. and H.P. Muller, *Microbiology of aggressive periodontitis.* Periodontol 2000, 2014. **65**(1): p. 46-78.
199. Mysak, J., et al., *Porphyromonas gingivalis: major periodontopathic pathogen overview.* J Immunol Res, 2014. **2014**: p. 476068.
200. van Essche, M., et al., *Bacterial antagonism against periodontopathogens.* J Periodontol, 2013. **84**(6): p. 801-11.

201. Fan, S., et al., *GC-MS Analysis of the Composition of the Essential Oil from Dendranthema indicum Var. Aromaticum Using Three Extraction Methods and Two Columns.* Molecules, 2018. **23**(3).
202. Schulz, K.F., et al., *CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials.* BMC Med, 2010. **8**: p. 18.
203. Ainamo, J. and I. Bay, *Problems and proposals for recording gingivitis and plaque.* Int Dent J, 1975. **25**(4): p. 229-35.
204. Tonetti, M.S., *The future of periodontology: new treatments for a new era.* J Int Acad Periodontol, 2002. **4**(3): p. 110-4.
205. Arbes, S.J., Jr., G.D. Slade, and J.D. Beck, *Association between extent of periodontal attachment loss and self-reported history of heart attack: an analysis of NHANES III data.* J Dent Res, 1999. **78**(12): p. 1777-82.
206. Sathian, B., et al., *Impact of COVID-19 on clinical trials and clinical research: A systematic review.* Nepal J Epidemiol, 2020. **10**(3): p. 878-887.
207. Clark, D.C., et al., *An empirically based system to estimate the effectiveness of caries-preventive agents. A comparison of the effectiveness estimates of APF gels and solutions, and fluoride varnishes.* Caries Res, 1985. **19**(1): p. 83-95.
208. Kamath, N.P., et al., *The effect of aloe vera and tea tree oil mouthwashes on the oral health of school children.* Eur Arch Paediatr Dent, 2020. **21**(1): p. 61-66.
209. Freires, I.A., et al., *Antibacterial Activity of Essential Oils and Their Isolated Constituents against Cariogenic Bacteria: A Systematic Review.* Molecules, 2015. **20**(4): p. 7329-58.
210. Ne, Y.G.S., et al., *Is nutritional intervention an improvement factor in the management of periodontitis? A systematic review.* Clin Nutr, 2020. **39**(9): p. 2639-2646.
211. Chapple, I.L., et al., *Adjunctive daily supplementation with encapsulated fruit, vegetable and berry juice powder concentrates and clinical periodontal outcomes: a double-blind RCT.* J Clin Periodontol, 2012. **39**(1): p. 62-72.
212. El-Sharkawy, H., et al., *Adjunctive treatment of chronic periodontitis with daily dietary supplementation with omega-3 Fatty acids and low-dose aspirin.* J Periodontol, 2010. **81**(11): p. 1635-43.
213. Garcia, M.N., et al., *One-year effects of vitamin D and calcium supplementation on chronic periodontitis.* J Periodontol, 2011. **82**(1): p. 25-32.
214. Leyva-Lopez, N., et al., *Essential Oils of Oregano: Biological Activity beyond Their Antimicrobial Properties.* Molecules, 2017. **22**(6).

215. Dagli, N., et al., *Essential oils, their therapeutic properties, and implication in dentistry: A review.* J Int Soc Prev Community Dent, 2015. **5**(5): p. 335-40.
216. Silva, G.L., et al., *Antioxidant, analgesic and anti-inflammatory effects of lavender essential oil.* An Acad Bras Cienc, 2015. **87**(2 Suppl): p. 1397-408.
217. Borges, R.S., et al., *Rosmarinus officinalis essential oil: A review of its phytochemistry, anti-inflammatory activity, and mechanisms of action involved.* J Ethnopharmacol, 2019. **229**: p. 29-45.
218. Capatina, L., et al., *Thymus vulgaris Essential Oil Protects Zebrafish against Cognitive Dysfunction by Regulating Cholinergic and Antioxidants Systems.* Antioxidants (Basel), 2020. **9**(11).
219. Anusha, D., et al., *Efficacy of a mouthwash containing essential oils and curcumin as an adjunct to nonsurgical periodontal therapy among rheumatoid arthritis patients with chronic periodontitis: A randomized controlled trial.* Indian J Dent Res, 2019. **30**(4): p. 506-511.
220. Marinelli, L., et al., *Carvacrol prodrugs as novel antimicrobial agents.* Eur J Med Chem, 2019. **178**: p. 515-529.
221. Miladi, H., et al., *Synergistic effect of eugenol, carvacrol, thymol, p-cymene and gamma-terpinene on inhibition of drug resistance and biofilm formation of oral bacteria.* Microb Pathog, 2017. **112**: p. 156-163.
222. Tardugno, R., et al., *Phytochemical composition and in vitro screening of the antimicrobial activity of essential oils on oral pathogenic bacteria.* Nat Prod Res, 2018. **32**(5): p. 544-551.
223. Amano, A., *Disruption of epithelial barrier and impairment of cellular function by Porphyromonas gingivalis.* Front Biosci, 2007. **12**: p. 3965-74.
224. Alexa, V.T., et al., *Synergistic/Antagonistic Potential of Natural Preparations Based on Essential Oils Against Streptococcus mutans from the Oral Cavity.* Molecules, 2019. **24**(22).
225. Lemes, R.S., et al., *Chemical composition and antibacterial activity of essential oils from Citrus aurantifolia leaves and fruit peel against oral pathogenic bacteria.* An Acad Bras Cienc, 2018. **90**(2): p. 1285-1292.
226. Aires, A., A.S. Barreto, and T. Semedo-Lemsaddek, *Antimicrobial Effects of Essential Oils on Oral Microbiota Biofilms: The Toothbrush In Vitro Model.* Antibiotics (Basel), 2020. **10**(1).
227. Koychev, S., et al., *Antimicrobial Effects of Mastic Extract Against Oral and Periodontal Pathogens.* J Periodontol, 2017. **88**(5): p. 511-517.
228. Pradebon Brondani, L., et al., *Evaluation of anti-enzyme properties of Origanum vulgare essential oil against oral Candida albicans.* J Mycol Med, 2018. **28**(1): p. 94-100.

229. Akkaoui, S., et al., *Chemical Composition, Antimicrobial activity, in Vitro Cytotoxicity and Leukotoxin Neutralization of Essential Oil from Origanum vulgare against Aggregatibacter actinomycetemcomitans.* Pathogens, 2020. **9**(3).
230. Manconi, M., et al., *Thymus essential oil extraction, characterization and incorporation in phospholipid vesicles for the antioxidant/antibacterial treatment of oral cavity diseases.* Colloids Surf B Biointerfaces, 2018. **171**: p. 115-122.
231. Fernandez-Rojas, B. and G. Gutierrez-Venegas, *Flavonoids exert multiple periodontic benefits including anti-inflammatory, periodontal ligament-supporting, and alveolar bone-preserving effects.* Life Sci, 2018. **209**: p. 435-454.
232. Kote, S., S. Kote, and L. Nagesh, *Effect of pomegranate juice on dental plaque microorganisms (streptococci and lactobacilli).* Anc Sci Life, 2011. **31**(2): p. 49-51.
233. Bhatia, M., et al., *Novel therapeutic approach for the treatment of periodontitis by curcumin.* J Clin Diagn Res, 2014. **8**(12): p. ZC65-9.
234. Asteriou, E., et al., *Curcumin for the Management of Periodontitis and Early ACPA-Positive Rheumatoid Arthritis: Killing Two Birds with One Stone.* Nutrients, 2018. **10**(7).
235. Grover, S., et al., *Effect of Subgingivally Delivered 10% Emblica officinalis Gel as an Adjunct to Scaling and Root Planing in the Treatment of Chronic Periodontitis - A Randomized Placebo-controlled Clinical Trial.* Phytother Res, 2016. **30**(6): p. 956-62.
236. Chava, V.K. and B.D. Vedula, *Thermo-reversible green tea catechin gel for local application in chronic periodontitis: a 4-week clinical trial.* J Periodontol, 2013. **84**(9): p. 1290-6.
237. Hrishi, T.S., et al., *Effect of adjunctive use of green tea dentifrice in periodontitis patients - A Randomized Controlled Pilot Study.* Int J Dent Hyg, 2016. **14**(3): p. 178-83.
238. Bailly, C., *The implication of the PD-1/PD-L1 checkpoint in chronic periodontitis suggests novel therapeutic opportunities with natural products.* Jpn Dent Sci Rev, 2020. **56**(1): p. 90-96.
239. Freires, I.A., et al., *The alveolar bone protective effects of natural products: A systematic review.* Arch Oral Biol, 2018. **87**: p. 196-203.
240. Mittal, A., et al., *The effect of various ultrasonic and hand instruments on the root surfaces of human single rooted teeth: A Planimetric and Profilometric study.* J Indian Soc Periodontol, 2014. **18**(6): p. 710-7.
241. Dahiya, P. and R. Kamal, *Rotary instruments in the treatment of chronic periodontitis: A randomized clinical trial.* J Indian Soc Periodontol, 2013. **17**(6): p. 748-52.
242. Iauk, L., et al., *Antibacterial, antioxidant and hypoglycaemic effects of Thymus capitatus (L.) Hoffmanns. et Link leaves' fractions.* J Enzyme Inhib Med Chem, 2015. **30**(3): p. 360-5.

243. Khumaedi, A.I., et al., *The relationship of diabetes, periodontitis and cardiovascular disease.* Diabetes Metab Syndr, 2019. **13**(2): p. 1675-1678.
244. Larvin, H., et al., *Risk of incident cardiovascular disease in people with periodontal disease: A systematic review and meta-analysis.* Clin Exp Dent Res, 2021. **7**(1): p. 109-122.
245. Sanz, M., et al., *Periodontitis and cardiovascular diseases: Consensus report.* J Clin Periodontol, 2020. **47**(3): p. 268-288.
246. Del Pinto, R., et al., *Periodontitis and Hypertension: Is the Association Causal?* High Blood Press Cardiovasc Prev, 2020. **27**(4): p. 281-289.
247. Orlandi, M., F. Graziani, and F. D'Aiuto, *Periodontal therapy and cardiovascular risk.* Periodontol 2000, 2020. **83**(1): p. 107-124.
248. Priyamvara, A., et al., *Periodontal Inflammation and the Risk of Cardiovascular Disease.* Curr Atheroscler Rep, 2020. **22**(7): p. 28.
249. Xue, W., J. Yu, and W. Chen, *Plants and Their Bioactive Constituents in Mesenchymal Stem Cell-Based Periodontal Regeneration: A Novel Prospective.* Biomed Res Int, 2018. **2018**: p. 7571363.
250. Pedrazzi, V., et al., *Herbal mouthwash containing extracts of Baccharis dracunculifolia as agent for the control of biofilm: clinical evaluation in humans.* ScientificWorldJournal, 2015. **2015**: p. 712683.
251. San Miguel, S.M., et al., *Use of antioxidants in oral healthcare.* Compend Contin Educ Dent, 2011. **32**(9): p. E156-9.
252. Krauss, A., *Why all randomised controlled trials produce biased results.* Ann Med, 2018. **50**(4): p. 312-322.
253. Liccardo, D., et al., *Periodontal Disease: A Risk Factor for Diabetes and Cardiovascular Disease.* Int J Mol Sci, 2019. **20**(6).
254. Bourgeois, D., et al., *Periodontal Pathogens as Risk Factors of Cardiovascular Diseases, Diabetes, Rheumatoid Arthritis, Cancer, and Chronic Obstructive Pulmonary Disease-Is There Cause for Consideration?* Microorganisms, 2019. **7**(10).
255. Minty, M., et al., *Oral microbiota-induced periodontitis: a new risk factor of metabolic diseases.* Rev Endocr Metab Disord, 2019. **20**(4): p. 449-459.