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Short Article

Treatment by *Moringa oleifera* extract can reduce gingival inflammatory cytokines in the rat periodontal model

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Abstract

Introduction: Overproduction of gingival pro-inflammatory cytokines have been implicated to play a noticeable role in the pathogenesis of periodontitis, which is characterized by host-mediated destruction of soft and hard periodontal tissues. *Moringa oleifera* (MO) is a highly valued medicinal plant which has a wide impressive range of traditional medical applications and is an alternative medicine for synthetic drugs which are accompanied with many downsides. The aim of this study was to investigate the effect of administration of the MO extract on gingival levels of TNF-α and IL1-β in the rat periodontal model.

Methods: Inflammatory periodontitis was induced using 0-3 ligatures around the neck of right mandibular first molar in male rats. MO leaf extract was solved in dimethyl sulfoxide and injected into the gum tissue directly (500mg/kg) as a pre/post-treatment. Positive control group gave indomethacin (5mg/kg) on a daily basis. Gingival levels of TNF-α and IL1-β were measured using ELISA.

Results: The results of this study revealed that levels of IL1-β and TNF-α increased in the gingival tissue in a model of periodontitis compared to control group ($P \leq 0.001$). Also, the results indicated that administration of MO extract could reduce production of TNF-α and IL1-β in the gum tissue of rat periodontal model ($P \leq 0.001$). There was no significant difference between MO extract and indomethacin anti-inflammatory effects.

Conclusion: It can be concluded that pre/post-treatment with MO extract due to its direct effect on inhibition of pro-inflammatory cytokines can alleviate inflammatory symptoms in a rat periodontal model.

Keywords:
Inflammation;
Periodontitis;
TNF-α;
IL-1β;
Moringa oleifera

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Introduction

Periodontitis is a chronic inflammatory condition of the periodontal tissues which is caused by dental plaques and is characterized by destruction of periodontal supporting connective tissue, alveolar bone loss, migration and formation of pathological

pockets around the affected teeth (Pellegrini et al., 2009; Struillou et al., 2010). Periodontal disease is a major cause of tooth loss in adults and one of the most common infectious diseases in all over the world which affected approximately 800 million people in 2016 mostly in upper-middle income countries (Ryan and Golub, 2000).

Lipopolysaccharide (LPS) is a fundamental molecule which comprises the outer membrane of gram-negative bacteria. It is obvious that bacterial LPS is a major effector in the periodontitis pathology which can increase production of pro-inflammatory cytokines, including interleukin-1beta (IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α) due to the motivation of the host's immune system cells (Yamaguchi and Kasai, 2005). Constitutive overproduction of these cytokines in the gingival tissue due to the periodontitis can cause soft and hard tissue destruction (Saliva and Lang, 2005; Bansal et al., 2012). Studies indicated that inhibition of these cytokines could be a promising and effective strategy for periodontitis treatment (Pellegrini et al., 2009; Graves, 1999).

Nowadays, there is a great number of drugs for treatment of periodontitis. Antibiotics which have antimicrobial effects on LPS and ability to decrease levels of inflammatory cytokines are widely used to manage periodontitis. Nonetheless, their overuse have led to the outburst of antibiotic-resistant pathogens (Rizzo et al., 2012). The use of synthetic compounds such as corticosteroids and non-steroidal anti-inflammatory drugs like indomethacin, which are commonly prescribed for the treatment of periodontitis, are associated with many side effects on human's health, for instance stomach discomfort, heartburn and nausea. Studies have demonstrated that long-term use of indomethacin in the treatment of periodontitis resulted in the lower bone loss, although disease progression returned when the drug was stopped. So, the wide range of research to define new therapeutic approaches, including herbal medicines and plant extracts therapy is in progress (Salvi and Lang, 2005; Pellegrini et al., 2009). Thus, herbal and natural products, as an alternative for synthetic drugs, are receiving extraordinary attention for using in medical remedy. In this regard, it seems that plant extracts which don't have any adverse effects on the host may be useful for treating inflammatory diseases (Bansal et al., 2012). Down the ages, plant-derived dietary constituents, such as polyphenols and resveratrol are profited for human's health and play a crucial role in the prevention of human periodontal disease due to their anti-inflammatory, anti-oxidant, chemo-prevention and anti-cancer effects (Rizzo et al., 2012; Bansal et al., 2012).

Moringa oleifera (MO), as a member of *Moringaceae* family, is a highly valued medicinal plant which is distributed in many tropic regions and used for treatment of various types of diseases. Different parts of MO including leaves, roots, seeds, fruits and flowers can act as antitumor, anti-inflammatory and antibacterial markers. A great number of chemicals are existed in the MO leaf extract such as phytosterols, glycosides, tannins and amino acids (Manaheji et al., 2011). The benefits of MO administration for the treatment or prevention of oral inflammatory diseases such as periodontal disease are not quite well-known and further investigations are needed (Ndiaye et al., 2001). Thus, because of the importance of offering effective treatments with fewer adverse effects and considering the anti-inflammatory and immuno-modulatory properties of MO due to the existence of saponin, terpenoids, alkaloids and also anti-inflammatory transcription factors, this study attempted to examine the anti-inflammatory effects of MO on gum tissue levels of IL-1β and TNF-α in the rat periodontitis model.

Materials and methods

Plant material

Aerial parts of MO were collected initially, identified in the Herbarium of Medicinal plants (the academic center for education, culture and research), Iran (Research Institute of Forest and Rangelands, Tehran). The voucher specimen was deposited there with number 66. After collection and identification by experts, plants were cleaned, air dried and powdered for extract preparation.

Extract preparation

In order to provide MO extract, 200g of the plant powder was exposed to 70% methanol for 72 hours in the percolator. After this time, the extract was collected and filtered using filter paper. The clear extracts were separated from their solvents (alcohol) by Rota vapor under vacuum at 55-65°C for 2 hours.

Then for dehydration, the freeze drier machine was used for 3 hours. Concentrated extracts were stored at 4°C in refrigerator until the examination. Dried extracts were solved in dimethyl sulfoxide (DMSO). So extract with a dose of 500mg/kg was prepared (Manaheji et al., 2011).

Qualitative phytochemical analysis of *MO*

The Moringa plant extract was standardized by phytochemical analysis method (Trease and Evans, 1983). For this purpose, the extract was examined for evaluating the presence of active ingredients such as saponin (saponins), terpenoids (terpenoidi) and alkaloids (alkaloids). Also according to our previous studies, for quantization of flavonoids, high performance liquid chromatography (HPLC) was used: KNAUER Chrome gate V3. (Manaheji et al., 2011; 2015; Vinoth et al., 2012). Extract was prepared in similar situation of previously described method and ultraviolet detector was set on 225nm (Aguilar-Sanchez et al. 2005).

Laboratory animals

Adult male Wistar rats (weighing 250-300g) were used in the present study. Rats were housed in standard environmental conditions (22-24°C, humidity 60-70% under a 12h light/dark cycle). The animals had access to standard food and water except experimental time. All the procedures were approved by the guidelines of the ethical standards for the examinations on animals. Rats were randomly divided into 6 experimental groups, as follow: (a) LI (ligature), (b) control (c) LI+MO (pre), (d) LI+MO (pos), (e) LI+DMSO and (f) LI+Indo (indomethacin).

Experimental procedure:

After induction of periodontitis using 0-3 suture around the crown of the right first maxillary molar ligatures were remained in the place for 7 days (LI group). In order to inject the extract, rats were anesthetized slightly with CO2. Then, extract was diluted in the DMSO at a concentration of 500mg/kg with volume of 30µl was injected into the gum tissue directly by Hamilton syringe. The LI+MO (pre) group was received MO extract daily from a day before ligature up to day 7 (pre-treatment). The LI+MO (pos) group was received MO extract daily from day 7 up to day 14 after ligature (post-treatment). The LI+DMSO group was received DMSO, as a vehicle, daily after ligature. The LI+Indo group was received indomethacin daily after ligature (5mg/kg, ip) (Shadnoush et al., 2016; Manaheji et al., 2011). In this study, gingival tissue levels of TNF-α and IL-1β were assessed by enzyme-linked immunosorbent assay (ELISA) for each group.

Induction of inflammatory periodontitis

General anesthesia was achieved through intraperitoneal injection of ketamine hydrochloride (50mg/kg) and xylazine hydrochloride (5mg/kg). Inflammatory periodontitis was induced using 0-3 ligatures around the crown of the right first maxillary molar in rats and in order to accumulate plaque in that area the ligature was pressed into the sulcus. The ligatures were studied before the sacrifice. Left mandibular first molar was considered as a control for each group (Ionel et al. 2015).

TNF-α and IL-1β measurements by ELISA

In order to identify the gingival tissue levels of TNF-α and IL-1β in different time points of the study, rats were anesthetized and gum tissues were removed. Samples in phosphate buffer saline with 10% fetal bovine serum inactivated by heating at pH=7 were homogenized, centrifuged (1200 rpm at 4ºC) and supernatant was collected. Levels of IL-1β and TNF-α were measured using rat ELISA standard kit (e-Bioscience). Assessment procedures were performed according to the manufacturer's protocol (Graves, 1999).

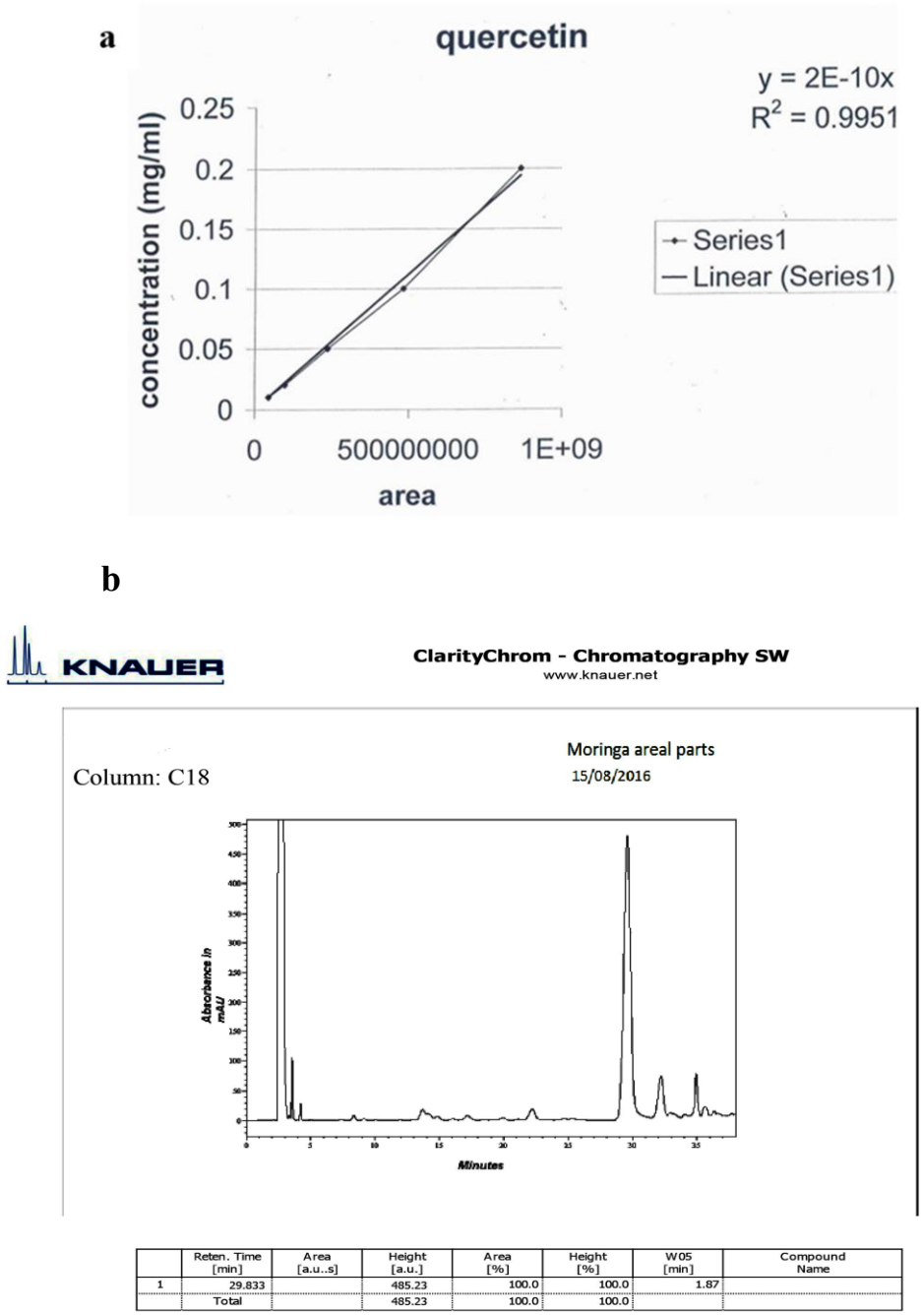
Statistical analyses

Results were expressed as mean ± SEM. Independent student *t*-test and one-way ANOVA (Post hoc Tukey) were used for comparison the variants within groups. To compare more accurately the changes of IL-1β levels and TNF-α expression on the same days between two groups, unpaired student *t*-test was used. Data analysis was performed using the SPSS software 19 and the charts were analyzed via Excel. Statistical significance was accepted at $P \leq 0.05$.

Results

Moringa extract phytochemical analysis

For standardization the Moringa plant extract phytochemical analysis method was used. Qualitative



	Reten. Time [min]	Area [a.u..s]	Height [a.u.]	Area [%]	Height [%]	W05 [min]	Compound Name
1	29.833		485.23	100.0	100.0	1.87	
	Total		485.23	100.0	100.0		

Fig.1. (a) Calibration curve for analysis of quercetin. Total flavonoid amounts of aerial parts of Moringa extract which represented as quercetin. (b) HPLC chromatogram of flavonoids in aerial parts and root of plant consequently.

phytochemical study of the Moringa leaves extracts showed the presence of saponins and rich in terpenoids factors. The assessment also showed that there is no trace of alkaloid in extract of leaves of Moringa. The calibration curve showed acceptable linearity with a regression value of 0.995 (Fig. 1a and b). The total flavonoids content as a percentage of quercetin content was 0.68% (w/w).

Tissue levels of IL-1β

Periodontitis caused by ligature, significantly increased gum tissue levels of IL1-β in the LI group compared with the control group on day 7 of the study ($P \leq 0.001$). There was no considerable difference in gum tissue levels of IL-1β between two groups of LI+MO (pos) and LI+MO (pre). Intraperitoneal administration of MO could significantly decrease levels of IL-1β in the gum tissue in the LI+MO (pos) group compared with the LI group ($P \leq 0.001$). Besides, administration of indomethacin with a dose of 5mg/kg significantly reduced levels of IL-1β in the LI+Indo group

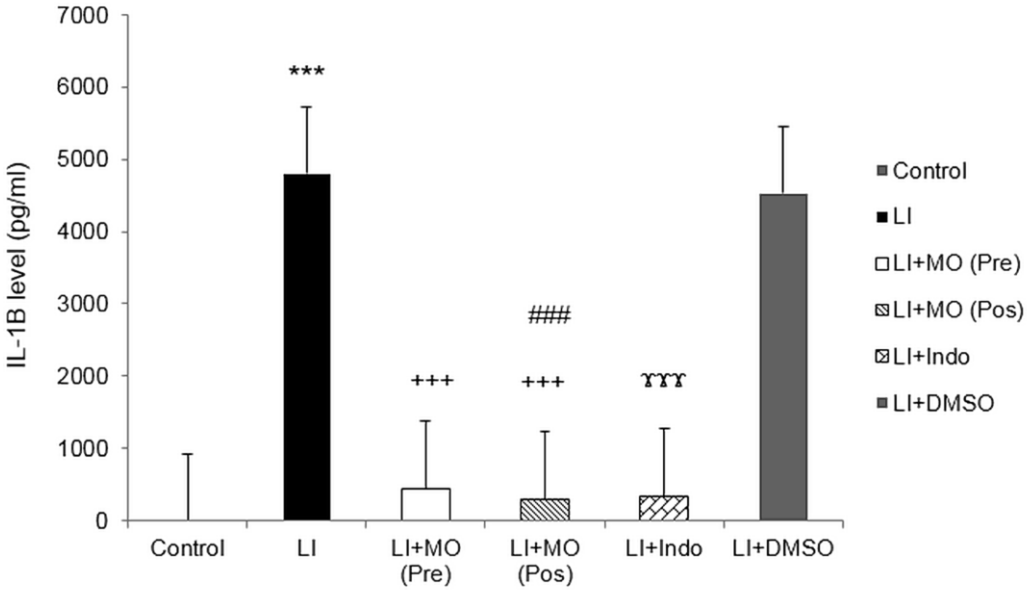


Fig.2. Changes in IL-1β levels in different experimental groups compared with LI and control groups. Results were expressed as mean ± SEM and n=6/group. *** (*P*≤0.001): comparison of gingival levels of IL-1β in LI group with control group. +++ (*P*≤0.001): comparison of gingival levels of IL-1β in LI+MO (pre) and LI+MO (pos) group with control group. ### (*P*≤0.001): comparison of gingival levels of IL-1β in LI+MO (pos) group with LI group. ϒϒϒ (*P*≤0.001): comparison of gingival levels of IL-1β in LI+Indo group with LI group.

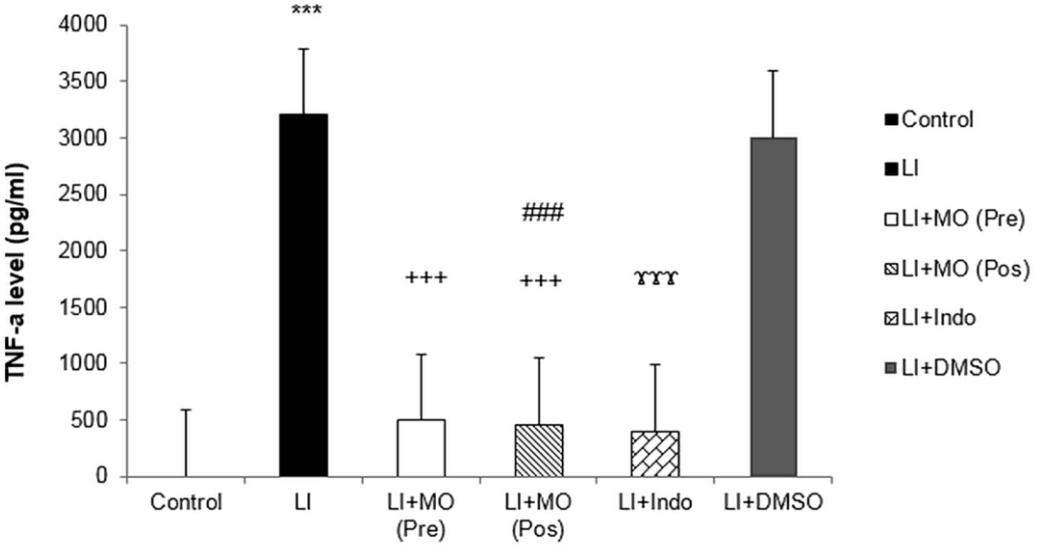


Fig.3. Variations of gingival tissue TNF-α level in different experimental groups compared with LI and control groups. Results were expressed as mean ± SEM and n=6/group. *** (*P*≤0.001): comparison of gingival levels of TNF-α in LI group with control group. +++ (*P*≤0.001): comparison of gingival levels of TNF-α in LI+MO (pre) and LI+MO (pos) group with control group. ### (*P*≤0.001): comparison of gingival levels of TNF-α in LI+MO (pos) group with LI group. ϒϒϒ (*P*≤0.001): comparison of gingival levels of TNF-α in LI+Indo group with LI group.

compared with the LI group (*P*≤0.001). Comparison of the two groups, LI+MO (pos) and LI+Indo illustrated that there was no noticeable difference in the tissue levels of IL-1β between these two groups. Injection of DMSO as a MO solvent in the LI+DMSO group had no significant effect on IL-1β levels compared with the LI group (Fig. 2). There was no significant difference in the tissue levels of IL1-β in the MO+LI group on days 7 and 14 of the study (only one of those shown graphically).

Tissue levels of TNF-α

Periodontitis induced by ligature, significantly elevated gum tissue levels of TNF-α in the LI group compared with the control group on day 7 of the study (P≤0.001). There was no considerable difference in gingival tissue levels of TNF-α between two groups of LI+MO (pos) and LI+MO (pre). Intraperitoneal injection of MO could considerably decline levels of TNF-α in the LI+MO (pos) group compared with the LI group (P≤0.001). Furthermore, administration of indomethacin with a dose of 5mg/kg significantly reduced levels of TNF-α in the LI+Indo group compared with the LI group (P≤0.001). Comparison of the two groups, LI+MO (pos) and LI+Indo illustrated that there was no significant difference in the tissue levels of TNF-α between these two groups. Administration of DMSO as a MO solvent in the LI+DMSO group had no significant effect on TNF-α levels compared with the LI group (Fig. 3). Our results in this study illustrated that there was no considerable difference in the tissue levels of TNF-α in the MO+LI group on day 14 of the study compared with day 7 after periodontitis induction (only one of those shown graphically).

Discussion

The results of this study revealed that MO extract could reduce gum tissue levels of IL-1β and TNF-α during different phases of periodontitis caused by ligature in male Wistar rats. It seems that there was no considerable differences in tissue levels of TNF-α and IL-1β due to the administration of the MO leaf extract with a dose of 500mg/kg and indomethacin with a dose of 5mg/kg.

Dental plaque is the main etiological reason of periodontitis, so this essay is focused on the prevention and treatment of periodontitis by administration of herbal products such as MO (Ndiaye et al., 2001). MO and antioxidant phenolic compounds are composed of loads of terpenoids, kampferol, querectin and flavonoids. It is believed that these compounds can act synergistically as free radical scavengers (Guardia et al., 2001). Hukeri et al. (2006) have shown that qualitative chemical examinations corroborated the existence of a great number of phytosterols, glycosides, tannins and amino acids in the various leaf extracts of MO. In this regard, they also showed that administration of the MO extract (300mg/kg) can be effective in wound healing. Gupta et al. (1999) have presented that MO extract not only could alleviate pain in mice, but also could enhance the analgesic effects of morphine and meperidine. Moreover, MO extract is cable to augment the sleeping time induced by pentobarbitone sodium and diazepam due to release of 5-hydroxytryptophan and GABA in the brain. A large body of evidence exists to substantiate that transient receptor potential vanilloid-1 (TRPV1) is a sensor for an extensive range of cellular and environmental signals and is involved in the pain process with the potential to induce cytokine production. Sashidhara et al. (2009) have illustrated that 1,3-dibenzyl thioureas which is a TRPV1 antagonist is one of the MO extract components and also can inhibit Ca^{2+} uptake in rat's dorsal root ganglia neurons. Ergo, MO has an extraordinary therapeutic potential for pain alleviation due to its anti-inflammatory and analgesic effects and inhibition of cytokine production. Scientists also demonstrated that the methanolic extract of MO can exert anti-inflammatory properties and decrease pro-inflammatory cytokine levels, which is effective in the treatment of acute and chronic inflammation (Manaheji et al., 2011). Transcription factors play a vital role in the regulation of expression of pro-inflammatory cytokines and their gene coding. Lampronti et al. (2008) have demonstrated that medicinal plants, including MO which are a major source of transcription factors can obviously alleviate inflammation. Mahajan et al. (2007) have indicated that the MO seed extract can reduce paw edema, serum levels of IL-6, rheumatoid factor, TNF-α and also can inhibit oxidative stress in the arthritis model in mice. Ezeamuzie et al. (1996) have shown that MO extract (600mg/kg) as a prototype of anti-inflammatory drug is more effective than indomethacin (5mg/kg) in the inhibition of acute and chronic pouch inflammation. Ndong et al. (2007) have reported that MO leaves extract due to its arginine and COX-2 inhibitors can exert anti-inflammatory effects and alleviate inflammation and pain.

It is vividly depicted in our study that Moringa extract noticeably reduced production of inflammatory cytokines namely IL-1β and TNF-α which are responsible for periodontitis induction and progression. Anti-inflammatory effects of MO (500mg/kg) in reduction of IL-1β and TNF-α levels are approximately equal to indomethacin (5mg/kg)

and there is no significant difference between their anti-inflammatory effects. Hence, it can be concluded that indomethacin can be replaced by Moringa extract. In our study it was shown that pre-treatment and post treatment with MO extract can decrease levels of IL1-β and TNF-α in the periodontitis model.

Conclusion

According to the results of this study it can be concluded that pre-treatment and post-treatment by MO extract can reduce inflammatory cytokines in gum tissue and alleviate inflammatory symptoms in a rat periodontal model.

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Conflict of interest

The authors declare that they have no competing interests.

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