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EFFECTS OF A FLAVONOID-RICH EXTRACT OF CITRUS BERGAMIA ON AN EXPERIMENTAL MODEL OF PERIODONTITIS IN RATS

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1. ABSTRACT

Objective: aim of the study was to investigate the effects of a flavonoid-rich fraction of Bergamot juice (BJe) in rats affected by LPS-induced periodontitis.

Main methods: fourteen days after LPS injection, radiographic analyses of periodontal sites were performed and gingivomucosal tissues surrounding the mandibular first molar were harvested for histological, immunohistochemical and molecular analyses.

Results: LPS significantly promoted edema formation, tissue damage and increased neutrophil infiltration. NF-κB translocation as well as TNF-α and IL-1β expression were found to be up-regulated. Additionally, the increased myeloperoxidase activity was associated with up-regulation of adhesion molecules. Immunohistochemical analysis for nitrotyrosine and poly ADP-ribose displayed an intense staining in the gingivomucosal tissue. Oral administration of BJe for 14 consecutive days was found to reduce tissue injury and lower several markers of gingival inflammation including nuclear NF-κB translocation, cytokines expression, myeloperoxidase activity and the expression of some adhesion molecules such as ICAM and P-selectin. Moreover, both nitrosative stress and PARP positive staining were reduced after BJe treatment, while Bax expression was downregulated and Bcl-2 expression was upregulated.

Conclusion: BJe improves LPS-induced periodontitis in rats by reducing the typical markers of inflammation, thus suggesting its potential in the treatment of periodontal diseases.

2. REVIEW OF LITERATURE

2.1 Oxidative stress

Oxidative stress is a pathological condition caused by the alteration of the physiological balance between production and removal of free radicals (R-), unstable molecular species containing one or more unpaired valence electrons (Hayyan et al., 2016). The presence of an unpaired electron confers certain properties common to most radicals, like instability and high reactivity (Lobo et al., 2010). To reach a more stable electronic configuration, they can behave as both oxidants or reductants, either donating an electron to or accepting an electron from other molecular species (Cheeseman et al., 1993).

Free radicals can interact with biological components, major targets being lipids, proteins, and nucleic acids.

Whenever a free radical interacts with a non-radical species, the latter becomes a free radical, in a mechanism leading to a chain reaction that can be halted only when two radicals interact between themselves.

This domino effect can be responsible for severe cellular damage, even resulting in tissues and organs deterioration (Cestaro, 1994; Wiseman and Halliwell, 1996; Berliner and Heinecke, 1996).

Highly reactive molecules containing oxygen are defined as “Reactive Oxygen Species” (ROS); it is important to note that some non-radical species, (e.g. hydrogen peroxide) are included under the ROS heading. The most important reactive oxygen species include hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical (Young and Woodside, 2001; Ahsan et al., 2003; Lobo et al., 2010).

These highly reactive compounds can either origin from normal essential metabolic processes or from exogenous sources. In the human body, ROS usually derive from both enzymatic and non-enzymatic reactions, such as respiratory chain, uric acid metabolism (e.g. xanthine oxidase), cellular signaling (e.g. nitric oxide synthase), phagocytosis, neutrophils oxidative burst, and cytochrome P450 system (Bagchi and Puri, 1998).

ROS can additionally be generated externally from exposure to cigarette smoking, air pollutants, X-rays, ozone, and various chemical compounds. Endogenous production of ROS occurs mostly inside mitochondria because of cellular respiration, in which oxygen is used as the final electron acceptor to produce energy. Human cells possess endogenous defense systems against reactive species to protect structural and functional biomolecules (Yu, 1994).

In case of hyperproduction of reactive species, these defense systems cannot fully prevent the subsequent damage and a condition of oxidative stress arises (Burton and Jauniaux, 2011). Oxidative stress is responsible for premature ageing of both cells and tissues, and it is usually associated with the onset of important chronic diseases, like atherosclerosis, cancer, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, diabetes mellitus, inflammatory diseases, as well as psychological diseases and aging processes (Durackova, 2010; Halliwell and Gutteridge, 1990; Ames and Shigenaga, 1992; Cestaro, 1994; Chen et al., 1995; Stocker, 1999; Benzie, 2000).

However, several studies demonstrated how an adequate dietary intake of antioxidants can play a role in the prevention of cell ageing (Rice-Evans et al., 1995), cardiovascular diseases, (Fuhrman et al., 1995), diabetes, cancer (Thole et al., 2006), and neurodegenerative pathologies (Zamaria, 2004).

2.2 Antioxidants

Antioxidants are compounds that inhibit or delay oxidation of biomolecules. They can be classified into two major categories: natural and synthetic antioxidants. Natural antioxidants are either produced endogenously or supplemented by natural sources. Depending on their chemical and physical properties and their mechanism of action, they can be divided into enzymatic and non-enzymatic antioxidants (Pal, 2014). Enzymatic antioxidants are further subdivided into primary and secondary antioxidants. Primary antioxidants are mainly chain breakers, able to scavenge radical species by hydrogen donation, like catalase, superoxide dismutase, and glutathione peroxidase. Secondary antioxidants are singlet oxygen quenchers, peroxide decomposers, metal chelators, oxidative enzyme inhibitors or UV radiation absorbers (G6PDH, glutathione reductase, etc.) (Pisoschi and Pop, 2015). Non-enzymatic antioxidants comprehend different compounds, like vitamins, carotenoids, polyphenols, and other molecules. Polyphenols are a structural class of phytochemicals, mainly studied for their marked antioxidant activity. They include different subclasses, among which phenolic acids and flavonoids are the most important (Ratnam et al., 2006).

Among the non-enzymatic antioxidants, it is possible to include macromolecules like albumin, ceruloplasmin, and ferritin, and micro-molecules like ascorbic acid, glutathione, uric acid, tocopherols, and hormones (estrogens, angiotensin, melatonin).

In physiological conditions antioxidants are able to end the chain reaction started by free radicals, thus halting the damaging processes involving biomolecules. Therefore, after its oxidation, the antioxidant in its radical form must not be not able to interact with other molecules.

The antioxidant effect does not usually depend on concentration, since it can be exerted on low concentrations; on the other hand, some compounds can elicit an oxidant action, thus favoring the formation of free radical species (Shahidi e Naczk, 1995). An example of this dual behavior is vitamin C, that possesses an antioxidant action versus the hydrogen peroxide (Duarte and Lunec, 2005), but at the same time it is capable of reducing metal ions, thus producing free radicals via Fenton's reaction (Carr e Frei, 1999; Stohs e Bagchi, 1995).

I) $2\ Fe^{3+} + \text{ascorbate} \rightarrow 2\ Fe^{2+} + \text{dehydroascorbate}$

II) $2\ Fe_2^{+} + 2H_2O_2 \rightarrow 2Fe^{3+} + 2OH^{\cdot} + 2OH^{-}$

2.3 Dietary intake of antioxidants

A wide variety of natural compounds demonstrates biological activity, either benefic or toxic.

Epidemiologic studies remarked how an adequate intake of food of plant origin plays a role in the prevention of many chronic diseases, like cardiovascular, metabolic, neurodegenerative, and inflammatory disorders, and even tumors of lung, mouth, pharynx, esophagus, stomach, and colorectum (World Cancer Research Fund / American Institute for Cancer Research, 2007).

Specifically, antioxidants are present in a significant amount in fruits and vegetables; together with olive oil, legumes, unrefined cereals, and fish, they constitute the base for Mediterranean diet, a nutritional model inspired by the eating habits of Crete, much of the rest of Greece, and southern Italy in the early 1960s (Willett et al., 1995). Mediterranean diet is associated with a lower risk of cardiovascular diseases, overall cancer incidence, neurodegenerative diseases, diabetes, and early death (Dinu M et al., 2017).

In animal model, it has been suggested that antioxidants (vitamin E in particular) can improve insulin resistance, a pathological condition determined by oxidative stress and recurring in diabetes type II. Moreover, low vitamin E serum levels can represent a risk factor for this widespread condition (Salonen et al., 1995).

As already stated above, the high consumption of fruits and vegetables may lower the risk of cancer, especially in the respiratory and alimentary tracts. A review on 200 epidemiological studies by Block et al. that examined the relationship between intake of fruits and vegetables and cancer of the lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas, and ovary found that the consumption of fruits and vegetables can offer a significant protection from cancer risk (Liu, 2004). Consumption of quercetin from onions and apples is inversely associated with lung cancer risk, with a particularly strong effect against squamous-cell carcinoma (Le Marchand et al., 2000). Increased quercetin plasma levels improve the DNA resistance to strand breakage and lower the amount of oxidative metabolites found in urine. (Boyle et al., 2000) Another study on a Netherlands population found that the risk of developing lung cancer is consistently reduced whenever fruits and vegetables are consumed together as a dietary habit (Voorrips et al., 2000).

Biomolecules found in fruits and vegetables can provide this effect through various means; proposed mechanisms include, inhibition of cell proliferation and differentiation, inhibition of oncogene expression, induction of tumor suppress gene expression, inhibition of cell adhesion and invasion, and prevention of DNA binding (Liu, 2004). Some antioxidants do not interrupt the chain reaction started by free radicals, but instead can delay the oxidant effect of some molecular species, like β-carotene and α-tocopherol. These compounds are able to interfere with LDL oxidation processes. Copper-induced LDL oxidation is characterized by three phases: *latency* (“lag phase” or “induction phase”), during

which eventual endogenous antioxidants are consumed; *propagation*, in which the polyunsaturated fatty acids associated with LDL are oxidized; *decomposition*, during which oxidized products are decomposed. While not able to arrest the propagation phase, β-carotene and α-tocopherol can prolong latency phase, thus delaying LDL oxidation (Schnitzer, 1995; Scoccia, 2001). Most important dietary antioxidants are polyphenols, carotenoids and vitamins (Liu, 2004).

2.4 Polyphenols

Polyphenols are the widest group of phytochemicals. It is difficult to identify polyphenols from a chemical point of view; in 2011, Quideau et al. defined them as compounds "*derived from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression*" (Quideau et al., 2011).

However, this definition may not fully reflect their high heterogeneity (more than 8000 polyphenols have been identified to date); therefore, it is possible to define them simply as a group of natural compounds with phenolic structural features (Tsao 2010).

They are commonly found in the fruits, vegetables, cereals and beverages. In plants, polyphenols usually represent secondary metabolites involved in defense against ultraviolet radiation or aggression by pathogens (Pandey, 2009; Beckman, 2000).

Cranberries, apples, strawberries, red or purple grapes, pineapples, bananas, peaches, lemons, oranges, pears, and grapefruit are considered high polyphenols fruits (Sun et al., 2002). Among vegetables, it is possible to list as main sources spinaches, onions, peppers, carrots, cabbages, potatoes, lettuce, celery, and cucumbers (Chu et al., 2002).

Polyphenols can be classified by their source of origin, chemical structure, or biological activity. According to the chemical structure of aglycones, it is possible to divide them in:

- Phenolic acids (benzoic acid derivatives, cinnamic acid derivatives), non-flavonoid polyphenolic compounds;
- Flavonoids (flavones, flavonols, flavanones, flavanonols, flavanols, proanthocyanidins, anthocyanidins, isoflavones, neoflavonoids, chalcones), that represent more than half of the totality of polyphenols;
- Polyphenolic amides (capsaicinoids, avenanthramides), that possess N-containing functional substituents (Davis et al., 2007; Bratt et al., 2003);
- Other polyphenols (lignans, resveratrol, etc.).

Biological activity of is strictly defined by the chemical arrangement and the types of functional groups contained inside the molecular structure (Carratu e Sanzini, 2005).

Carotenoids are natural liposoluble isoprenoid pigments, commonly found in every photosynthetic organism. In humans and animals, which are uncapable of synthetizing carotenoids, they can still be detected in blood and tissues (Fiedor and Burda, 2014). Carotenoids are precursors of vitamin A (retinol), and act proficiently as physical and chemical quenchers of singlet oxygen and scavengers of other reactive oxygen species. Carotenoids can be classified as xanthophylls (lutein, zeaxanthin, etc.), containing oxygen, and carotens (β-carotene, lycopene, etc.), not containing oxygen. Carrots, potatoes, papayas, mangoes, and melons are labeled as carotenoid-rich foods.

2.5 Flavonoids

Flavonoids are secondary metabolites of plants that constitute the larger subcategory of polyphenols. They are characterized by a benzo-γ-pyrone structure linked to a phenyl substituent (Fig. 1).

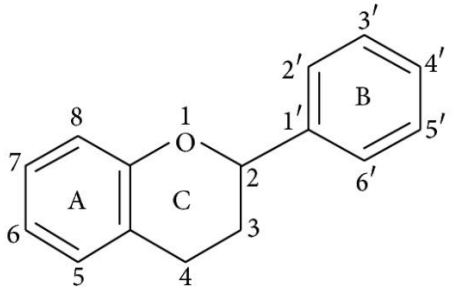


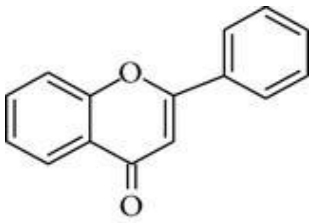
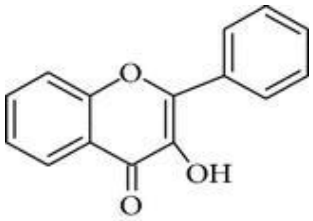
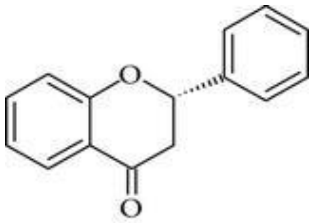
Fig.1. Basic flavonoid structure (Kumar et al., 2013)

Basic flavonoid structure is made of a fifteen-carbon skeleton presenting two benzene rings (A and B) linked via a γ-pyrone (C). Differences in the level of oxidation and pattern of substitution of the C ring define flavonoids classes, while specific patterns of substitution of the A and B rings identify individual compounds within a class (Middleton, 1998; Kumar and Pandey, 2013).

Flavonoids can be classified as:

- flavones (e.g. flavone, apigenin, and luteolin);
- flavonols (e.g. quercetin, kaempferol, myricetin, and fisetin);
- flavanones (e.g. flavanone, hesperetin, and naringenin);

- others (e.g. proanthocyanidins, anthocyanidins, isoflavones, neoflavonoids, chalcones) (Tab. 1).

Class	Basic structure
Flavones	
Flavonolos	
Flavanones	

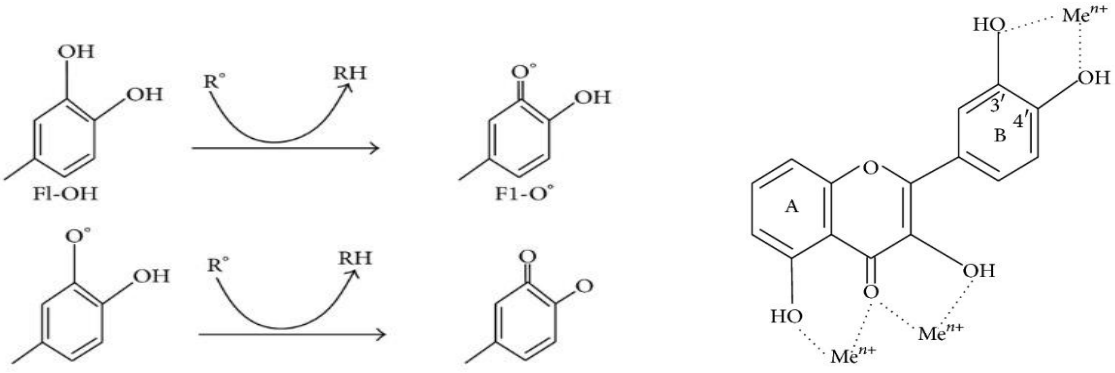
Tab.1. Class structural patterns

In plants, flavonoids are present as glycones, usually conjugated to glucose, or even arabinose, galactose, glucuronic acid, rhamnose, and xylose. Eventually, an aglycone can be linked to multiple saccharides (Williams et al., 1994). In plants, flavonoids play a role in the response to oxidative stress caused by both biotic and abiotic stimuli, and act as growth

regulators stimulating the production of auxin (Agati et al, 2012; Kumar and Pandey, 2013). Therefore, they help mitigating the damage caused by UVA and UVB exposure, and represent a defense mechanism in response to bacterial infection, since the most lipophilic flavonoids are able to interact with the membranes of the bacteria thus causing their lysis (Cowan, 1999, Mishra et al., 2009).

In human, flavonoids cannot be synthetized, therefore need to be absorbed from exogenous sources in nutrition, like fruits and vegetables. With the exception of catechins, they are usually present in the diet as β-glycosides. Since glycosides are considered too hydrophilic to be effectively absorbed by the intestinal mucosa, they need to be broken down through hydrolyzation into aglycones and sugar chains (Hollman, 2004).

Flavonoids present a remarkable biological activity and are consequently considered among the most useful compounds in the prevention and treatment of several diseases. As antioxidants, they behave as "radical scavengers", inhibiting the formation of both reactive oxygen species and reactive nitrogen species (RNS), and "metal chelators", removing metals from enzyme metabolism and thus lowering oxidative stress (Heim et al., 2002) (Fig.2).



(A) (B)

Fig.2. Radical scavenging (A) and metal chelation (B)

Their chelating properties greatly enhance their protective effect in inflammatory, cardiovascular and neurodegenerative disorders. Some flavonoids show hepatoprotective effects, like silymarin, a natural compound derived from Silybum marianum. Silymarin contains taxifolin and different flavolignans, the most important being silybin, silydianin, and silychristine. It stimulates RNA- and DNA-polymerases, thus promoting cellular reparation, stabilizes hepatocytes membrane, prevents the entrance of toxic metabolites, and shows antiviral activity (Vargas-Mendoza et al., 2014; Karimi et al., 2011). Anti-inflammatory activity of flavonoids is due to interaction with specific kinase receptor and inhibition of prostaglandin synthesis and release (Manthey et al., 2000).

Several studies pointed how *Citrus spp*. downregulate inflammatory response inhibiting the synthesis of prostaglandin E2, prostaglandin F2, and thromboxane A2 (Benavente-García et al., 2008). Naringin, naringenin, and hesperidin, three of the most important and well-represented *Citrus* flavonoids (Tripoli et al., 2007), limit the ROS production by granulocytes downregulating caspase-3 (Zielinska-Przyjemska and Ignatowicz, 2008). Naringenin and hesperidin reduce TNF-α levels in LPS/IFN-gamma stimulated glial cells. Moreover, naringenin inhibits LPS/IFN-gamma-induced inducible nitric oxide synthase (iNOS) expression in glial cells, protects neuronal cells from inflammatory-induced cell death, downregulates LPS/IFN-gamma-induced p38 mitogen-activated protein kinase (MAPK) phosphorylation and interferes with STAT-1 transcription factor in LPS/IFN-gamma stimulated primary mixed glial cells (Vafeiadou et al., 2009). *Citrus*' flavonoids can additionally behave as metal chelators, thus playing a role in the prevention of cancer development, much like curcumin (Ak and Gülcin, 2008; Miller et al., 2008; Batra and Sharma, 2013).

Aspects of potential antitumoral activity of flavonoid-rich foods include:

- Inhibition of protein kinase activity (Huang et al., 1999; Lee et al., 2004; So et al., 1996);
- Antiestrogenic activity (Singhal et al., 1995);
- Antimutagenic activity (Edenharder R et al., 1993; Miyazawa and Hisama, 2003);
- Free radicals scavenging (Sawa et al., 1999);
- p53 downregulation (Davis et al., 2000);
- Antiproliferative activity (Kandaswami et al., 2007);
- Inhibition of heat shock proteins (HSP) induction (Hosokawa et al., 1990; Davis et al., 2000);
- Interference with cell cycle progression and induction of apoptosis (Wang et al., 1999; Mahmoud et al., 1999; Shukla and Gupta, 2004).

Finally, some flavonoids show antiviral activity, lowering viral infectivity and/or intracellular replication (Zandi et al., 2011). More precisely, their antiviral activity was demonstrated toward herpes simplex virus type 1 (HSV-1), polio-virus type 1, parainfluenza virus type 3 (Pf-3), respiratory syncytial virus (RSV) (Kaul et al., 1985), and human immunodeficiency virus (HIV) (Wang et al., 1998). Over the past years, flavonoids have received much attention due to a variety of potential beneficial effects derived from their consumption and their potential positive impact on human health.

2.6 *Citrus bergamia* (bergamot)

Citrus bergamia Risso et Poiteau is a plant belonging to *Rutaceae* family, first mentioned in 1646 by Ferrari in his monography “Hesperides sive de malorum aureorum cultura et usu”, as an “aurantium stellatum et roseum” (Maruca et al., 2017). The term “bergamot”, with

whom *C. bergamia* is commonly addressed, has a disputed etymology, deriving either from Berga, a Spanish city, or from the Turkish locution bey *armudu* or *bey armut* ("prince's pear" or "prince of pears"). *C. bergamia* is a middle-sized tree, with large ovate leaves that resemble lemon's in shape and color, white star-shaped flowers and round and yellow fruits. From the outermost to the innermost, the fruit present three parts: *exocarp*, full of utricles containing essential oil; *mexocarp*, white and spongy; and *endocarp*, usually divided in 10 to 15 lodges containing fruit pulp, few seeds, and a sour and bitter juice (Fig.3).

Fig.3. *Citrus Bergamia* (Köhler 1897)

The botanical and geographical origins of *C. bergamia* is, to date, disputed (Rapisarda and Germanò, 2013). While *Citrus* species are most likely originated from the Southeast Asia area, before being spread globally (Calabrese, 1992), *C. bergamia* probably originated in Southern Italy as seedling (Chapot, 1962). It is a hybrid between *C. aurantium*, the sour orange and either *C. limon*, *C. aurantifolia*, or *C. medica*, respectively limon, lime, and citron (Rapisarda and Germanò, 2013; Li et al., 2010). *C. bergamia* trees are cultivated almost exclusively along the southern coast of the Calabria region (more than 90% of the world production of bergamot comes from this region). However, small numbers of bergamot plants grow in other countries, such as Greece, Morocco, Iran and Ivory Coast, Argentina, and Brazil.

In Southern Calabria there are three main cultivars of *C. bergamia*:

- *Femminello*: the most aromatic one, it is a fast-growing plant with spherical fruits and thin exocarp;
- *Castagnaro*: A broadleaf tree with a thick canopy, resistant to winds, that produces medium size globular fruit with a medium thickness exocarp containing average quantities of essential oils;
- *Fantastico*: despite being the last introduced, its cultivation is the most represented (circa 80% of total) because its pear-shaped fruits possess a thick exocarp full of essential oils with an excellent aroma (Maruca et al., 2017).

C. bergamia is usually not grown from seeds, because of different phytopaties that may affect the plant root system (Crispo e Dugo, 2003). The plant needs rich soil that holds moisture well (e.g. alluvial or argillic-calcareous), good sun exposition, and frequent watering, while does not well tolerate freezing and intense heat. These requirements justify the exclusive nature of bergamot cultivation sites, with the Southern Calabrian coast showing an ideal microclimate for the plant growth and cultivation. In 2001, the European

Communities granted Calabrian bergamot production the protected designation of origin (PDO) (Reg. CE n. 509/01, GUCE L.76 del 16/03/01).

Bergamot fruit is mainly used for its essential oils (bergamot essential oil: BEO), obtained by rasping and cold pressing the fruit peel, whose first uses can be dated back to 1686 when a Sicilian gentleman, Francis Procopius, introduced in France the so-called "bergamot water", an essence then used in most perfumes (Amato, 2005). Still today, BEO is widely used in perfumery, cosmetics, food, and confectionery industries for its intense fragrance and freshness (Mannucci et al., 2017). BEO is volatile, greenish or brownish-yellowish depending on the stage of the productive season, and possesses a bitter aromatic taste and a characteristic pleasant odor. Recently, further interest was added to BEO because of its neuroprotective and anti-cancer biological activities (Corasaniti et al., 2007; Celia et al., 2013; Navarra et al., 2015). It is included in various countries' official Pharmacopoeias. While the essential oil has managed to find several applications, bergamot juice has long time been considered just a waste of the BEO production, being less appealable than other *Citrus* juices due to its bitter taste. Despite its not so appreciated organoleptic properties and being used just to fortify other fruit juices in place of synthetic additives, it lately gained attention because of its hypolipemic and hypoglycaemic activity (Mollace et al., 2011), as well as its anti-inflammatory (Impellizzeri et al., 2015; Impellizzeri et al., 2016; Risitano et al., 2014), and anti-cancer properties (Delle Monache et al., 2013; Navarra et al., 2014; Visalli et al., 2014).

After fruit industrial processing, the remaining biomass, addressed as "*pastazzo*", is further used to feed farm animals. Branches and leaves, instead, can be harvested to extract a different kind of essential oil, "petit grain", mostly intended for aromatherapy (Hanneguelle et al., 1992, Setzer et al., 2009, Navarra et al., 2015).

2.7 Bergamot juice (BJe)

Thanks to the interest showed by the scientific community toward antioxidant compounds, fruit juices extracted from *Citrus spp*. drew considerable attention because of their biological properties. Therefore, bergamot juice changed his status of disposable waste product to that of flavonoid-rich compound possibly usable to increase the flavonoid quota in other *Citrus* juices (Pernice et al., 2009).

The most represented flavonoids in BJe are neoeriocitine, naringin, and neohesperidin. Furthermore, BJe contains five C-glucosides (lucenin-2, stellarin-2, isovitexin, scoparin, and orientin 4‘-methyl ether), four O-glycosides (rhoifolin 4‘-O-glucoside, chrysoeriol 7-O-neohesperidoside-4‘-O-glucoside, neodiosmin, and chrysoeriol 7-O-neohesperidoside), a flavanon-O-glycoside, eriocitrin (Caristi et al., 2006; Gattuso et al., 2006; Gattuso et al., 2007; Gidaro et al., 2015), oleic, linoleic, and palmitic acids, and linear furanocumarins (psoralens, like bergapten) shared by *Rutaceae* and *Apiaceae* (Eisenbrand, 2007) (Tab.2).

N°	Compounds	Bergamot juice
1	Apigenin-6,8-di-*C*-glucoside	n.a.*
2	Diosmetin-6,8-di-*C*-glucoside	n.a.*
3	Crisoeriol-7-*O*-neohesperidoside-4′-glucoside	n.a.*
4	Eriocitrin	85
5	Neoeriocitrin	115
6	Naringin	84
7	Neohesperidin	108

*n.a.: standard was not available in sufficient amount for quantitative calculation.

Tab.2. Concentration (mg/L) of flavonoids in bergamot juice (Delle Monache et al., 2013).

Recently, BJe has been demonstrated to decrease cell proliferation in different tumor cell lines.

In human hepatocellular carcinoma HepG2 cells, BJe induces apoptosis via both intrinsic and extrinsic pathways (Ferlazzo et al., 2016).

Inhibition of cell adhesion, migration and invasion may be responsible for the decreased metastatic pulmonary colonization observed in an experimental rat model producing spontaneous metastases (Navarra et al., 2014).

In human neuroblastoma SH-SY5Y cells, antiproliferative effect of BJE is not due to cytotoxicity or induction of apoptosis, as previously hypothesized. Instead, BJE freezes cell cycle in G1 phase, decreasing cell adhesion and consequently inhibiting cell invasion and migration. The concentration-dependent loss of adhesion in different physiological substrata seems to be linked to the damage of actin filaments and the decrease of the active form of focal adhesion kinases (FAK) (Delle Monache et al., 2013).

Since cytoskeleton reorganization, actin remodeling, and cell cycle progression represent key mechanisms of an aggressive phenotype in cancer cells, these findings suggest bergamot potential role as a supplement or an adjuvant to conventional therapies (Delle Monache et al., 2013).

Studies conducted on human colon cancer cells found that the antiproliferative activity of BJe is due to its flavonoid fraction, via concentration-dependent mechanisms (Visalli et al., 2014). The same research group also demonstrated the anti-inflammatory effect of the BJe flavonoid fraction in both in vitro (Risitano et al., 2014; Currò et al., 2016) and in vivo models (Impellizzeri et al., 2015; Impellizzeri et al., 2016).

Moreover, oral administration of bergamot juice lowered the seric levels of low-density lipoproteins (LDL) and triglycerides in rats fed on a high cholesterol diet, while increasing

high density lipoproteins (HDL). Supposed mechanism for this behavior is the BJe-mediated increase in fecal neutral sterols and total bile acids excretion (Miceli et al., 2007), but it remains still uncertain (Giglio et al., 2016).

Moreover, in similar experimental conditions, antioxidant activity of BJe exerted protective effects on the kidney, with an observed significant reduction of hypercholesterolemic diet-induced renal damage (Trovato et al. 2010).

BJe hypolipemic activity was further demonstrated with a study on 237 patients affected by metabolic syndrome. Besides significantly lowering plasmatic levels of cholesterol and triglycerides, it additionally showed hypoglycaemic activity (Mollace et al., 2011).

2.8 Periodontal disease

Periodontitis is a chronic multifactorial inflammatory disease that leads to the progressive destruction of the teeth supporting structures of the tooth. Over the years, periodontal disease has attracted much interest because of its high prevalence: in fact, it affects from 20 to 50 percent of the general population of developed and developing countries, thus representing a challenge for public health (Nazir, 2017).

Periodontitis may cause tooth loss, impairment of stomatognathic function, aesthetics, and ultimately worsen quality of life (Papapanou et al., 2018). Despite its constant association with a plaque “metabolic shift”, termed “dysbiosis”, bacteria alone cannot be accounted for periodontitis etiopathogenesis. While it is possible to identify specific pathogens in association with various forms of the disease, the very same microbes can also be detected in subjects with no evidence of periodontitis. Therefore, it is possible to affirm that the pathological mechanisms behind periodontal disease result from both plaque dysbiosis and

unbalance in the host inflammatory and both innate and acquired immune responses, ultimately failing to return to homeostasis (Cekici et al., 2014) (Fig.4).

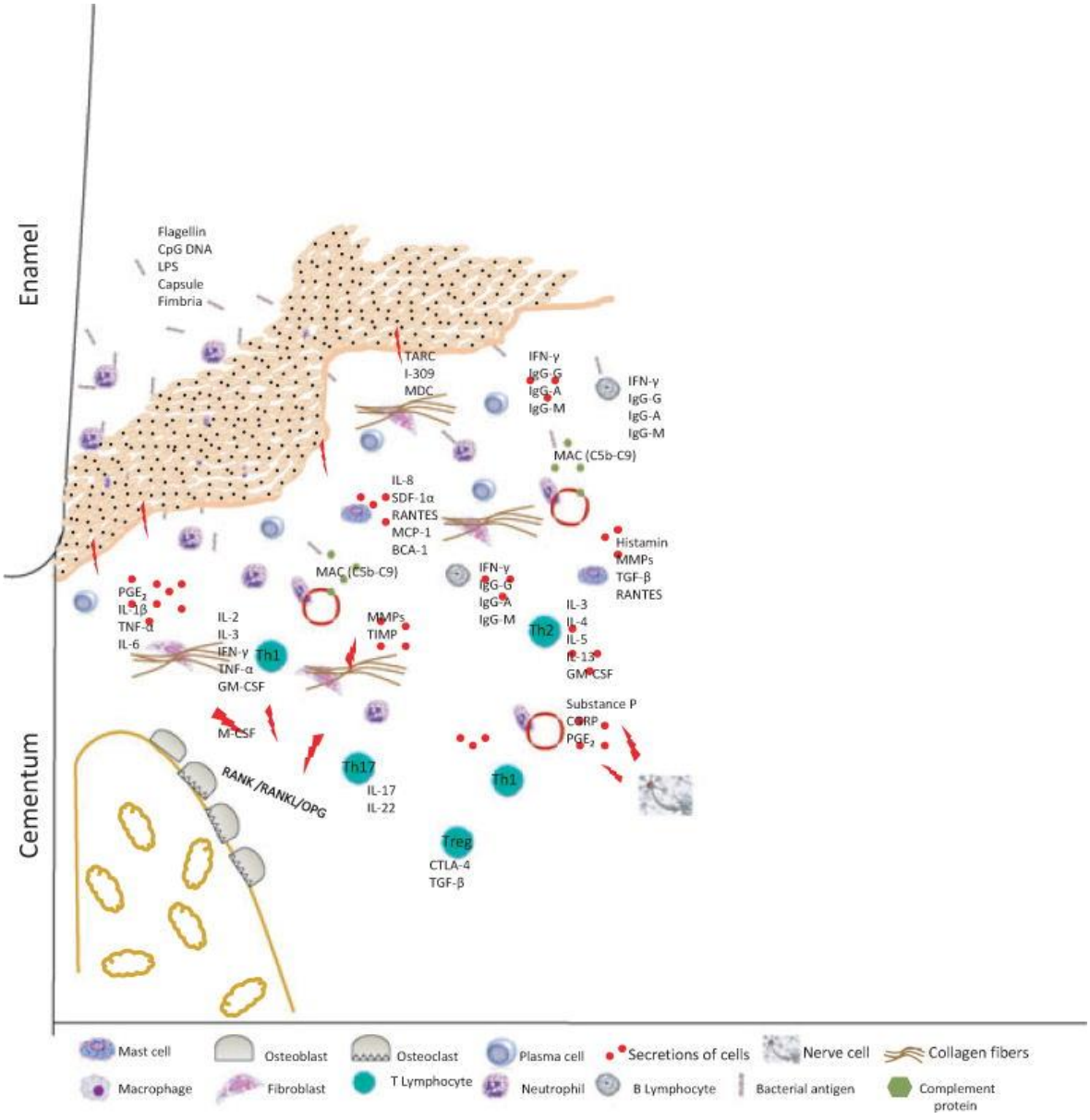


Fig.4. Overview of the effector molecules and effector cells in the pathogenesis of periodontitis (Cekici et al., 2014)

The host prominent role may account for periodontitis individual susceptibility, and it is possibly influenced by environmental factors (Seymour, 1991; Seymour and Gemmell, 2001; Uitto et al., 2003).

2.8.1 *Onset and staging*

Bacterial insults exert the host response, that starts with inflammatory cell recruitment in the subjacent to the periodontal pocket (Page and Schroeder, 1976). The resulting gingival inflammation, along with supra- and sub-gingival plaque formation, (Novak and Novak, 2006) when persisting, ultimately evolves into periodontal disease. Four stages can be identified in the pathogenesis of periodontitis (Page and Schroeder, 1976):

- the *initial lesion*, consisting of the response of resident leukocytes and endothelial cells to the bacterial biofilm. At this stage, there are no signs of clinical inflammation, but the microbial challenge promotes production of cytokines and neuropeptides, resulting in vasodilatation of local blood vessels. Neutrophils follow chemokines concentration gradients to reach the inflammation site, thus leaving the vessels.

- the *early lesion*, in which it is possible to detect signs of clinical inflammation, like gingival bleeding, and a raise in neutrophil quota, along with changes in the epithelium, and the appearance of macrophages, lymphocytes, plasma cells and mast cells.

- the *established lesion*, showing a worsening in the previous observed clinical and histological phenotype;

- the *advanced lesion*, also termed destructive phase, representing the transition from gingivitis to periodontitis, characterized by irreversible attachment and bone loss (Fiorellini et al., 2006).

2.9 Cells and mediators of periodontal inflammation

The host response first acts through the innate immune system. It includes hematopoietic and nonhematopoietic cells, and a complex of humoral mechanisms (Zadeh et al., 1999). This immediate line of defense is supported by specific signaling molecules, the cytokines, the chemokines, and the neuropeptides (Tracey, 2002), and triggers the inflammatory response; its purpose is to recruit competent cells to deal with the irritating stimuli. Whenever the "noxa patogena" is not removed, the chronic lesion emerges as early lesion, and innate immune response can induce the activation of a more complex and specific line of defense, the adaptive immune response. Innate immunity was formerly thought to be nonspecific and mostly acting through macrophages and neutrophils, cells able to phagocyte and digest foreign compounds and microorganisms (Medzhitov and Janeway, 1997; Janeway and Medzhitov, 2002). In fact, phagocytes like macrophages and neutrophil can discern between the host (self) and bacteria (non-self) thanks to surface receptors able to recognize recurring surface patterns of microbes (Zadeh et al., 1999; Rietschel and Brade, 1992). Among the pathogen recognition receptors, the toll-like receptors play a fundamental role and seemingly represent the link between innate and adaptive immunity (Pasare and Medzhitov, 2005; Werling and Jungi, 2003).

After non-self recognition, chemokines are secreted to attract phagocytes via concentration patterns. The complement system also participates attracting monocytes, lymphocytes and neutrophils, and directly killing certain microbes through the membrane attack complex, that leads to pore formation and membrane destruction. Phagocytes recruitment is further favored by vasodilatation induced by mast cells.

2.9.1 *Activation of complement*

The complement cascade activation classically follows three pathways:

- the *classical pathway*, promoted by immunoglobulins, either IgG or IgM;
- the *lectin pathway*, in which a mannose-binding lectin is employed to bind carbohydrate on the bacterial cell-surface to form mannose-associated serine protease-2;
- the *alternative pathway*, activated by bacterial polysaccharides, such as zymosan, lipopolysaccharide or aggregated IgA, through factor P (properdin).

Interestingly, even though pathogen-specific antibodies are formed in chronic periodontitis, majority of complement activation is due to the alternative pathway (Wingrove et al., 1992).

2.9.2 *Neuropeptides*

Peptide neurotransmitters – neuropeptides – can be secreted by neurons as an alternative to electric impulses into the extracellular fluid to interact with other neurons or immune cells. Most neuropeptides act on nonneuronal targets (Hoyle, 1996), implying a pivotal role in immunomodulation (Hartung et al., 1986; Lundy and Linden, 2004; McGillis et al., 1991). Additionally, over the last few years, a growing body of evidence is emerging showing how the inflammatory response in periodontitis is modulated by the nervous system. (Tracey, 2002). The link between immune and neurological system may be explained by the fact that, under certain circumstances, some neuropeptides are synthesized and released from inflammatory cells (Metwali et al, 1994; Hartung et al., 1986; McGillis et al., 1991).

The major involvement of neuropeptides in periodontitis is supported by their detection in gingival crevice fluid (Hanioka et al., 2000; Linden et al., 1997; Linden et al., 2002; Lundy et al., 1999; Lundy et al., 2000).

The main effects of neuropeptides in inflammation are vasodilatation, vasoconstriction and the recruitment and regulation of immune cells (Awawdeh et al., 2002; Byers and Taylor, 1993; Kvinnsland and Heyeraas, 1992). Three major neuropeptides have modulatory effects in periodontal inflammation: substance P, calcitonin gene-related peptide and vasoactive intestinal peptide.

2.9.3 *Toll-like receptors*

In mammals, toll-like receptors are a family of transmembrane proteins, characterized by a leucin-rich repeat (LRR) domain in their extracellular domain and a Toll/IL-1 receptor (TIR) domain in their intracellular domain (Takeda et al., 2003). TLRs represent the best-characterized class of PRR, and are expressed on a wide variety of cell types, like lymphocytes, osteoclast precursors, macrophages, osteoblasts, and stromal, epithelial and dendritic cells, each presenting a different toll-like receptor expression profile (Hayashi et al., 2003; Iwasaki and Medzhitov, 2004; Cekici et al., 2014) (Tab.3). TLRs can discern recurring molecular patterns shared by microorganisms, termed "pathogen-associated molecular patterns", from host components, like lipopolysaccharide, bacterial lipoproteins and lipoteichoic acids, flagellin, CpG DNA of bacteria and viruses, double-stranded RNA and single-stranded viral RNA (Iwasaki and Medzhitov, 2004). The interaction between a TLR and its PARP ligand leads to a signaling cascade which results in production of cytokines, chemokines and antimicrobial peptides (Kagnoff and Eckmann, 1997). Each type of TLR induces a specific response pathway, even with the same ligand: for example, when dendritic cells recognize LPS, TLR-4 promotes cytokines production (like IL-12), while TLR-3 induces type-I interferon production (Cekici et al., 2014). The most represented TLR in periodontal tissue during periodontitis are TLR-2 and TLR-4 (Hatakeyama et al., 2003).

Receptor	Location	Cells	Bacteria
Toll-like receptor 1	Cell membrane	Myeloid dendritic cells, monocytes	Not specified
Toll-like receptor 2	Cell membrane	Monocytes, natural killer cells, myeloid dendritic cells, mast cells, T-cells, epithelial cells	Porphyromonas gingivalis Escherichia coli Tannerella forsythia Prevotella intermedia Prevotella nigrescens Treponema denticola
Toll-like receptor 3	Intracellular	Myeloid dendritic cells, natural killer cells, epithelial cells	Not specified
Toll-like receptor 4	Cell membrane	Monocytes, mast cells, neutrophils, T cells, epithelial cells, endothelial cells	Aggregatibacter actinomycetemcomitans, Veillonella parvula
Toll-like receptor 5	Cell membrane	Monocytes, natural killer cells, myeloid dendritic cells epithelial cells	Not specified
Toll-like receptor 6	Cell membrane	Myeloid cells, mast cells, B-cells, myeloid dendritic cells	Escherichia coli
Toll-like receptor 7	Intracellular	Plasmacytoid dendritic cells, B-cells, eosinophils	Not specified
Toll-like receptor 8	Intracellular	Natural killer cells, T-cells, myeloid cells, myeloid dendritic cells	Not specified
Toll-like receptor 9	Intracellular	Plasmacytoid dendritic cells, B-cells, natural killer cells	Porphyromonas gingivalis Aggregatibacter actinomycetemcomitans
Toll-like receptor 10	Cell membrane	B-cells, plasmacytoid dendritic cells, myeloid dendritic cells	Not specified
Toll-like receptor 11	Intracellular	Macrophages, dendritic cells, epithelial cells	Not specified

Tab.3. The source cell, location and associated bacteria for toll-like receptors (Cekici et al., 2014)

2.9.4 *Antigen presentation and activation of acquired immunity*

If the early lesion does not regress, the host's innate immune cells process bacterial antigens to develop a specific and more efficient response to the pathogens. This process, defined as antigen presentation, ultimately leads to two different types of lymphocytes:

- T-lymphocytes, effectors of cell-mediated immunity (delayed hypersensitivity);
- B-lymphocytes, responsible for humoral immunity.

The T-cell antigen receptor is a membrane-bound immunoglobulin-like molecule which recognizes peptide fragments of pathogens. Standard classification of T-lymphocytes is based on the expression of specific cell-surface major histocompatibility complex molecules, namely CD4 and CD8. CD4+ T-cells (T-helper cells) are designed to support cell-mediated response, and are subdivided in two additional categories, T-helper 1, secreting the cell-response enhancers interferon and IL-2 and T-helper 2, which are responsible for production of IL-4, 5, 6, 10, and 13 (Murphy and Reiner, 2002). IL-4, their signature interleukin, acts suppressing cell-mediated response (Modlin and Nutman, 1993). Both T-helper categories produce interleukin-3, tumor necrosis factor alpha and granulocyte–macrophage colony-stimulating factor (Kelso, 1995; Zadeh et al., 1999). CD8+ T-cells are the cytotoxic T-cells and behave as immune effectors, while displaying the ability to secrete cytokines like T-helpers.

B-lymphocytes can differentiate into plasma cells, able to secret into the extracellular environment a soluble form of immunoglobulins, the antibodies, that bind to pathogens and foreign compounds as molecular markers, thus making them easily detectable as non-self by immune system, or directly compromise sensible structures of the pathogen.

In periodontal disease, plasma cells are the most represented cell type, about 50% of all cells, while B cells comprise about 18%. The proportion of B cells is larger than that of T cells, and T helpers surpass in numbers T cytotoxic cells (Berglundh and Donati, 2005).

2.9.5 *Cytokines and chemokine networks*

Cytokines are low-molecular-weight proteins with a specific effect on the interactions and communications between cells. They play a pivotal role in the regulation of immune response to infection and foreign stimuli, and are involved in the initiation and progression of inflammatory reactions, in which they modulate the amplitude and the duration of the response. The secretion of proinflammatory cytokines mostly depends on nuclear factor kappa-B transcription (Baldwin, 1996; Hanada and Yoshimura, 2002), which is activated by pathogen-associated molecular patterns, such as lipopolysaccharide, through the toll-like receptor pathway (Hanada and Yoshimura, 2002).

Timing in the lesion development identifies specific sources of cytokine production. Therefore, different cell types resident cells, such as epithelial cells and fibroblasts, and phagocytes (neutrophils and macrophages) secret cytokines in the acute and early chronic phases of inflammation, while immune cells (lymphocytes) produce them in established and advanced lesions (Ara et al., 2009). First to appear are cytokines of the innate response, including tumor necrosis factor alpha, interleukin-1beta and interleukin-6, are the in the periodontal disease pathogenesis pathways (Garlet, 2010). Interleukin-1beta and interleukin-6 are signature innate cytokines, typically associated with inflammatory cell migration and osteoclastogenesis (Fonseca et al., 2009; Graves et al., 2008). Tumor necrosis factor alpha is a cytokine that serves many purposes, from cell migration to tissue destruction. It favors cell migration by promoting production of chemokines and inducing the up-regulation of

adhesion molecules, thus determining rolling and adhesion of neutrophils to the vessel wall with subsequent extravasation (Dinarello, 2000; Kindle et al., 2006; Peschon et al., 1998; Wajant et al., 2003). It also enhances the production of interleukin-1beta and interleukin-6 (Dinarello, 2000; Graves et al., 2008, Wajant et al., 2003; Cekici et al., 2014) and the secretion of matrix metalloproteinases and RANKL, thus leading to extracellular matrix degradation and bone resorption (Graves et al., 2008; Cekici et al., 2014) and coupled bone formation (Behl et al., 2008). The dual key role of TNF-alpha in periodontitis pathogenesis and progression is further confirmed by an experimental model of periodontal disease in tumor necrosis factor alpha p55 receptor-deficient mice, in which the periodontal lesions resulted less severe, supposedly because of the heavily impaired host response, but at the same time infection was characterized by higher bacterial load and C-reactive protein levels (Garlet et al., 2007).

2.9.6 *Lipid mediators of inflammation*

Prostaglandins are derived from the hydrolysis of membrane phospholipids. Cyclo-oxygenase 1 and 2 catalyze the conversion of arachidonic acid into prostaglandins, prostacyclins and thromboxanes. Prostaglandins have 10 subclasses, of which D, E, F G, H and I are the most important (Gemmell et al., 1997). Prostaglandin E2 heavily promotes alveolar bone resorption (Dietrich et al., 1975; Goodson et al., 1974). Within gingival lesions, macrophage-like cells are mainly accounted for its secretion following LPS exposition (Loning et al., 1980). Periodontal ligament cells also produce prostaglandin E2, even when unstimulated, and interleukin-1beta, tumor necrosis factor alpha and parathyroid hormone may enhance this secretion (Cekici et al., 2014).

2.10 Destruction of periodontal tissues

2.10.1 *Destruction of bone*

Periodontitis-induced bone loss results from an unbalance in the homeostasis of bone remodeling processes. The bacterial insults and the inflammatory cytokines disturb the osteoblast-osteoclast equilibrium, resulting in inflammation-induced periodontal bone destruction (Liu et al., 2010). Lipopolysaccharide is considered an important mediator of bone loss in periodontitis, directly stimulating bone resorption when added to osteoclast precursor cultures containing osteoblasts and / or stromal cells (Cekici et al., 2014). In the course of periodontal disease, different pathways lead to osteoclastogenesis, with a combined action of both toll-like receptors and inflammatory mechanisms (The American Academy of Periodontology, 1999). During the inflammatory response, cell signaling effected through RANKL, interleukin-1beta, interleukin-6, tumor necrosis factor alpha and prostaglandin E2 promotes osteoclastic activity (Henderson et al., 2003).

RANK, its ligand (RANKL) and its antagonist (osteoprotegerin) are the main regulators of bone remodeling, modulating differentiation, activation and survival of osteoclasts and osteoclast precursors. RANKL is expressed by osteoblasts, stromal cells, activated T-cells and B-cells, and other mesenchymal cells (Cekici et al., 2014), while RANK is expressed by osteoclast progenitors, mature osteoclasts, chondrocytes, monocytes / macrophages and dendritic cells (Anderson et al., 1997). Its antagonist, a decoy receptor named osteoprotegerin, is expressed by periodontal tissue cells, including fibroblasts and periodontal ligament cells (Liu et al., 2010).

LPS action is dual: on one hand it triggers toll-like receptor 2 and toll-like receptor 6, enhancing expression of RANKL through a myeloid differentiation primary response protein (MyD88)-dependent mechanism (Sato et al., 2004), and toll- like receptors 2, 4 and 9,

promoting osteoclastic differentiation and activation (Hayashi et al., 2003); on the other hand, lipopolysaccharide well-known induction of IL-1 beta production promotes RANKL upregulation while suppressing osteoprotegerin expression, resulting in osteoclast formation in a prostaglandin E2-dependent manner (Suda et al., 2004).

2.10.2 *Destruction of extracellular matrix*

There is significant evidence showing that collagenases, along with other matrix metalloproteinases, play an important role in periodontal tissue destruction. Matrix metalloproteinases are a group of 23 enzymes that degrade extracellular matrix and basement membrane components, termed after their main substratum (e.g. collagenases, gelatinases, etc.). While structurally related, these enzymes are genetically distinct. Matrix metalloproteinases, especially collagenases, are considered among the main actors of periodontal damage caused by periodontitis. Normally involved in physiological processes such as tissue development, remodeling and wound healing, strong evidences demonstrate their up-regulation in periodontal disease (Uitto et al., 2003). Their activation is favored by tissue and plasma proteinases and bacterial proteinases, together with oxidative stress (van den Steen et al., 2002).

Several periodontal cells display matrix metalloproteinases, including gingival epithelial cells, fibroblasts, endothelial cells, monocytes / macrophages and plasma cells, shattering the old paradigm of their expression and pathologic release being specific to neutrophils (Uitto et al., 2003).

Matrix metalloproteinase basal expression is very low in healthy periodontal tissue. In periodontal disease, it is modulated by cytokines, the main stimulatory ones being tumor necrosis factor alpha, interleukin-1 and interleukin-6.

The major collagen-degrading enzyme in periodontitis is matrix metalloproteinase-8, which is mainly produced by neutrophils. It is found in gingival crevice fluid and saliva in diseased periodontal tissue. The main function of this enzyme is the degradation of interstitial collagen (Sorsa et al., 2006).

Cells of the inflamed pocket epithelium strongly express matrix metalloproteinase-2 (gelatinase A), involved in epithelial cell migration, and matrix metalloproteinase-13 (collagenase 3), that degrades type I, type III and type IV collagens, fibronectin, tenascin and some proteoglycans, and supports the growth of pocket epithelium into periodontal connective tissue (Cekici et al., 2014). Fusobacterium nucleatum, one of the most important bacteria of the dysbiotic periodontal plaque, induces expression and release of matrix metalloproteinase-13 (Uitto et al., 2003). Matrix metalloproteinase-7 (matrilysin) is another enzyme well-represented in periodontal lesions; it degrades fibronectin, laminin, type IV collagen, gelatin, elastin, entactin, tenascin and proteoglycans.

2.10.3 *Role of neutrophils*

Neutrophils are the most abundant of the leukocytes. Formerly considered simple suicide killers at the bottom of the hierarchy of the immune response (Mócsai et al., 2013), these myeloid-derived cells are now believed to represent one of the most complex tools available to the host. Neutrophils behave as efficient antimicrobial phagocytes, are able to kill pathogens extracellularly, favor host's return to homeostasis promoting tissue healing and inflammation resolution, and constitute a bridge between innate and adaptive immune responses. The neutrophil role is fundamental in human, to the point that a total absence of neutrophils or a significant decrease in their number leads to death or severe immunodeficiency, respectively (Kolaczkowska and Kubes, 2013).

In periodontal tissues, neutrophils are heavily represented and play a main role in host defense (Meng et al., 2007; Deas et al., 2003). Their behavior is peculiar, with histological evidence suggesting their assembling to form a "wall" to shield the junctional epithelium from the dysbiotic dental plaque (Schenkein, 2006; Ryder, 2010). This cellular wall is not assembled to act as a mere barrier function, but rather a coordinate apparatus with both secretory (reactive oxygen species [ROS] and bacteriocidal proteins) and phagocytic functions (Scott and Krauss, 2012).

The secretory component of neutrophils' activity can be defined as oxygen-dependent (the "respiratory or oxidative burst") and oxygen-independent (lytic and proteolytic enzymes) mechanisms.

However, efficiency comes to a cost: in fact, considerable observational, genetic and experimental data have assessed a clear association between neutrophil periodontal infiltration severity of periodontal diseases (Ryder, 2010; Scott and Krauss, 2012).

Three mechanisms can explain this association. The first two (Ryder, 2010) admit the presence of cells with an altered behavior, namely:

- the *impaired neutrophil*, defective cells, mostly on a genetic basis (even though environmental factors, like cigarette smoking, can exacerbate the impairment), that underperform in both phagocytosis and secretory mechanisms (Palmer et al., 2005);

- the *hyperactive neutrophil*, cells showing elevated function that overperform in their antimicrobial activities, especially respiratory burst.

A third mechanism can be set up alongside the first two, to better justify the pivotal role of neutrophils in periodontal disease development:

- *Chronic recruitment and activation of the normal neutrophil*, that progressively deteriorates because of its prolonged activation because of the chronic insult represented by periodontal bacteria (Scott and Krauss, 2012).

3. EXPERIMENTAL STUDY

3.1 Aim of the study

The present study was designed to investigate the anti-inflammatory effect of BJe on an animal model of LPS-induced periodontitis in rats, through radiological, histopathological and molecular analyses.

3.2 Materials and methods

3.2.1 *Materials*

Study materials were obtained, unless otherwise specified, from Sigma-Aldrich Company Ltd (Poole, Dorset, UK), and every stock solution was kept in non-pyrogenic saline solution (0.9% NaCl; Baxter Healthcare Ltd, UK).

3.2.2 *Drug*

The "Agrumaria Corleone" company (Palermo, Italy) provided the bergamot juice. Bergamot fruits (*C. bergamia*) harvested in the orchards of Reggio Calabria province (Italy) were processed: the juice was extracted, converted into dry powder via lyophilization, and stored at -20°C. This experimental model employed the same BJe already tested in other studies (16, 21-26). However, as an additional check, a qualitative and quantitative analysis of the BJe obtained for this study was performed to define the flavonoid quota; results from a high-performance liquid chromatography system (HPLC) reflected a chemical composition of BJe in accord with previous reports (Visalli et al., 2014; Ferlazzo et al., 2015; Risitano et al., 2014). The main flavonoids detected (in order of concentration expressed in

mg/g) were neohesperidin (96.24), naringin (93.73), melitidin (65.82), hesperetin (52.02), neoeriocitrin (51.80) and naringenin (39.7).

3.2.3 *Animals*

For this study, 40 Sprague-Dawley male rats (200–230 g) were obtained from Harlan Italy. Animals were housed in a facility with food and water ad libitum, while humidity, temperature, and day/night light cycle were constantly monitored and kept stable. The University of Messina Review Board approved the study for the care of animals. Experiments were performed according to both the new legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and ARRIVE guidelines.

3.2.4 *LPS-induced periodontitis*

Induction of periodontitis was accomplished reproducing the method of Gugliandolo et al. (Gugliandolo et al., 2018). Animals were anaesthetized with sodium pentobarbitone (35 mg/kg), and then periodontitis was induced by a single intragingival injection of 1 µl LPS (10 µg/µl) derived from Salmonella typhimurium (Sigma-Aldrich) in sterile saline solution. Designed injection site was the mesolateral side of the interdental papilla between the first and the second molar. It was performed slowly and the needle kept in place for some seconds after the injection to guarantee that LPS was not lost through needle extraction. Additionally, LPS injection was performed bilaterally to double investigation sites, totaling 20 samples per group and 5 samples per analysis. In order to control food intake and masticatory behavior, animals were constantly monitored and weighted at regular time intervals. Rats were sacrificed at 14 days after LPS injection to evaluate periodontitis lesions.

3.2.5 *Experimental groups*

Rats were randomly assigned to the following groups (n = 10 for each):

-LPS + saline group: rats underwent periodontitis induction by LPS as described above (N=10).

- LPS + BJe group: rats underwent periodontitis induction similarly to LPS + saline group and were administered BJe (20 mg/kg) by oral gavage every 24 h for 14 days, starting from 1 h after the injection of LPS (N=10).

- Sham + saline group: animals received a single intragingival injection of saline solution in the same fashion as LPS + saline group (N=10).

- Sham + BJe group: same as the Sham + saline group, with the addition of BJe (20 mg/kg) administrations by oral gavage every 24 h (1h after LPS-injection) for 14 days (N=10). The tested dose for BJe was chosen in agreement with previous findings (25, 26).

3.2.6 *Radiographic analysis*

Radiographic analyses of mandibles of rats belonging to the four experimental groups above were performed with an X-ray machine (Bruker MS FX Pro, Billerica, Massachusetts, USA). The X-ray tube was operated at 30 kW, with a current of 6 mA, an exposition time of 0.01s, and a source-to-sensor distance of 50 centimeters. Fourteen days after the LPS/saline inoculation, an evaluation of dental alveolar bone level, defined as the distance from the cemento-enamel junction (CEJ) to the maximum coronal level of the alveolar bone crest (CEJ–bone distance), was performed, using IMAGE J processing software (Image J software, National Institutes of Health, Bethesda, MD, USA) (Chiang et al., 2016).

3.2.7 *Measurement of Vascular Permeability*

Vascular permeability was assessed via Evans blue extravasation, as previously reported (Györfi et al., 1994). Briefly, Evans blue (2.5% dissolved in saline, at a dose of 50 mg/kg) was administered to rats through a femoral venous catheter. Gingivomucosal tissues were then embedded for 48 h at room temperature with 1 mL formamide to extract the extravasated Evans blue. Spectrophotometric determination was assessed at 620 nm and evaluated as μg/g gingivomucosal tissue.

3.2.8 *Histological examination*

Gingivo-mucosal tissues were surgically removed 14 days after LPS/saline injection. Sample fixation was performed with 10%(w/v) PBS-buffered formaldehyde solution at 25°C for 24 h, followed by dehydration by graded ethanol solutions and subsequent inclusion in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Hence, samples were deparaffinised with xylene and colored with hematoxylin and eosin. For each animal, five series of sections were produced, and then investigated using the Axiovision Zeiss (Milan, Italy) microscope. All the histological studies were executed in blinded fashion. A semiquantitative scale based on morphological criteria was used to determine the histological injury score for the collected samples, as described below:

- grade 0, normal gingivomucosal tissue (0–2+);
- grade 1, minimal edema or infiltration (0–2+);
- grade 2, moderate edema and inflammatory cell infiltration without obvious damage to gingivomucosal architecture (0–2+);
- grade 3, severe inflammatory cell infiltration with obvious damage to gingivomucosal architecture (0–2+).

In order to evaluate fibrosis degree, gingiva-mucosal sections were positioned parallel to the longitudinal axis of teeth and then stained with the Masson trichrome stain, according to the manufacturer's instructions (Bio-Optica, Milan, Italy).

3.2.9 *Measurement of cytokines*

Prior to the measurement of cytokines, gingivomucosal tissues were homogenized in 2 mmol/L of PBS containing phenylmethylsulfonyl fluoride (Sigma-Aldrich). Tissue levels of TNF-α and IL-1β were assessed using a colorimetric kit (Calbiochem-Novabiochem Corporation, USA), according to manufacturer's protocol. All cytokine determinations were carried out in duplicate serial dilutions. Results are showed as pg/100 g wet tissue.

3.2.10 *Immunohistochemical localization of nitrotyrosine, PAR, ICAM and P-selectin*

Fourteen days after the LPS/saline injection, samples were fixed in 10% (w/v) PBS-buffered formaldehyde and embedded in paraffin. Each paraffin block was then processed to produce 7 μm sections in which, after deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Slide permeabilization was achieved using 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was lowered by incubating sections in 2% (v/v) normal goat serum in PBS for 20 min. Sequential incubation was performed for 15 min with avidin and biotin to block endogenous avidin and biotin sites(Vector Laboratories, Burlingame, CA), respectively. Sections were incubated overnight with the different antibodies dissolved in PBS (v/v), either anti-nitrotyrosine (Merck Millipore, Milan, Italy; 06-284), or anti-PAR (Santa Cruz Biotechnology, H-250: sc-7150, 1:350), or anti-ICAM (Santa Cruz Biotechnology Inc., Dallas, Texas USA; C-20:

sc-8439, 1:460), or anti-P-selectin (Santa Cruz Biotechnology, G-5: sc-6941, 1:460). After this step, slides were washed with PBS, and incubated with secondary antibodies. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vector Laboratories). To further verify antibody-binding specificity, control slices were incubated with only primary antibody or secondary antibody, to whom no positive staining was recorded. Images were collected using a Zeiss microscope and Axio Vision software. Computer-assisted color image analysis (Leica QWin V3, UK) was used to quantify the positively-stained area, expressed as percentage of total area within five random fields at 40x magnification, thus providing graphic display of densitometric analyses. This method involved a first step in which the colors of the images that have been stained to the molecule of interest were defined. Once these colors were defined, the software automatically detected them in all samples, analyzing the stained area in relation to the total area. This semi-quantitative analysis is only able to measures areas, but not intensities (Rodríguez-Sanabria et al., 2010; Ferrè et al., 2006; Hernández-Aguilera et al., 2015).

3.2.11 *Western blot analysis for IκBα, NF-κB, Bax and Bcl-2*

Gingivo-mucosal samples collected from each rat were processed with suspension in extraction buffer A containing 0,15 µM pepstatin A, 0.2 mM PMSF, 1 mM sodium orthovanadate and 20 µM leupeptin, homogenization at the highest setting for 2 min, and centrifugation at 1000×g at 4 °C for 10 min. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, underwent re-suspension in buffer B containing 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 10 mM Tris–HCl pH 7.4, 0.2 mM PMSF, 1 mM EDTA, 0.2 mM sodium orthovanadate and 20 µm leupeptin. After centrifugation at 15.000 g and 4°C for 30 min, the nuclear protein contained in the supernatants were stored at -80°C

for further analysis. Cytosolic fraction was used to measure the levels of IκB, while nuclear fraction was utilized to quantify NF-kB p65 levels. Membranes were blocked with 1× PBS, 5% (w/v) nonfat dried milk at room temperature for 40 min, and subsequently probed with the following antibodies: specific anti-IκBα (Santa Cruz Biotechnology, C-21: sc-371, 1:550), or anti-Bax (Santa Cruz Biotechnology, P-19: sc-526, 1:530), or anti-Bcl-2 (Santa Cruz Biotechnology, N-19: sc-492, 1:510), or anti-NF-κB p65 (Santa Cruz Biotechnology, F-6: sc-8008, 1:400) dissolved in 1× PBS, 5% w/v dried milk, 0.1% Tween-20 (PMT) at 4 °C overnight. Then, membrane incubation was performed with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA, US) for 1 h at room temperature. The blots were also probed with primary antibody against β-actin protein or laminin (both 1:10.000; Sigma-Aldrich), employed as internal standards for cytosolic or nuclear fraction, respectively. The relative expression of the protein bands of IκB-α (37 kDa), Bax (23 kDa), Bcl-2 (29kDa) and NF-κB p65 (65 kDa) was quantified by densitometric scanning of the X-ray films (GS-700 Imaging Densitometer, Bio-Rad Laboratories, Milan, Italy), by densitometric analysis (Molecular Analyst, IBM), and standardized for β-actin or laminin levels.

3.2.12 *Statistical Evaluation*

Experimental data, reported in both text and figures, are expressed as mean ± standard error of the mean (S.E.M.) of the number of employed animals (N). In case of histology or immunohistochemistry, the photos are representative of at least three different experiments. Results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 *Effects of BJe administration on bone destruction induced by LPS in gingival tissues*

As shown in Fig. 5 the CEJ-bone radiographic distance was significantly larger in the LPS-treated rats (Fig. 5B and 5D) than in the sham-group animals (Fig. 5A and 5D), whereas the CEJ–bone distance in BJe-treated animals was significantly shorter than in LPS + saline group (Fig. 5C and 5D).

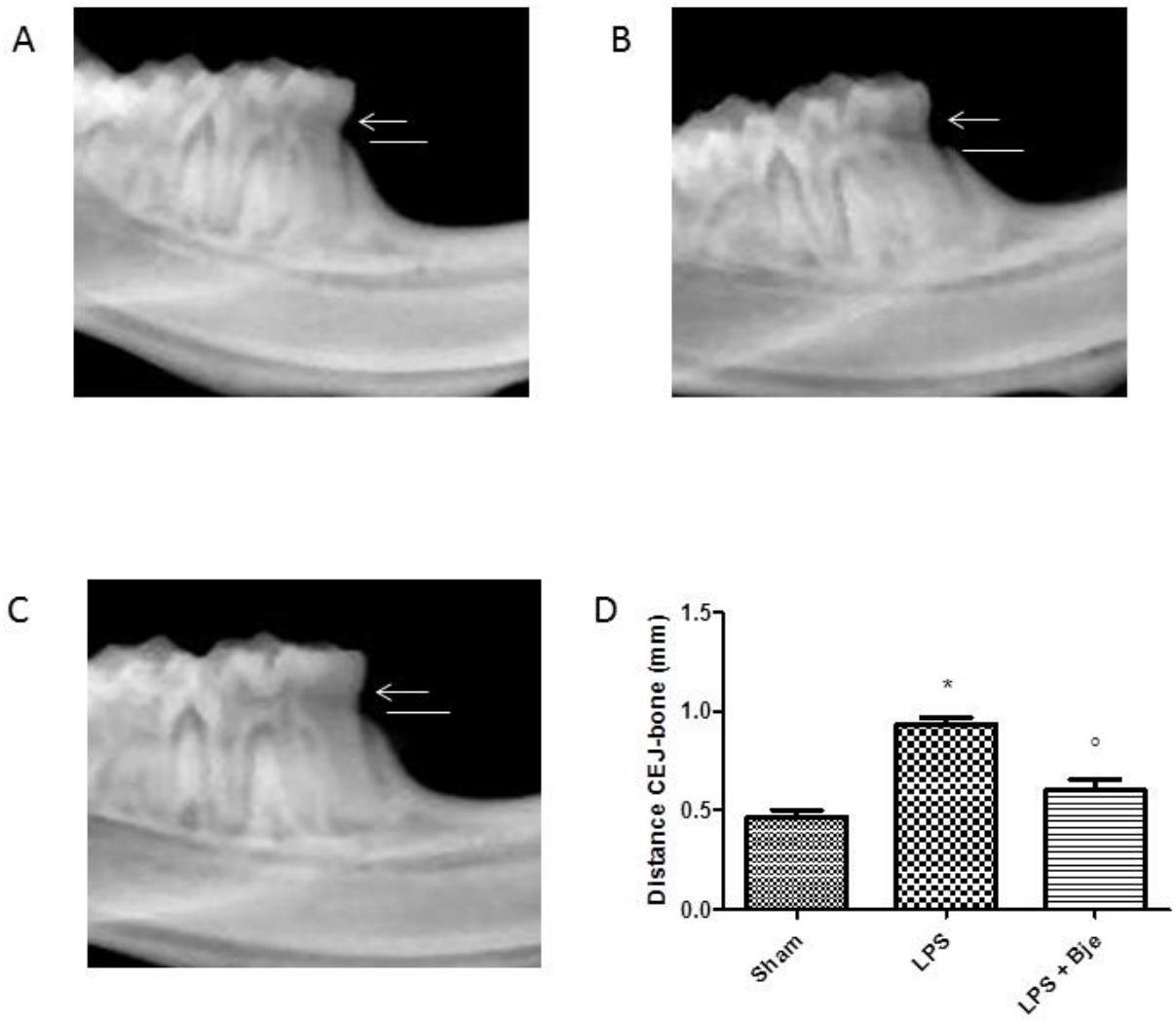


Fig. 5. Radiological analysis after 14 days from the LPS administration. LPS-injected rats (B and D) showed a bigger CEJ-bone distance when compared to sham group rats (A and D), while BJe-treated animals showed a shorter distance (C and D). Scale bar 1.5 mm. Values reported in the histogram are expressed as mean ± SEM of 10 rats for each group. ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.3.2 *Histological examination indicates that BJe treatment reduces LPS-induced periodontitis*

Histological examination of gingivo-mucosal tissues harvested from sham and LPS groups are shown in Fig. 6A and 6B, respectively. Samples belonging to LPS-injected animals (Fig. 6B) showed a significant increase in edema and tissue damage (Fig. 6G) when compared to those of sham + saline group (Fig. 6A), while a significant decrease of these indexes was observed after BJe administration (Fig. 6C, see histological score in Fig. 6G). Similarly, Masson's trichrome stain highlighted an increase of collagen formation in LPS-injected rats (Fig. 6E) when compared to tissue samples collected from sham rats (Fig. 6D). The increase of collagen was less pronounced in animals treated with BJe (Fig. 6F).

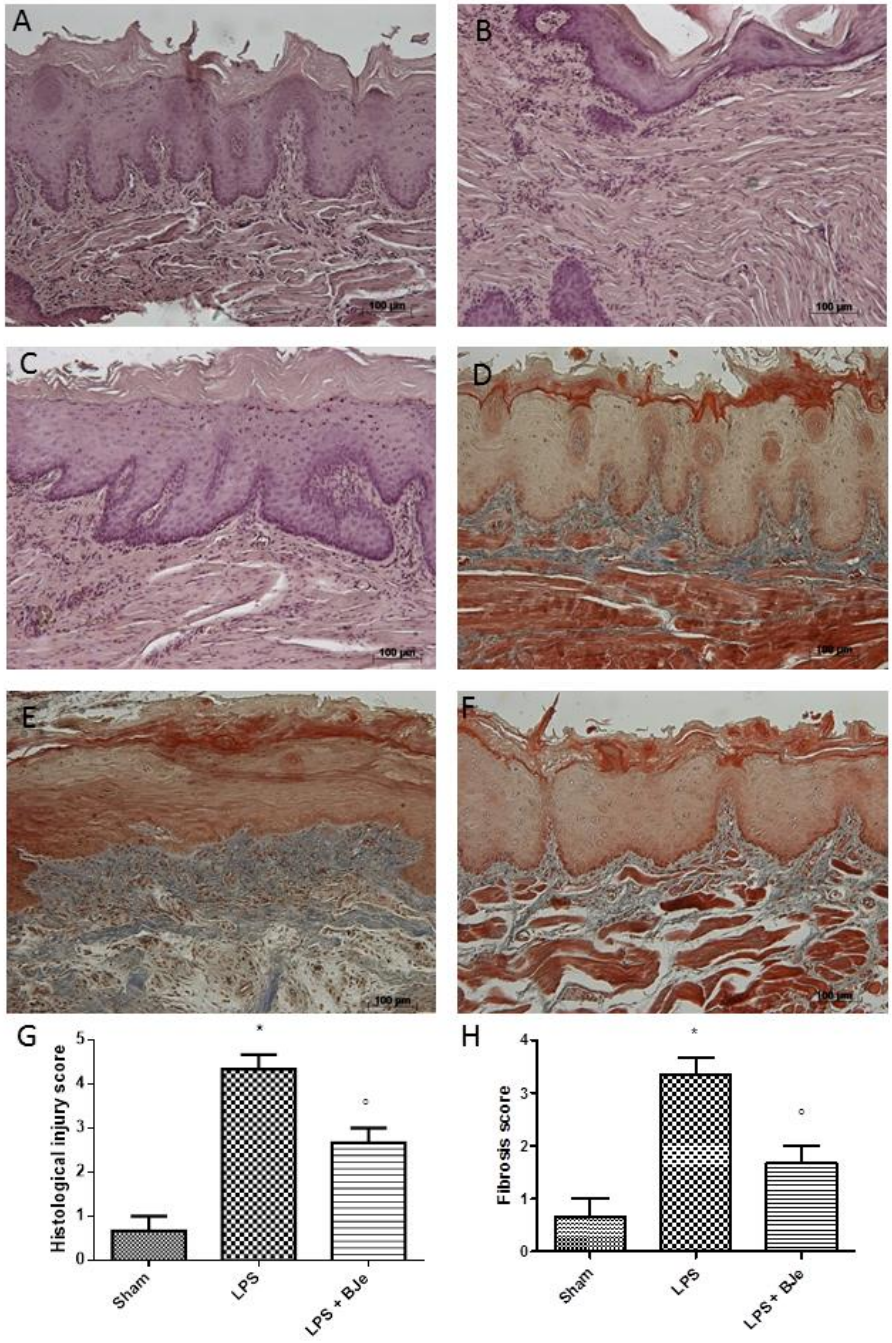


Fig. 6. Histopathological analysis. Samples from LPS-injected rats showed edema, tissue injury and inflammatory cells infiltration (B and G) when compared to the rats of sham-group (A and G). BJe treatment significantly reduced the inflammatory picture (C and G). Masson's trichrome stain highlighted an increased concentration of collagen fibers in gingivomucosal tissues in vehicle group (E and H) when compared to sham group (D and H). BJe treatment significantly lowered collagen formation (F and H). Values are expressed as mean ± SEM (N =10 rats in each group). ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.3.3 *BJe administration decreases IkB-α and NF-kB activation induced by LPS in gingivomucosal tissues*

In order to comprehend the molecular mechanisms involved in BJe effects on LPS-induced periodontitis, the involvement of IκB-α and NF-κB was first analyzed. IκB-α acts as an inhibitor of NF-κB. IκB-α degradation leads to the nuclear translocation of p65/p50 subunits of NF-κB and its consequential activation. Therefore, basal expression of IκB-α was detected in gingiva-mucosal tissues from rats of sham-group; in comparison, samples from animals belonging to LPS-group showed a marked reduction of IκB-α (Fig. 7A and 7C), resulting in an increased LPS-induced NF-κB activation in the nuclear fractions of rats belonging to LPS-group (Fig. 7B and 7D). BJe treatment prevents LPS-induced IκB-α degradation (Fig. 7A and 7C), inhibiting NF-κB activation and consequently reducing NF-κB levels in the nucleus (Fig. 7B and 7D) in a statistically significant way. To underline the purity and quality of subcellular fractionation of the proteins, we included actin and laminin as internal standards for cytosolic (Fig. 7A) or nuclear (Fig. 7B) fraction, respectively.

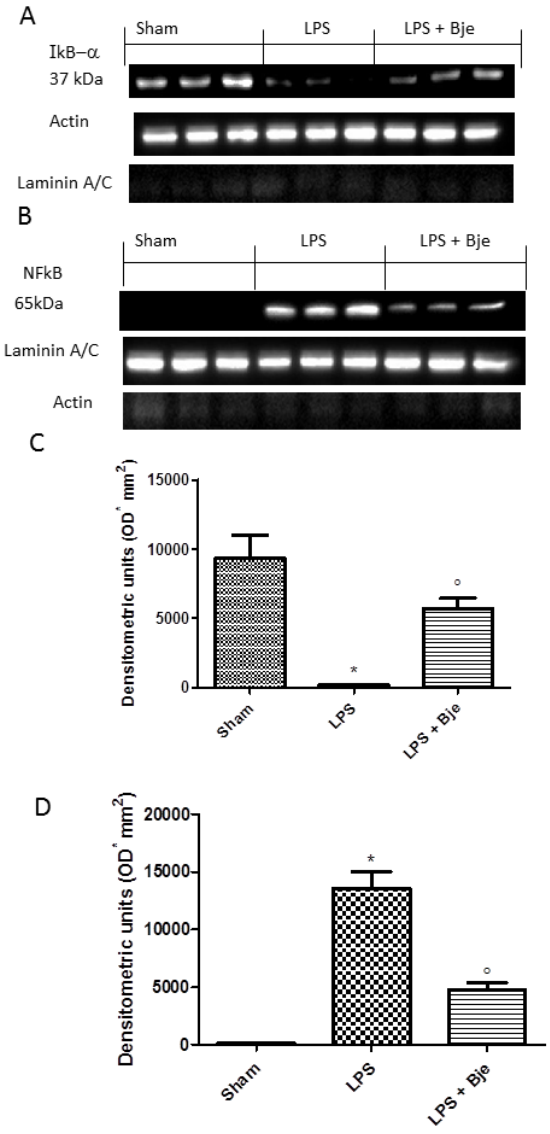


Fig. 7. IκB-α expression (A and C) and NF-κB translocation in the nucleus (B and D). Blot in A and its densitometric analysis in C showed BJe-treatment significantly decreasing IκB-α degradation (A and C). Blot B displayed that LPS injection increased NF-κB translocation in the nucleus (B and D), whereas BJe significantly reduced the presence of NF-κB in the nuclear fraction (B and D). Levels of IκB-α and NF-κB presented in the densitometric analyses of protein bands were normalized for β-actin and laminin, respectively. We also included β-actin and laminin as internal standards for cytosolic or nuclear fraction, respectively. Blots in A and B are representative of 3 different gels. Data in C and D are means ± SEM of 10 rats for each group. Data reported in C and D are presented as mean ± SEM (N =10 rats for each group). ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.3.4 *BJe reduces TNF-α and IL-1β generation, MPO activity and plasma extravasation LPS-induced*

TNF-α and IL-1β sample levels after 14 days from LPS/saline injection were evaluated. In LPS group a significant increase in these cytokines was detected (Fig. 8A and 8B), while, in contrast, a significant reduction was found in the tissues of animals from the LPS + BJe group (Fig. 8A and 8B). Moreover, in the LPS-group, myeloperoxidase (MPO) activity was significantly raised in comparison to that detected in the samples from rats of sham-group (Fig. 8C). BJe treatment appeared to significantly reduce MPO activity (Fig. 8C). Furthermore, LPS injection significantly increased Evans blue extravasation in comparison to sham-group animals (Fig. 8D), while treatment with BJe administration seemingly limited this behavior (Fig. 8D).

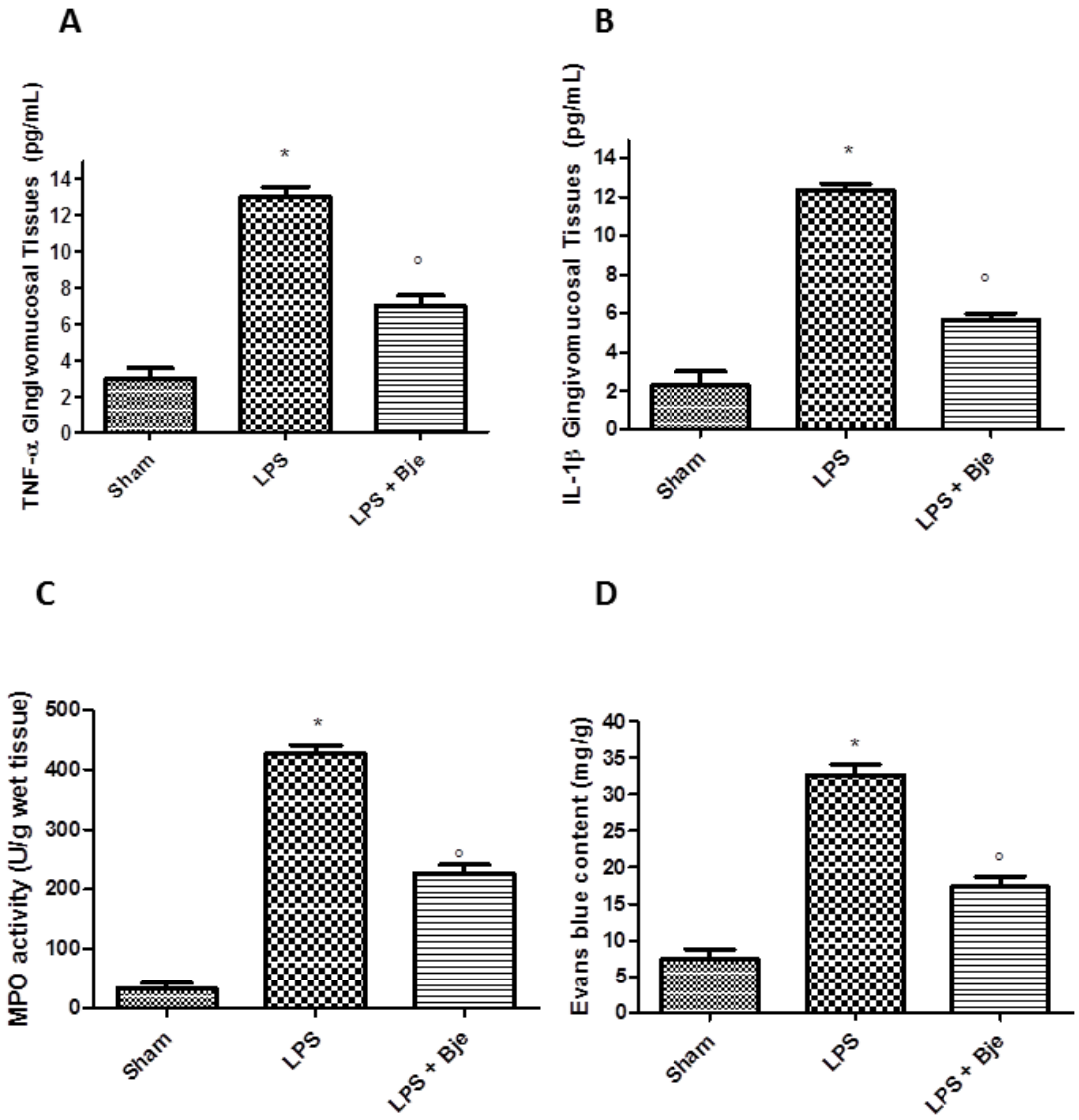


Fig. 8. TNF-α and IL-1β levels were increased in samples collected from LPS+saline group when compared to samples from sham-group (A and B), while BJe treatment significantly down-regulated the production of these cytokines (A and B). LPS significantly promoted myeloperoxidase activity (C), whereas MPO activity was significantly reduced in BJe-treated rats (C). Moreover, BJe administration inhibited the increase in Evans blue extravasation induced by LPS (D). Data are mean ± SEM of 10 rats for each group). ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.3.5 *Treatment with BJe counteracts ICAM and P-selectin expression induced by LPS-injection in gingival tissue*

Gingiva-mucosal tissues from sham-group showed a basal staining for ICAM (Fig. 9A); while LPS injection increased this staining (Fig. 9B), BJe treatment significantly decreased it (Fig. 9C and 9G). In a similar fashion, immunohistochemical analysis of LPS-treated group samples showed positive staining for P-selectin (Fig. 9E and 9H) when compared to tissues from sham-treated group (Fig. 9D and 9H), while BJe-treated groups reduced this staining (Fig. 9F and 9H).

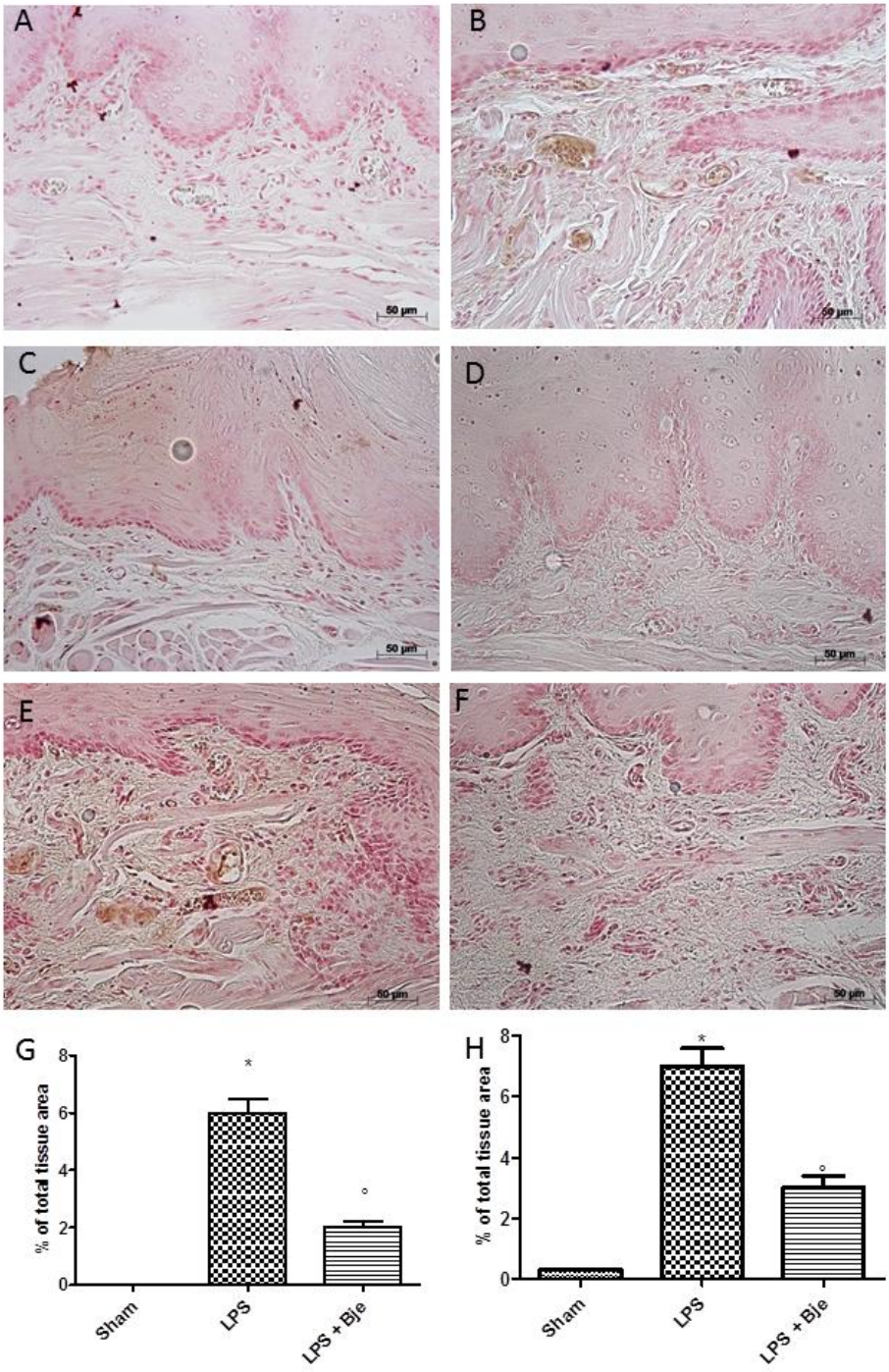


Fig. 9. No positive staining for ICAM (A and G) or P-selectin (D and H) was detected in rats of sham group that, instead, was found in samples from LPS-subjected animals (B and G for ICAM; E and H for P-selectin). Staining for ICAM (C and G) as well as P-selectin (F and H) was decreased for BJe-treated animals. Values of densitometric analyses are presented as mean ± SEM of 10 rats for each group). ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.3.6 *BJe decreases nitrotyrosine and PAR expression in LPS-induced periodontitis*

LPS injection determined positive staining for nitrotyrosine (Fig. 10B and 10G). BJe treatment after LPS injection decreased this positive staining (Fig. 10C and 10G). Additionally, the immunohistological staining for poly ADP-ribosylated proteins showed a positive staining for the PAR in rats of LPS-group (Fig. 10E and 10H), less pronounced in LPS + BJe group (Fig. 10F and 10H).

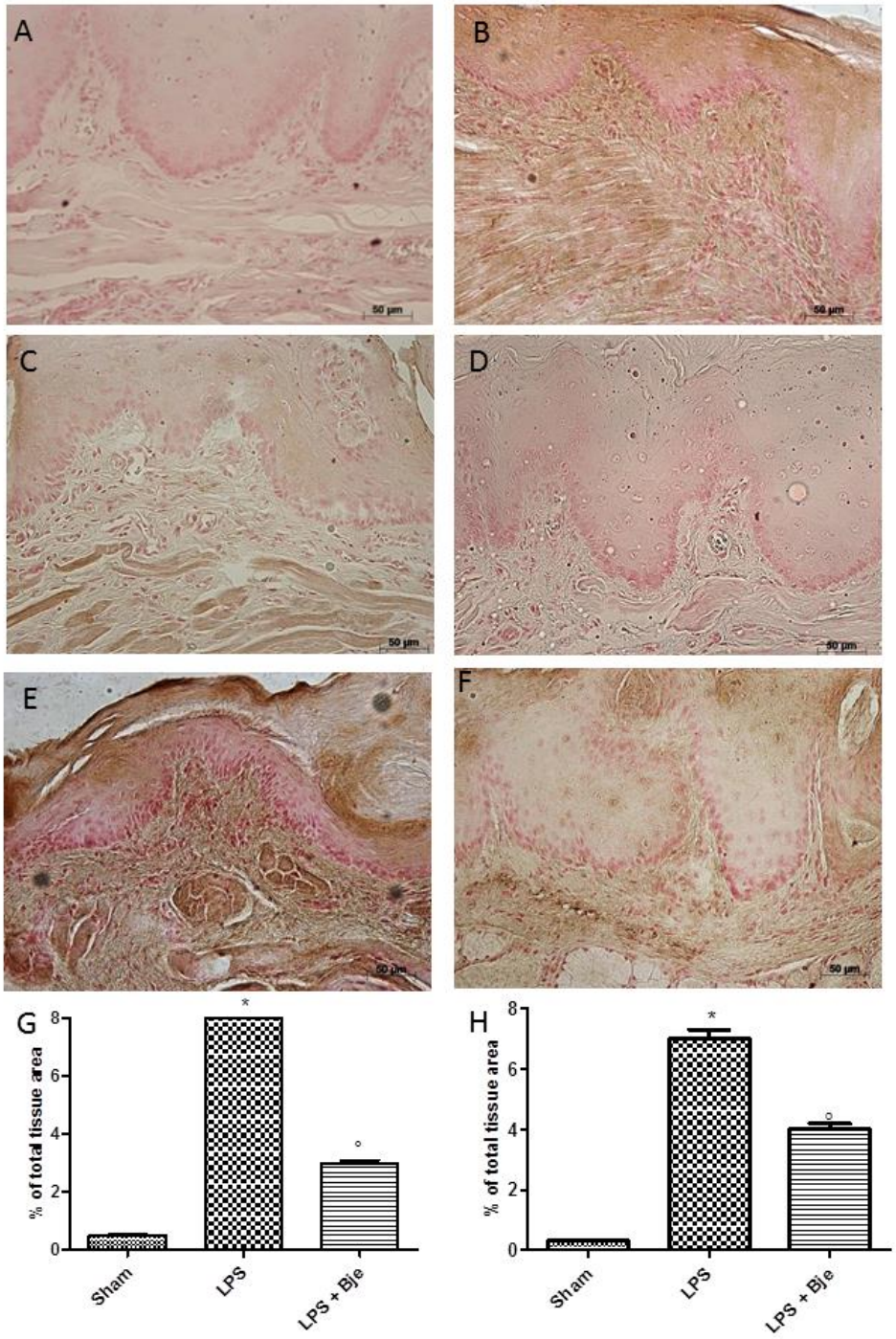


Fig. 10. LPS-injected rats showed intense positive staining for nitrotyrosine (B and G) and PAR (E and H). Staining was significantly reduced in BJe-treated rats (nitrotyrosine, C and G; PAR, F and H), and absent in sham-group (nitrotyrosine, A and G; PAR, D and H). Values in graphs are expressed as mean ± SEM (N =10 rats per each group). ∗P <0.05 vs sham group. ◦P <0.05 vs LPS.

3.3.7 *Effects of BJe on Bax and Bcl-2 expression in rats subjected to a gingival injection of LPS*

In order to find an association between LPS-induced tissue damage and apoptosis, Western blot analyses were performed. Figure 11 show that LPS significantly promoted Bax (a pro-apoptotic protein) expression (Fig. 11A and 11C) while decreasing Bcl-2 (an anti-apoptotic protein) levels (Fig. 11B and 11D). BJe treatment significantly down-regulated LPS-induced Bax expression (Fig. 11A and 11C), increasing Bcl-2 levels (Fig. 11B and 11D).

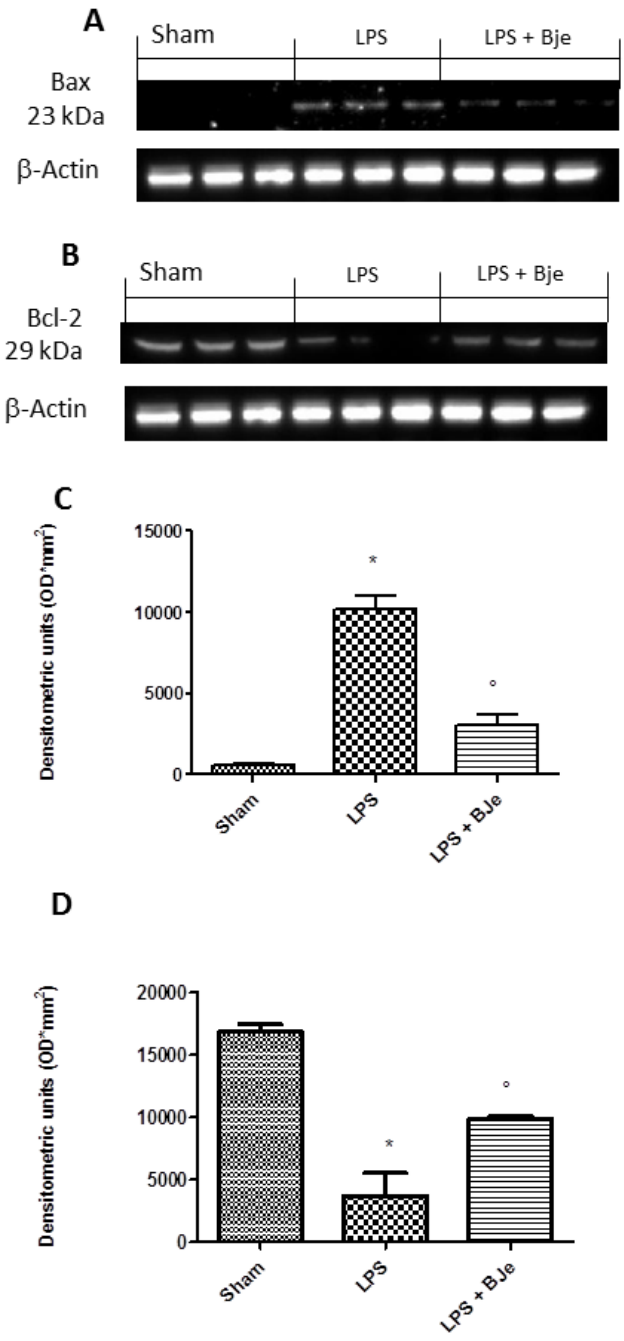


Fig. 11. Western blot analyses for Bax and Bcl-2. LPS-injected rats, compared to sham group, displayed an increase of Bax expression, that was found reduced in BJe-treated rats (A and C). Conversely, LPS decreased expression of Bcl-2 was found to be raised in the rats treated with BJe (B and D). Densitometric analyses of blots were performed normalizing bands for β-actin. Blots in A and B are representative of 3 different gels. Data in C and D are means ± SEM of 10 rats for each group. ∗P <0.05 vs sham group. ◦P <0.05 vs LPS group.

3.4 Discussion

Periodontitis is a chronic multifactorial inflammatory disease that affects the supporting structures of the teeth, progressively leading to their destruction. Because of its high prevalence, periodontal disease is addressed as a major public health problem, leading to tooth loss, compromising stomatognathic function, aesthetics, and ultimately impairing quality of life (Papapanou et al., 2017). Despite its constant association with a plaque metabolic shift, periodontitis recognizes oxidative stress as a major component of the pathologic mechanisms accounted for periodontal damage.

Citrus spp. flavonoids are acknowledged for a wide spectrum of biological properties, behaving as antioxidants through radical scavenging and metal chelation (Sawa et al., 1999; Ak and Gülcin, 2008; Miller et al., 2008; Batra and Sharma, 2013), inhibitors of protein kinase activity (Huang et al., 1999; Lee et al., 2004; So et al., 1996), antiestrogenic and antimutagenic agents (Singhal et al., 1995; Edenharder R et al., 1993; Miyazawa and Hisama, 2003), p53 down-regulators (Davis et al., 2000), HSP induction inhibitors (Hosokawa et al., 1990; Davis et al., 2000), inhibitors of cell proliferation (Kandaswami et al., 2007), cell cycle blockers and apoptosis promoters (Wang et al., 1999; Mahmoud et al., 1999; Shukla and Gupta, 2004).

During inflammation, *Citrus* flavonoids can inhibit cytokines synthesis (Benavente-García et al., 2008) and polymorphonucleocytes ROS production downregulating caspase-3 (Zielinska-Przyjemska and Ignatowicz, 2008).

As suggested by Blignaut and Grobler in 1992 in a study on fruit-farm workers, a fruit-rich diet may improve periodontal health. Additionally, increasing vitamin C plasma levels through intake of grapefruit can improve bleeding of probing in periodontopathic patients, especially smokers (Staudte et al., 2005). These statements are further strengthened by evidence that a lower antioxidant capacity may be an innate feature of periodontitis patients

(Brock et al., 2004; Sculley and Langley-Evans, 2003). In consideration of the above findings and the previous reports on the antioxidant properties of *Citrus bergamia* juice (Ferlazzo et al., 2016; Marino et al., 2015), BJe was investigated to better comprehend its effects and the underlying related mechanisms of action using an experimental model of periodontitis. To researchers' best knowledge, no other study tried to prove the antioxidant and anti-inflammatory activity of a *Citrus* derivative against periodontal diseases. In particular, BJe demonstrated to significantly improve the inflammatory findings of gingiva-mucosal tissues of rats subjected to LPS-induced periodontitis. Its main effects comprehended downregulation of NF-κB activation, pro-inflammatory cytokines, ICAM, P-selectine, nitrotyrosine, and PAR expression as well as apoptosis. BJe administration toned down LPS-induced periodontitis manifestations in rats, as suggested by the radiographic analysis of the mesial root surface displaying a clear reduction of periodontal bone-supporting ratio caused by LPS. Besides its effects on periodontal bone, BJe protective action extended at tissue level, deeply improving the histological picture when compared to non-treated LPS-injected group. Masson's trichrome staining showed how LPS injection increased collagen formation thus improving the fibrosis score. This finding goes along literature reports suggesting that simultaneously with collagen destruction, wound repair occurs, resulting in fibrosis and scarring coexisting at the foci of inflammation (Bartold and Narayanan, 2006). There are evidences showing that both ROS and reactive nitrogen species (RNS) play a key role in the development of the periodontitis (Di Paola et al., 2004). Both ROS and RNS can either inhibit or promote NF-κB signaling; this crosstalk is accounted for regulation of gene transcription in many and pathophysiological conditions, ultimately resulting in regulation of cell death (Morgan and Liu, 2011). Normally, IκBs proteins inhibit NF-κB in multiple ways, either by masking its DNA binding sites or through nuclear export signals, thus removing NF-κB from the nucleus. Infection, hypoxia, oxidative stress,

inflammation and other extracellular stimuli lead to the activation of IκB kinase that phosphorylates the regulatory proteins IκBs. As a consequence, the NF-κB dimers are released and able to translocate into the nucleus. In this study a significant increase in the translocation of NF-κB in the nucleus of epithelial cells of gingivomucosal tissues from LPS-injected rats was observed, with a simultaneous IκB-α degradation. Furthermore, it is well known that NF-κB is atop of a signaling pyramid responsible for promotion of several proteins and mediators in inflammation such as TNF-α and IL-1β. Herein LPS-injection increased TNF-α and IL-1β production, confirming the role of these cytokines in the periodontitis pathogenesis. On the other hand, oral treatment with BJe for 14 consecutive days reduced LPS-induced NF-κB activation and counteracted TNF-α and IL-1β expression in gingivomucosal tissues from LPS-injected rats, suggesting a role in periodontitis reduction. These findings are in accord with previous reports regarding antioxidant and anti-inflammatory properties of BJe in in vitro models, in both abiotic and cell culture models (Ferlazzo et al., 2015; Ferlazzo et al., 2016). Moreover, BJe was found able to affect cellular pathways normally activated during inflammatory response, thus restraining both gene expression and secretion of cytokines such as IL-1β, interleukin-6 (IL-6) and TNF-α induced by LPS in THP-1 monocytes, the key mechanism behind being the inhibition of NF-κB (Risitano et al., 2014). Others experimental research suggested the involvement of both NF-κB and cytokines in the anti-inflammatory mechanisms exerted by bioactive molecules against periodontitis. For instance, daily curcumin oral gavage inhibited IL-6, TNF-α and PGE2 LPS-induced tissue flogosis, and suppressed NF-κB activation and innate immune responses associated with periodontal disease (Guimarães et al., 2012). Green tea catechins behave in a similar way, downregulating expression of pro-inflammatory cytokines and reducing oxidative stress in experimental periodontal inflammation induced by topical application of LPS and proteases in the gingival sulcus in rats (Maruyama et al., 2011). Like

IκB proteins, green tea catechins also prevented nuclear translocation of NF-κB and IL-1β expression induced by LPS in gingival tissue, thus limiting bone resorption (Nakamura et al., 2010).

Whenever an inflammatory response is triggered, polymorphonuclear neutrophils are recruited and, mainly through oxidative burst, generate a large part of the total quota of ROS and RNS, leading to various kind of tissue damage including protein folding/unfolding and fragmentation, lipid peroxidation and subsequent release of bioactive molecules, and DNA strands break and mutations (Dahiya et al., 2013).

PMN activity possibly amplifies inflammatory response, in a vicious cycle of escalating tissue destruction.

Adhesion molecules are a key factor in the polymorphonuclear cells infiltration. This study showed that treatment with BJe decreases Evans blue extravasation caused by LPS, reduces MPO activity, and limits inflammatory cellular infiltration in the gingivomucosal tissues of LPS-injected rats. Additionally, BJe administration down-regulated expression of ICAM and P-selectin, thus suggesting a possible mechanism for the reduced polymorphonuclear cells LPS-induced infiltration. The role of ROS and RNS in the pathogenesis of periodontal disease, as well as the involvement of PARP in experimental periodontitis have been already described (Di Paola et al., 2004). BJe treatment reduced the LPS-induced nitrosative stress while increasing PARP activity in the LPS-injured gingivomucosal tissue.

Several studies displayed apoptosis role in periodontal disease. In this study, BJe administration demonstrated to condition apoptotic cell response by both down-regulating expression of the pro-apoptotic protein Bax and up-regulating the anti-apoptotic Bcl-2, confirming the involvement of programmed cell death in periodontitis (Song et al., 2017) and BJe ability to modulate the signaling pathways leading to apoptosis.

Overall, these findings concord with previous reports regarding the anti-inflammatory

activity of BJe in experimental models of both colitis (Impellizzeri et al., 2015) and intestinal ischemia (Impellizzeri et al., 2016), and strengthen the potentiality of natural products in periodontal diseases. In these regards, very recently, treatment with a *Salvia sclarea* extract was reported as significantly reducing IL-1β, IL-6 and TNF-α tissue levels, preventing damage to gingival tissue and bone alveolar resorption (Kostić et al., 2017).

3.5 Conclusion and future perspectives

In recent years, BJe has demonstrated a wide spectrum of biological activities, ranging from antimicrobial properties to antioxidant and anti-inflammatory effects in several in vitro and in vivo experimental models. In this study, through several methodological approaches, BJe was found able to improve LPS-induced periodontitis in rats, suggesting its potential in the treatment of periodontal disease.

Further investigations are required to support this new-found role of BJe in the treatment of inflammatory pathologies in a contest of a multitarget pharmacological strategy.

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