



## Article

# Mouthwash with *Tagetes lucida Cav.* for Control of Chronic Periodontitis in Older Adults

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**Abstract:** Background: *Tagetes lucida Cav.* (*T. lucida*) is an herbaceous plant from the family *Asteraceae*, native to Mexico, Guatemala, and other Mesoamerican countries. Their common names are: (i) Spanish names: “hierbanís”, “jericón”, “pericón vomol”, “Santa María”, “cuchrucumín”, “falso hipericón”, “hierba añil”, “pericón”, “periquillo”; (ii) indigenous names: “ichka”, “cuahuyauhtli”, “yahuhtli”, “naná uarhi”, and (iii) English names: “sweet-scented marigold”, “sweet mace”, and “sweet marigold”. Its chemical composition of coumarins, estragole, quercetin, and flavonoids has antioxidant, anti-inflammatory, and antimicrobial effects. This study aimed to evaluate the effect of a mouthwash made with *T. lucida* for control of chronic periodontitis (CP) in older adults. Methods: We carried out a quasi-experimental study with a convenience sample of 60 older adults with CP. The sample was divided into a placebo group (PG)  $n = 30$ , administered with a mouthwash made with alcohol diluted with purified water (10%), and the experimental group (EG)  $n = 30$  treated with a mouthwash made with *T. lucida* extract in 10% alcohol; both groups were administered three times a day for three months. Results: We found a significant improvement in the EG group compared with the PG group in: probing pocket depth (baseline,  $5.2 \pm 0.81$  vs. post-treatment,  $3.1 \pm 0.43$ ,  $p < 0.05$ ), the clinical attachment loss (baseline,  $6.6 \pm 1.9$  vs. post-treatment,  $2.1 \pm 1.5$ ,  $p < 0.05$ ), lipoperoxides (baseline,  $0.056 \pm 0.03$  vs. post-treatment,  $0.034 \pm 0.02$   $\mu\text{mol/L}$ ,  $p < 0.05$ ), and chronic inflammation markers (IL-1, IL-8, and TNF- $\alpha$ ) ( $p < 0.05$ ). Conclusions: Our findings suggest that mouthwash made with *T. lucida* extract, administered three times a day, clinically improved chronic periodontitis in older adults, associated to a significant decrease in lipoperoxides and proinflammatory markers.

**Keywords:** chronic periodontitis; *Tagetes lucida Cav.*; older adults; oxidative stress; inflammation



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## 1. Introduction

Chronic periodontitis (CP) is a multifactorial disease, which is associated with the activation of polymorphonuclear leukocytes. Consequently, it generates a high concentration of reactive oxygen species (ROS) and a decrease in the antioxidant system, which causes oxidative stress in the periodontal tissue [1–4]. CP has a high prevalence and incidence in the world; in Mexico it occurs in more than 70% of the older adult population ( $\geq 60$  years) [5,6].

If this biochemical disorder is maintained, the epithelial adhesion is destroyed and the alveolar crest loses its height, which translates clinically into dental mobility and formation of periodontal pockets, causing the accumulation of bacteria that increases the inflammation, destroying the periodontal ligament, and consequently, the alveolar bone atrophies, and the tooth is lost. This disease is associated with the activation of

polymorphonuclear leukocytes, which in turn can generate a high concentration of reactive oxygen species (ROS) and a decrease in the antioxidant system, which causes oxidative stress in the tissue periodontal [1–4]. It has been observed that CP has a high prevalence and incidence in the world; in Mexico it occurs in more than 70% of the population of older adults [5,6].

To avoid the damage related to CP, conventional treatment focuses on stopping the progression of periodontal tissue destruction by eliminating local factors through oral hygiene instructions combined with scaling and root smoothing, to maintain and/or restore the function and aesthetics [5]. However, even when this treatment is carried out rigorously, the results are not entirely satisfactory. For this reason, various ethnobotanical therapies have been developed in the treatment of CP, with favorable effects [7–9].

In this context, we propose that *Tagetes lucida* could be useful as a therapeutic option for CP, considering its chemical composition of coumarins, estragole, and quercetin, with antioxidant, anti-inflammatory and antimicrobial effects [10,11].

*T. lucida* is an herbaceous plant from the family *Asteraceae*, native to Mexico, Guatemala, and other Mesoamerican countries, with a self-supporting growth habit, size up to 80 cm tall, usually several branched stem, simple leaves, and yellow flowers. Its common names are: (i) in Spanish: “hierbanís”, “jericón”, “pericón vomol” “Santa María” “cuchrucumín”, “falso hipericón”, “hierba añil”, “pericón”, and “periquillo”; and (ii) its indigenous names are “ichka”, “cuahuyauhtli”, “yahuhli”, and “naná uarhi”. In English, it is known as: “sweet-scented marigold”, “sweet mace”, or “sweet marigold”. This plant is registered at the FES Zaragoza Herbarium UNAM (N° 156999) and the U.S. FDA Unique Ingredient Identifier (UNII: N4052J8NFR) [12,13].

Experimentally, it has been shown that *T. lucida* has antifungal and antibacterial properties. Additionally, it has tranquilizing, antidepressant activity, as well as anti-inflammatory and antioxidant activities [14–19]. Therefore, this study aimed to evaluate the impact of *T. lucida* on oxidative stress and chronic periodontitis in older adults.

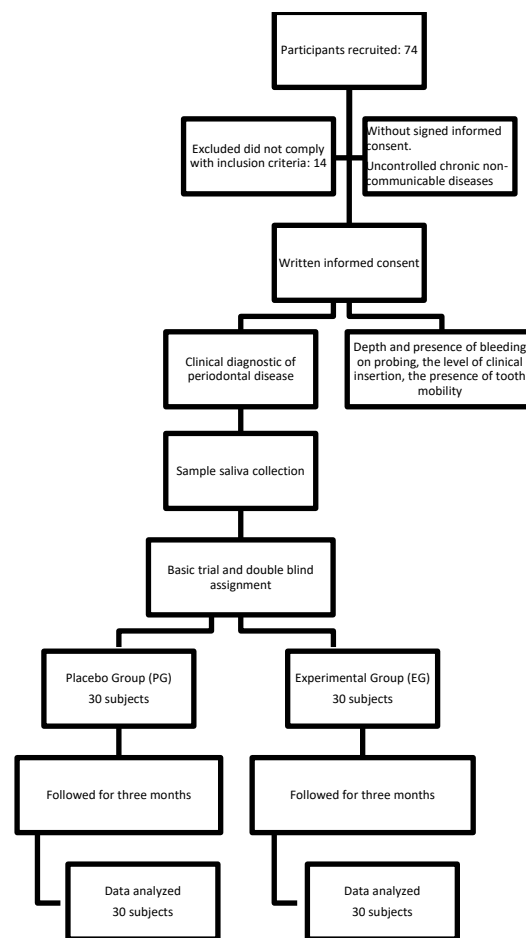
## 2. Materials and Methods

### 2.1. Subjects and Design

After informed consent, a quasi-experimental, double-blind study was conducted in 60 older adults, 17 (28%) men and 43 (72%) women with an average age of  $68 \pm 6.52$ , who attended the odontology service at Zaragoza Clinic, UNAM. They had controlled chronic non-communicable diseases, without consumption of antioxidant supplements in the last 6 months, all with clinical diagnosis of periodontal disease. The patients were not under periodontal treatment or used a supplement for oral cleaning, no change was made in their hygienic and dietary habits. The Ethics Committee of the Universidad Nacional Autónoma de México (UNAM), Zaragoza Campus approved the research protocol (FESZ/DEPI/325/16).

Subsequently, a clinical evaluation for the diagnosis of CP was achieved by the following parameters: the depth and presence of bleeding on probing, the level of clinical insertion, and the presence of tooth mobility.

For the study, a convenience sample of 60 subjects who met the inclusion criteria were randomly divided into 2 groups, the placebo group (PG,  $n = 30$ ) was administered mouthwash of absolute alcohol (10%) in purified water and the experimental group (EG,  $n = 30$ ) was treated with a mouthwash treatment based on the extract of *T. lucida* (10%), 3 times a day for 3 months (5 mL every 8 h) (Figure 1).



**Figure 1.** General scheme for study.

Saliva samples were taken before and after treatment. Lipoperoxides were quantified by the TBARS (Thiobarbituric acid reactive substance) technique, total antioxidant capacity by the ABTS<sup>®</sup> method. Superoxide dismutase enzyme (SOD) activity by the xanthine and xanthine oxidase method; inflammation markers (interleukin-1 (IL-1), IL-8, and tumor necrosis factor- $\alpha$ ) were quantified by flow cytometry.

## 2.2. Sample Size

The sample size was calculated based on the formula for the difference of means by the lipoperoxide marker.

$$N = 2 \left[ \frac{(Z\alpha - Z\beta)SD}{\mu_1 - \mu_2} \right]^2$$

Assuming that:

$z\alpha$  = Z value corresponding to alpha error ( $p$  0.05 = 1.96)

$z\beta$  = Z value corresponding to beta error (20%)

$SD$  = Standard deviation (0.01)

$\mu_1$  = Group A mean (0.029  $\mu\text{mol/L}$ )

$\mu_2$  = Group B mean (0.025  $\mu\text{mol/L}$ )

$n$  = 28 per group

## 2.3. Extraction and Preparation of *T. lucida*

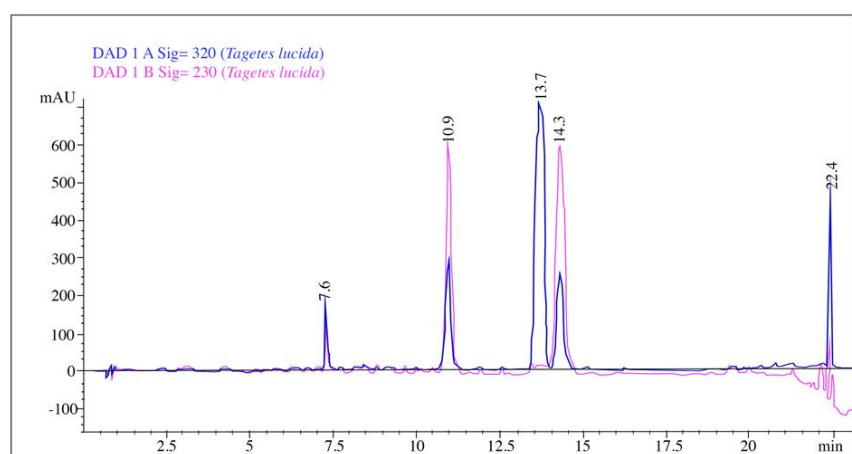
The plant specimen was collected full flowering in October, in Xochimilco, a rural area of Mexico City. It was kept on brown paper to preserve temperature and humidity. It was weighed thoroughly (flowers, leaves, and stems) and cut into proportions of approximately 10 cm.

The alcoholic extraction of active ingredients of *T. lucida* was performed under the NOM-O72 standard of the General Health Law of herbal supplements using absolute ethyl alcohol. Later, in a 250 mL amber glass crystal, 10% of the mother stain (extract) of *T. lucida* was diluted with 225 mL of purified water [18].

#### 2.4. Phytochemical Characterization

High performance liquid chromatography (HPLC) of *T. lucida* analysis was performed using an Agilent Technologies 1260 Infinity Chromatograph coupled to a DAD G1315C diode array detector and a 1290 Infinity G1316C oven; A Luna Omega 1.6  $\mu\text{m}$  Polar C18 100  $2.1 \times 50$  mm Phenomenex column. Chromatograms were recorded at wavelengths of 230 and 320 nm, processed using an Open Lab software program. Chromatographic conditions were developed using 0.1% formic acid (A) and acetonitrile (B) as a mobile phase. For the sample preparation, 11 mg of the dry extract were dissolved in 2 mL of a mixture of methanol: acetonitrile in a 1: 1 ratio. The injection volume was 1  $\mu\text{L}$ , the temperature was kept constant at 35  $^{\circ}\text{C}$ , and the flow was 0.25 mL/min.

The chromatographic profile showed five main compounds compared with the literature through the retention times obtained, and using the UV spectrum at 230 and 320 nm it can be considered that the peaks obtained in the retention times (Rt) of 10.9, 13.7, 14.3, and 22.4 min possess the characteristic spectra of phenolic compounds with antioxidant activity. Additionally, the 7.6 min peak corresponds to the peak of some coumarins. Therefore, it would have anti-inflammatory activity [16,18–20] (Figure 2).



**Figure 2.** Chromatogram of *Tagetes lucida* extract at 230 and 320 nm, showering four phenolic compounds and a coumarin-type compound with concentrations with pharmacological activity.

#### 2.5. Clinical Evaluation

The probing pocket depth (PPD) of all dental organs in each individual was evaluated, using a graduated periodontal probe between teeth and gums at a standard force to measure pocket depth. The clinical attachment loss (CAL) was determined measuring the distance between the pouch base and the cement-enamel junction. To evaluate the bleeding on probing, the probe was inserted in the pocket bottom; this procedure was repeated after 30 s [21].

Dental mobility tests were performed with vestibular-palatine, vestibular-lingual, and mesiodistal movements, considering physiological mobility.

#### 2.6. Saliva Samples and Biochemical Techniques

All participants were asked not to eat, drink, or brush their teeth 1 h prior to the trial, and not to consume any alcoholic beverages 24 h prior to sample collection. To stimulate saliva production, participants chewed a piece of paraffin wax for 7 min. Saliva produced during the first 2 min was discarded. Then, the patients chewed the paraffin during the

time of saliva collection until 5 mL [22]. Saliva samples were stabilized and conserved after centrifuged at 3000 rpm  $\times$  20 min, kept in 400  $\mu$ L aliquots at  $-70$   $^{\circ}$ C to preserve them, and then processed. Oxidative stress was quantified using the following techniques:

### 2.7. TBARS

The TBARS assay was performed using the whole saliva according Jentzsch et al. (1996) [23]. In this assay, one molecule of malondialdehyde reacts with two molecules of thiobarbituric acid (TBA), producing a pink pigment with an absorption peak of 535 nm. Amplification of peroxidation during the assay was prevented by adding the chain-breaking antioxidant butylated hydroxytoluene (BHT).

### 2.8. Saliva Total Antioxidant Status (TAS)

Antioxidant quantification was performed by monitoring 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS+) radical formation (Randox Laboratories, Ltd., Crumlin Co., UK). The antioxidants present suppressed the bluish-green staining of the ABTS+ cation, proportional to the antioxidant concentration. The reaction kinetic was measured using a colorimetric technique in an Autoanalyzer Vitalab Eclipse Merck (Dieren, The Netherlands) [24].

### 2.9. Saliva Superoxide Dismutase (SOD)

Xanthine and xanthine oxidase (XOD) were used to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to produce a red formazan dye. SOD activity was assessed by measuring the reaction inhibition using a commercial method (Randox Laboratories Ltd., Crumlin Co., UK). The kinetics of SOD activity was measured using a colorimetric technique in an Autoanalyzer Vitalab Eclipse Merck (Dieren, The Netherlands) [25].

### 2.10. Quantification of Cytokines

The levels of interleukin 1-beta (IL-1 $\beta$ ), interleukin 8 (IL-8), and tumor necrosis factor-alpha (TNF- $\alpha$ ) were assessed in saliva aliquots by flow cytometry (CBA Kit, Human Inflammatory Cytokine, BD Biosciences, Becton, Dickinson and Company, Baltimore, MD, USA) [26].

### 2.11. Statistical Analysis

Data were analyzed using descriptive statistics, the mean and standard deviation (SD), frequencies (f), and percentages (%). We performed a chi-square comparison test and repeated measures analysis of variance (repeated measures ANOVA) [27]. A  $p$  value of  $<0.05$  was considered statistically significant.  $p$  values were determined using SPSS program SPSS, version 16.0.

## 3. Results

### 3.1. Clinical Changes Related to Treatment

In the clinical evaluation, after 3 months of treatment, we observed a significant improvement in the EG compared to the PG in PPD (baseline,  $5.2 \pm 0.81$  vs. post-treatment,  $3.1 \pm 0.43$ ,  $p < 0.05$ ) and CAL (baseline,  $6.6 \pm 1.9$  vs. post-treatment,  $2.1 \pm 1.5$ ,  $p < 0.05$ ) (Table 1).

**Table 1.** Clinical changes baseline and post-treatment by group.

	Placebo Group ( $n = 30$ )			Experimental Group ( $n = 30$ )		
	Baseline	Post-Treatment	Differences	Baseline	Post-Treatment	Differences
PPD (mm)	$5.1 \pm 0.64$	$5.7 \pm 0.87$	$0.6 \pm 0.23$	$5.2 \pm 0.81$	$3.1 \pm 0.43$ *	$-2.1 \pm 0.38$
CAL (mm)	$6.6 \pm 1.10$	$7.4 \pm 1.30$	$0.8 \pm 0.20$	$6.6 \pm 1.90$	$2.1 \pm 1.50$ *	$-4.5 \pm 0.40$

Abbreviations: PPD, probing pocket depth; CAL, clinical attachment loss. Values are means  $\pm$  SD. \* Repeated measures analysis of variance  $p < 0.05$ .

Regarding bleeding by the dental probe and dental mobility, a significant decrease in both clinical symptoms was observed after treatment in the EG compared with PG ( $p < 0.001$ ) (Table 2).

**Table 2.** Periodontal clinical changes baseline and post-treatment by group.

	Placebo Group (n = 30)		Experimental Group (n = 30)	
	Baseline	Post-Treatment	Baseline	Post-Treatment
DM f (%)				
Yes	17 (57)	21 (70)	20 (68)	2 (8) *
No	13 (43)	9 (30)	10 (32)	28 (92)
BOP f (%)				
Yes	16 (52)	12 (40)	16 (53)	2 (7) *
No	14 (48)	18 (60)	14 (47)	28 (93)

Abbreviations: f, frequency; DM, dental mobility; BOP, bleeding on probing.  $\chi^2$ -test control (3 months) vs. treatment (3 months) \*  $p < 0.0001$ .

### 3.2. Effect of *T. lucida* on Oxidative Stress Markers

Concerning oxidative stress markers in saliva, at the end of the three-month treatment with the *T. lucida* mouthwash, a significant decrease in lipoperoxides levels was observed in the EG group compared with PG ( $p < 0.05$ ). In contrast, there was an increase in both total antioxidant capacity and superoxide dismutase activity, although these were not statistically significant ( $p > 0.05$ ) (Table 3).

**Table 3.** Oxidative stress markers baseline and post-treatment by group.

	Placebo Group (n = 30)		Experimental Group (n = 30)	
	Baseline	Post-Treatment	Baseline	Post-Treatment
LPO ( $\mu\text{mol/L}$ )	0.049 $\pm$ 0.02	0.054 $\pm$ 0.06	0.056 $\pm$ 0.03	0.034 $\pm$ 0.02 *
TAS (mmol/L)	0.55 $\pm$ 0.39	0.66 $\pm$ 0.42	0.58 $\pm$ 0.36	0.71 $\pm$ 0.47
SOD (IU/L)	1.60 $\pm$ 0.52	1.50 $\pm$ 0.54	1.56 $\pm$ 0.52	1.64 $\pm$ 0.49

Values are means  $\pm$  SD. \* Repeated measures analysis of variance  $p < 0.05$ . LPO: lipoperoxides TAS: total antioxidant status, SOD: superoxide dismutase.

### 3.3. Effect of *T. lucida* on Inflammatory Markers

A significant decrease in interleukin 1 $\beta$ , interleukin IL-8, and TNF- $\alpha$  concentration in saliva was found after three months of treatment with *T. lucida* compared with PG ( $p < 0.05$ ) (Table 4).

**Table 4.** Inflammatory markers, baseline, and post-treatment by group.

	Placebo Group (n = 30)		Experimental Group (n = 30)	
	Baseline	Post-Treatment	Baseline	Post-Treatment
IL-1 $\beta$ (pg/mL)	774 $\pm$ 1332	661 $\pm$ 483	871 $\pm$ 1126	462 $\pm$ 800.9 *
IL-8 (pg/mL)	448 $\pm$ 343	571 $\pm$ 310	827 $\pm$ 647	624 $\pm$ 494 *
TNF- $\alpha$ (pg/mL)	3.7 $\pm$ 1.5	4.4 $\pm$ 1.6	5.2 $\pm$ 2.6	3.3 $\pm$ 0.84 *

Values are means  $\pm$  SD. \* Repeated measures analysis of variance  $p < 0.05$ . IL-1 $\beta$ : interleukin 1 $\beta$ ; IL-8: interleukin 8; TNF- $\alpha$ : tumor necrosis factor alpha.

## 4. Discussion

Chronic periodontitis (CP) is a degenerative and inflammatory disease with damage and loss of connective tissue, which in general is associated with the activation of

polymorphonuclear leukocytes that generate reactive oxygen species (ROS) [28,29]. It has also been linked with physiopathological mechanisms of numerous aging-related diseases which lead to the production of ROS during inflammatory conditions [30–32]. In this sense, medicinal herbal medicine can be a therapeutic alternative in the dental field, since research has been carried out to support the use of various plant species such as *T. lucida*, which, due to its chemical composition, has anti-inflammatory and antioxidant properties. It could have a positive effect on chronic periodontitis, as an alternative treatment or in parallel with another dental treatment [18,33].

The results showed that older adults with CP treated with the *T. lucida* mouthwash for 3 months had a significant decrease in concentration of lipoperoxides associated with an improvement in the signs and symptoms of CP compared to untreated older adults. This finding supports the proposal of the antioxidant effect of *T. lucida* [11]. Additionally, the decrease in ROS and the increase in the efficiency of the antioxidant system agree with results reported by some authors who found an association between decrease of oxidative stress and CP [34–38]. Consequently, the excess of ROS can selectively damage the proteoglycans associated with soft periodontal tissues and alveolar bone, activating nuclear factor kappa B (NF- $\kappa$ B) that triggers signaling cascades that activate osteoclasts, leading to inflammation [35,39–42].

On the other hand, an increase in the total antioxidant capacity and in the activity of the SOD enzyme was observed, although not statistically significant, probably due to the sample size. Similarly, there have been studies conducted with different treatments based on plant species, such as *Commiphora myrrha*, *Mentha piperita*, *Bursa Pastoris*, *Clematis ternifolia*, *Timus vulgaris*, and *Aquillea millefolium*, at different times of intervention and in some cases, changing hygienic-dietary habits of the patients [32–37]. In our study, we observed that the post-treatment concentration of lipoperoxides decreased significantly ( $p \leq 0.05$ ). In this regard, other studies have reported a statistically significant decrease in the concentration of lipoperoxides, when performing periodontal treatments; however, such treatments increase damage to periodontal tissues caused by the scraping and curettage procedure [43].

Likewise, some studies have shown that the salivary system is formed by some enzymes such as SOD. This enzyme is capable of increasing in response to different inflammatory diseases such as pulpitis and periodontitis. SOD is able to protect the oral cavity against the effects of ROS, which could be considered a partial compensatory response when there is oxidative stress in the cavity, and SOD represents the first defense in the antioxidant tissues [44,45].

In this work, the results of oxidative stress markers show a global improvement in the EG by decreasing lipoperoxidation, preserving the activity of SOD, and increasing the total antioxidant activity.

Regarding the levels of pro-inflammatory interleukins, it was observed that they decreased significantly in the treatment group with *T. lucida*, an effect due to the decrease in the periodontal infectious process. Therefore, the decrease in the secretion of bacterial lipopolysaccharides is caused by a decrease in the secretion of these interleukins of the macrophages, and consequently the decrease in osteoclastic activity and expression of adhesion molecules to the vascular endothelium [46,47].

Accordingly, some animals studies where CP has been induced and treated with some plants rich in curcumin, tannins, and phenolic compounds showed a significant decrease in TNF- $\alpha$  [48].

In this sense, it is essential to consider that extracts of *T. lucida* have a potential antiseptic effect; this bactericidal activity decreased the microorganisms number leading to anti-inflammatory effects and decreased oxidative stress [11,14,19].

This was clinically observed with the favorable modifications found in the clinical evolution of CP in the group treated with *T. lucida*, since the CAL and the PPD decreased in this group ( $p < 0.05$ ); probably due to the decrease in periodontopathogens and leukocyte infiltrate associated with the reduced concentrations of pro-inflammatory cytokines. Additionally, the decrease in periodontal pockets was probably due to the osteoclastic action

did not continue because the secretion of IL-1 $\beta$  and TNF- $\alpha$  decreased [49,50]. Furthermore, mobility and gingival bleeding reduced significantly in the EG ( $p < 0.05$ ) due to the restoration of periodontal tissue integrity and the fibroblastic protective action of *T. lucida* quercetin [50].

Finally, it is important to point out as limitations of the study: (i) the reduced sample size to measure the effect on all oxidative stress markers; (ii) the groups were not proportional by sex, consequently, the influence of this variable could not be evaluated; (iii) study follow-up was only once at 3 months, so the effectiveness over time cannot be guaranteed; (iv) the plaque index was not measured; and (v) a comparison group with a mouth rinse as the gold standard was not included, such as 0.12% chlorhexidine [51,52]. In this sense, it is advisable to carry out a longitudinal study with subsequent clinical evaluations considering the indicated limitations.

## 5. Conclusions

Our findings suggest that *T. lucida* has an antioxidant and anti-inflammatory effect in older adult subjects with CP, presumably due to the presence of curcumin and quercetin contained in this plant.

**Author Contributions:** T.M.-C.: investigation, methodology, visualization, and writing—original draft. R.R.-U.: conceptualization, investigation, methodology, project administration, resources, supervision, writing—original draft. M.L.-H.: data curation, formal analysis, methodology. M.R.-R.: data curation, formal analysis, methodology. V.M.M.-N.: formal analysis, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of Universidad Nacional Autónoma de México (UNAM), Zaragoza Campus (protocol code FESZ/DEPI/325/16).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are because they are owned by UNAM.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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