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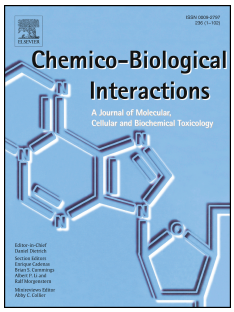

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Abstract

Preclinical repeated-dose toxicity and efficiency studies developed by our group suggest the potential of FITOPROT in treating mucositis. This serious limiting side effect is observed at a rate of 40–100% in patients under antineoplastic therapy and despite different palliative measures and therapeutic agents have been investigated, still no therapy was completely successful. Therefore, this study aimed to establish the safety and recommended phase II dose of FITOPROT for the prevention and treatment of chemoradiotherapy-induced oral mucositis (OM) in patients with head and neck cancer. Twenty healthy adult participants were randomized into two groups that received pre-established concentrations of the collutory: group 1 (FITOPROT A - mucoadhesive formulation containing 10 mg/mL of curcuminoids extract plus 20% v/v of *Bidens pilosa* L. extract) and group 2 (FITOPROT B - mucoadhesive formulation containing 20 mg/mL of curcuminoids extract, plus 40% v/v of *Bidens pilosa* L. extract). Participants rinsed their mouths with FITOPROT, three times daily, for ten consecutive days. No participant experienced toxicity or unacceptable discomfort and/or adverse reactions (CTCAE v5.0), with laboratory and clinical parameters under normal conditions. Side effects observed were low intensity and temporary mucosa/dental surface pigmentation (n=7) and tooth sensitivity (n=4), which disappeared after formulation use ceased. No significant cellular genotoxic effects were observed ($p>0.05$), and micronuclei frequencies were not changed ($p>0.05$). Biochemical assays reveled no altered levels of myeloperoxidase ($p=0.2268$), malondialdehyde ($p=0.1188$) nor nitric oxide ($p=0.5709$) concentration, and no significant difference were found in the levels of pro-inflammatory cytokines ($p>0.05$). Thus, FITOPROT demonstrated to be safe and tolerable in both tested doses and is suitable for evaluation in a phase II trial as treatment against OM.

Keywords: Phase I Clinical Trial, Oral mucositis, Chemoradiotherapy, Curcuminoids, *Bidens pilosa* Linn extract.

1. Introduction

Oral mucositis (OM) is described as a frequent and painful debilitating inflammation of the oral mucosa that varies from mild mucosal erythema to severe ulceration [1]. It is one of the main side effects of antineoplastic therapies and is observed at a rate of 40–100%, conditioned to cancer type, treatment regimen, neutrophil count, oral hygiene, nutritional status and patient's age [2,3]. Depending on severity, OM may trigger an inability to tolerate food or fluids, which leads to malnutrition, dehydration and weight loss [4]. Furthermore, it limits the effectiveness of anticancer therapy, increases hospitalization costs, and may even lead to interruption in chemoradiotherapy protocols, which reduces the chances of healing and patients' survival [5,6].

Different palliative measures and therapeutic agents have been investigated for prevention and treatment of OM [7,8]. Some interventions like basic oral care protocols, analgesics, cryotherapy, anti-inflammatories, antimicrobial agents, growth factors, probiotics, antioxidants, coating agents, laser therapy and herbal drugs were found to aid in preventing or reducing the severity of mucositis, but still no approach has been completely successful [9-11]. Therefore, there is a need to develop therapeutic strategies to prevent and treat OM.

Plant extracts such as curcuminoids and *Bidens pilosa* Linn have received great attention in the last decades due to their wide-ranging properties [12,13]. Curcuminoids are a mixture of antioxidant polyphenols from the root of *Curcuma longa* (Zingiberaceae). This plant is commonly used as a spice component, as well as in traditional herbal medicine. Pharmacological effects of curcuminoids have aroused interest in the last decades based on their application in treating inflammatory diseases [14,15] and antiproliferative and proapoptotic effects against tumors [16]. *Bidens pilosa* L. (Asteraceae) is a South American ruderal plant by origin and can also be found in tropical and subtropical regions worldwide [13]. Nearly 200 different bioactive

compounds present in *Bidens pilosa* L., mainly flavonoids, diterpenes, hydrocarbons and terpenoids, have been reported to exert anti-inflammatory, antioxidative, immunomodulatory, and antiulcerogenic activities [17,18].

Non-clinical (*in vitro*) and preclinical studies using the mucoadhesive formulation addressed here based on curcuminoids and *Bidens pilosa* L. extract were performed in HaCaT human immortalized keratinocytes, and in mice bearing intestinal mucositis induced by 5-fluorouracil (5-FU). Promising results were observed in relation to both safety and effectiveness. Formulations exerted protective action against mucosal damage by stimulating cell proliferation, reducing neutrophilic infiltrate and consequently inflammation, and decreasing levels of reactive oxygen species (ROS) as a result of antioxidant action [19-22].

Therapy based on mucoadhesive formulations containing curcuminoids and *Bidens pilosa* L. extract have their advantages. These include, accessibility, low cost and easy to find constituents which prolong the contact time between extracts components and mucosa, and provide interesting topical alternatives to prevent and treat mucositis. Given this evidence, the goals of the present phase I study were to assure the safety dose to healthy individuals and to strengthen the next step, a phase II study using the mucoadhesive formulation containing curcuminoids and *Bidens pilosa* L. extract (FITOPROT) with prophylactic and curative purposes in patients bearing OM.

2. Methods

This study was designed as a randomized double-blinded clinical trial - phase I. It was approved by the Institutional Research Ethics Committee at the Federal University of Goiás (protocol 206/2012) and registered in the Brazilian Clinical Trials Database - ReBEC (protocol RBR□8rqy3z). This investigation was also conducted in full accordance with the ethical principles of the World Medical Association Helsinki Declaration. All participants were 18 years and over and signed an informed consent form.

2.1. Mucoadhesive formulations containing curcuminoids and *Bidens pilosa* L. extract (FITOPROT)

The pilot batch of FITOPROT used in this study was produced in cooperation with FBMFARMA Indústria Farmacêutica, a Brazilian Pharmaceutical Company. The preparation started by mixing *Bidens pilosa* L. glycolic extract (Chemyunion - Ecobidens®, Sorocaba, SP, Brazil) and 40% of the total mass of poloxamer 407 (Basf, Ludwigshafen, RP, Germany) in a reactor under mechanical stirring. Then, sodium metabisulphite and sodium bisulphite were added under stirring until complete dispersion. This mixture (called mixture 1) was then reserved in suitable container.

A second mixture was prepared by adding the total volume of polyethylene glycol 400 and propilenglycol (1:1, v/v) in a heated reactor (65–70°C). Next, 60% of the total mass of poloxamer 407 was added to the mixture under mechanical stirring until complete dispersion. Later, Macrogol (15)-hydroxystearate (Basf - Soluplus®, Ludwigshafen, RP, Germany) and curcuminoids from *Curcuma longa* Linn extract (>95% curcuminoid content, Gamma

Comércio Importação & Exportação LTDA, São Paulo, SP, Brazil) were added, and the resulting homogenate (called mixture 2) was maintained under stirring for 30 minutes. After this period, mixture 1 was poured into mixture 2 and the resulting material was kept stirring until complete dispersion for more 30 minutes. Lastly, pH was adjusted by using 0.1 M citric acid (pH 6.5) and formulations were then bottled and stored at room temperature.

FITOPROT A consisted of a mucoadhesive formulation containing 10 mg/mL of curcuminoids extract plus 20% v/v of *Bidens pilosa* L. extract; and FITOPROT B consisted of a mucoadhesive formulation containing 20 mg/mL of curcuminoids extract plus 40% v/v of *Bidens pilosa* L. extract. The patent application for FITOPROT has been requested in Europe, Brazil, Russia, Canada, USA and Japan. All petitions are under analysis.

Collutory concentrations were established by non-clinical and preclinical multiple repeated-dose safety and efficacy studies as described by ÁVILA et al. [19], BASTOS et al. [20], SANTOS FILHO et al. [21] and SANTOS FILHO et al. [22]. To determine the two human doses, the no-observed-adverse-effect-level (NOAEL) 1.000 mg/kg of extracts per kg of body weight were considered. Maximum daily concentration of curcuminoids extract were 900 mg, and 18.000 mg for *Bidens pilosa* L. Pharmaceutical manufacturing technology was applied to all samples to achieve identical color, flavor, and texture. Additionally, phytochemical characterization by liquid chromatography-mass spectrometry (LC-MS) identified the following flavonoids: rutin, glucoronylated quercetin and dimethylquercetin rutenoside. Other results on phytochemical characterization are described by SANTOS FILHO et al. [22].

Participants were randomly assigned to receive either FITOPROT A or FITOPROT B by selection of a computer-generated randomization list. No stratification was performed for either cohort of volunteers. FITOPROT was stable under normal conditions (60% relative humidity,

25°C) for the period of time covering the duration of the trial and under conditions of accelerated decomposition (75% relative humidity, 40°C) for at least 180 days.

Each participant received 100 mL of FITOPROT provided in amber glass bottle and were instructed to keep the formulation out of the reach of children, to store it at room temperature, do not freeze and protect from sunlight. Furthermore, participants should return all unused medication to subsequent incineration.

2.2. Participant demographics and experimental design

Thirty healthy adult eligible participants, both genders, were signed to the Head and Neck Department at *Hospital Araújo Jorge/Associação de Combate ao Câncer de Goiás* (HAJ/ACCG) to comparatively analyze the possible potential adverse effects of FITOPROT in two pre-established concentrations. Participants from group 1 received FITOPROT A, and group 2 received FITOPROT B (Fig. 1).

Participants eligible criteria were as follow: 18–65 years of age; no prior medication use; adequate bone marrow function (white blood cell count 4.500–11.000/mm^3, neutrophil count 1.800–7.700/mm^3, platelet count 150.000–400.000/mm^3, and hemoglobin 12.8–17.8 g/dL); adequate liver function (aspartate aminotransferase/alanine aminotransferase (AST/ALT) <2.0 times the upper limit of normal); adequate renal function (urea 10–40 mg/dL, creatinine 0.60–1.30 mg/dL, uric acid 3.0–7.0 mg/dL); adequate glycemic level (blood glucose 70–99 mg/dL); adequate heart function (normal resting heart rates range from 60–100 bpm); adequate oral intake and health (gingival indices and standard plaque), and participants were asked to abstain from the consumption of foods containing the examined vegetal extracts during the study period.

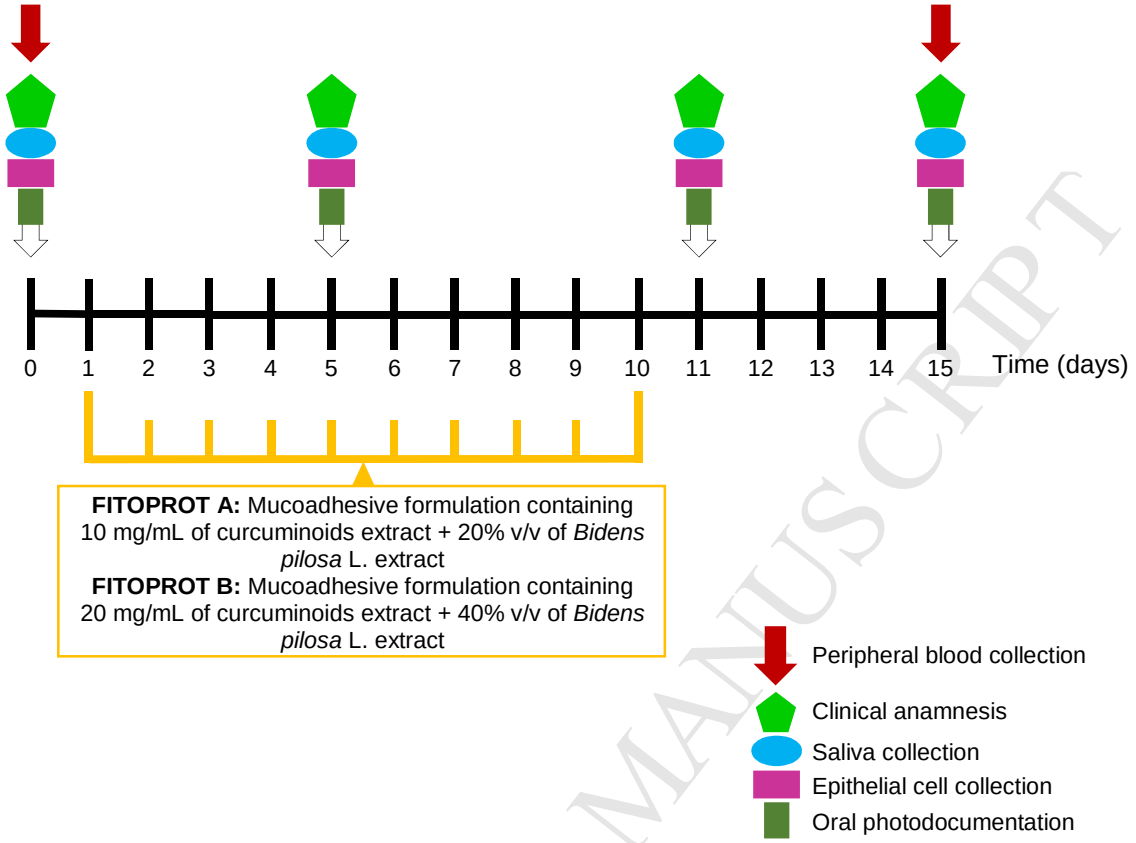
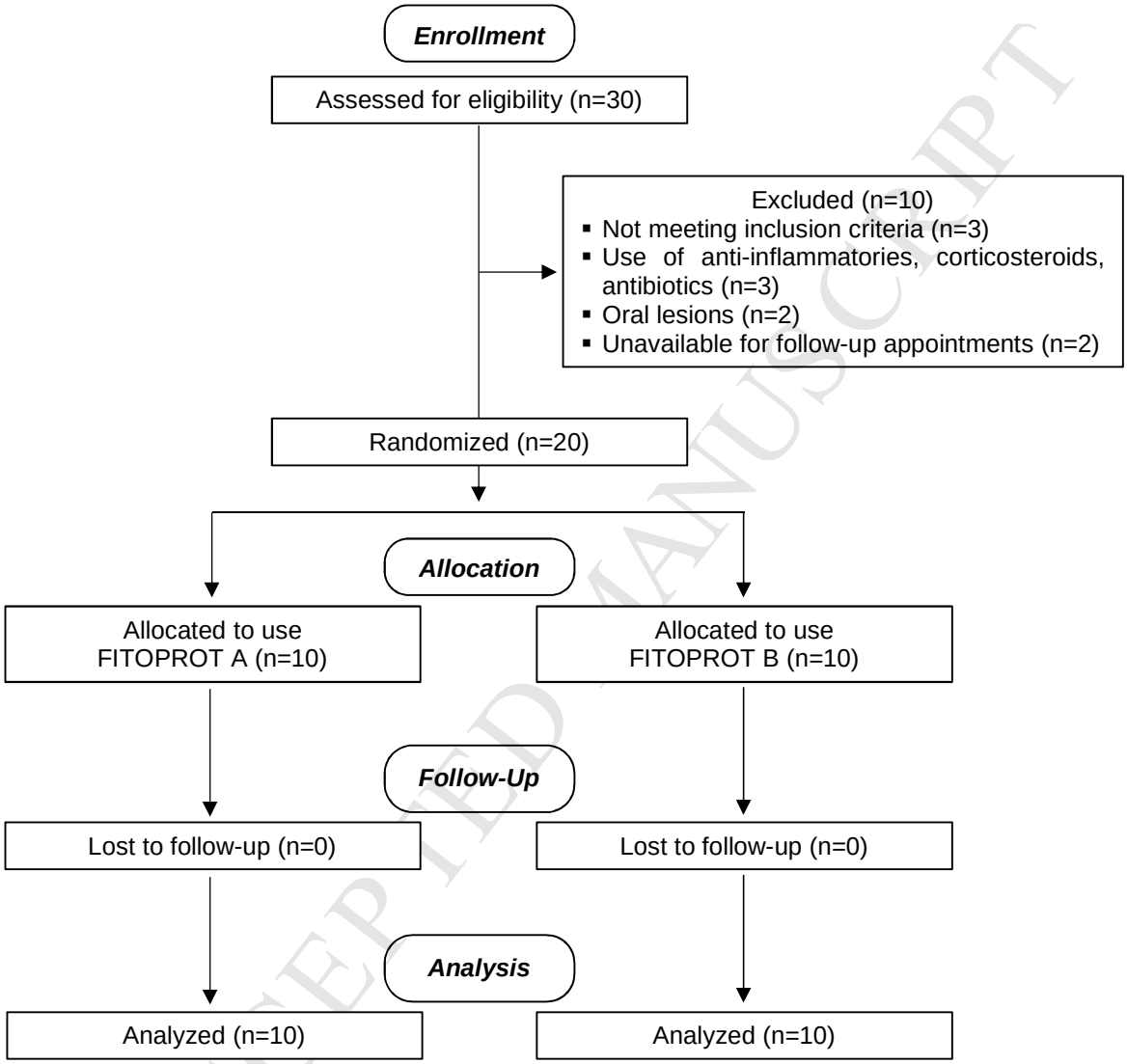


Fig. 1. Experimental study design.

Exclusion criteria included: pregnancy or nursing infants; smoking; major surgery within the past 4 weeks; a history of hypersensitivity or allergy to the vegetal extracts used in this study; periodontal disease; autoimmune diseases; any medical condition that would impair the administration of collutory, including viral infection; and any uncontrolled serious medical or psychiatric illness.

Selected participants were randomly assigned on a 1:1 ratio using Research Randomizer website (http://www.randomizer.org/). Calculated sample size per group was 15, considering α =

204 0.05, study power = 95%, and effect size = 2.0 [23,24]. The study randomization is shown in Fig.
205 2.



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207 **Fig. 2.** Study flow diagram.
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210 A statistician provided both randomization lists to the main supervisor, so participants
211 and the clinical research team (who assessed outcomes) were blinded to the treatment assignment

throughout the study. At participant enrollment, the research team notified the main supervisor, which ascribed the participant to sequential slot and treatment from the appropriate randomization list. Right after, the main supervisor supplied the research team with the blinded study medication and at the study completion, the main supervisor transferred the statistician with the two-randomization lists for analysis, which included individual treatment assignments.

Participants rinsed their mouths and had a gargle with 15 mL of FITOPROT for 60 seconds, three times daily, for ten consecutive days. Right after each use, the solution was expelled, and they were told not to eat or drink for at least 20 minutes. Furthermore, participants were asked to follow a standard oral hygiene regimen throughout the study.

2.3. Toxicity Evaluation

To assess possible local and systemic toxicity effects, all participants were subjected to four clinical evaluations. The first was performed one day before participants started using FITOPROT; the second was on the fifth day; the third took place one day after collutory use had stopped; and the fourth was five days after exposure had concluded (Fig. 1).

During each evaluation, a complete anamnesis was carried out to identify potential discomfort and/or adverse reactions related to formulation use. Clinical outcomes were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0, based on mild (Grade 1), moderate (Grade 2), severe (Grade 3), or life-threatening (Grade 4), with specific parameters according to the organ system involved. Safety was assessed throughout the study by physical and chemistry laboratory examination, including hematologic, hepatic, renal and glycemic evaluation. Inspection of oral tissues, change in salivary flow, and extraoral and intraoral photo documentation were collected to record possible damage and/or adverse events in

facial region, lips, mucosa and tooth structure. Additionally, participants received a form to report possible clinical occurrences daily over the 10-day span of FITOPROT use.

2.3.1. Oral mucosal epithelial cells collection

After each clinical examination, the oral mucosa was dried with a gauze swab to remove surface debris and excess saliva. Smears were taken from the buccal mucosa (cheek) of each participant using a swab and transferred to clean, dry glass slides. These were then immediately sprayed with a commercial fixative solution containing 95% ethyl alcohol (Kolplast ci Ltda, Itupeva, SP, Brazil), and processed for cytomorphological assessment by 4',6-diamidino-2-phenylindole (DAPI) and Papanicolaou staining as modified methods described by ALBERTI et al. [25], NERSESYAN et al. [26] and PALASKAR & JINDAL [27]. All stains were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analysis for possible genotoxic effects of FITOPROT upon oral mucosal epithelial cells included the formation of micronuclei, cellular binucleation, changes in morphology and/or cell size, broken eggs induction, cytoplasmic granules and apoptosis. Slides were assessed by two experience scorers, using both staining procedure and considering at least 1.000 cells per slide. Papanicolaou-stained slides were evaluated under a light microscope (Axioscope A1; Carl Zeiss, Jena, Germany); and DAPI-stained slides were evaluated under a fluorescence microscope (Axioscope A1; Carl Zeiss, Jena, Germany, filter UV-2a, 330–380nm) both with 1.000-fold magnification.

2.3.2. Saliva collection

Participants were told not to eat or drink for an hour before the procedure. Saliva samples were collected in the morning during each clinical evaluation according to a modified method

proposed by NAVAZESH [28]. They were instructed to wash their mouths with purified water, swallow all saliva in the mouth and then split for five continuous minutes into a plastic sterile tube.

Then, saliva samples were centrifuged at 10.000 rpm, 4ºC for 10 minutes, and supernatants were frozen at -80°C to proceed with myeloperoxidase (MPO) activity assay, malondialdehyde (MDA) determination, nitric oxide (NO) concentration and measurement of inflammatory cytokines.

2.3.2.1. Myeloperoxidase (MPO) activity assay

MPO is a hemic enzyme stored primarily in the azurophilic granules of the neutrophils. MPO behaves as a host defense mechanism mediating microbial killing substances and contributes to the initiation and propagation of inflammatory reactions [29]. It has been reported that MPO may be used as a biomarker for oral mucosal damage, such as periodontal disease [30]. In a 96-well microplate (TPP, Trasadingen, Switzerland), 10 µL of saliva were mixed with 100 µL of 0.3 M Tris-HCl buffer (pH 7.5), 50 µL 0.53 mM *O*-dianisidine, and 100 µL 0.15 mM H_2O_2. The microplate was then incubated for 15 minutes at room temperature and the absorbance reading was performed at 450 nm. Results were expressed as active MPO unit per milliliter of saliva [31].

2.3.2.2. Malondialdehyde (MDA) determination

MDA is the main and the most widely studied indicator of lipid peroxidation. Its interaction with DNA and proteins are correlated to several diseases and is often referred as

potentially mutagenic [32]. The method described by OHKAWA et al. [33] was used for salivary MDA determination assay, and data were expressed as nanomoles of MDA per milliliter saliva.

2.3.2.3. Nitric Oxide (NO) concentration

Salivary NO concentration is an interesting approach to detect tissue injury since NO is synthesized in substantial amounts by activated macrophages and can cause damaging effects against cell organelles eventually leading to cell death, tissue damage and organ injury [34].

Salivary NO concentration was determined according to the method described by MIRANDA et al. [35]. Microplates with pink colored chromophore were measured at 540 nm. Pure water was used as blank, and a sodium nitrite standard curve was established to calculate nitrite in saliva.

2.3.2.4. Measurement of inflammatory cytokines

Concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF were quantified in saliva samples of all participants using a Cytometric Bead Array Human Inflammation kit (CBA, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, all six Capture Beads were pooled immediately before the assay, processed, homogenized and incubated for 30 minutes at room temperature, protected from light. Thereafter, participants saliva samples and PE Detection Reagent were added to each assay tube (to form sandwich complexes) and incubated for 3 hours at room temperature, protected from light. Lastly, samples were washed twice and suspended in washing buffer.

The acquisition of the events was performed by BD FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA), the individual cytokine concentration (pg/mL) was

303 determined using the standard reference curve BD CellQuest, and analysis of results were
304 accomplished by FCAP Array software.
305
306 **2.4. Statistical analysis**
307 Paired T-test and one-way ANOVA were used for statistical analysis for the clinical
308 parameters and genotoxic effects data. In biochemical analyses, all values were presented as
309 mean ± standard deviation and statistical analysis was performed by Kruskall-Wallis Test and
310 Mann-Whitney Test. A p-value <0.05 was regarded as statistically significant.
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3. Results

3.1. Participant demographics and treatment

Twenty participants (11 female, 9 male; 17 white, 3 brown) of age range 19 to 46 years (mean, 26.5 years) were enrolled and used FITOPROT as a collutory daily, in the two predetermined concentration for ten consecutive days. The main demographics characteristics of the study sample are presented in Table 1.

Table 1. Baseline demographics and participant characteristics selected for research.

	n=20	
Characteristics	*n*	%
Sex		
Male	9	45
Female	11	55
Median age	26.5 (range 19–46)	
Skin color		
White	17	85
Brown	3	15
Tobacco		
Yes	0	0
No	20	100
Alcohol consumption		
Yes	6	30
No	14	70
Toothbrushing frequency per day		
0	0	0
1-2	6	30
3-4	14	70
Dental flossing frequency per day		
0	1	5
1	14	70

2	5	25
Consumes pigmented food		
Yes	16	80
No	4	20
Eventual gingival bleeding		
Yes	7	35
No	13	65
Stomach Disorder		
Yes	2	10
No	18	90
Respiratory Disorder		
Yes	3	15
No	17	85
Headache		
Yes	5	25
No	15	75
Allergies		
Yes	1	5
No	19	95
Familial diabetes		
Yes	7	35
No	13	65

No volunteer experienced toxicity or unacceptable discomfort and/or adverse reactions (Fig. 3). Two participants who used FITOPROT A and two who used FITOPROT B related tooth sensitivity (CTCAE, grade 1). Temporary dental surface pigmentation was observed in two participants (20%) who used FITOPROT A and in five participants (50%) who used FITOPROT B during the ten days of collutory use (CTCAE, grade 1). This pigmentation was no longer observed in the fourth clinical examination. Only one participant from each group experienced nausea (CTCAE, grade 1) reported from day 3 to day 6 of FITOPROT use. Interestingly, this symptom was spontaneously solved. Hematologic evaluation of one participant who used FITOPROT B detected thrombocytopenia (CTCAE, grade 1), and another participant from the same group exhibited hyperglycemia (CTCAE, grade 1). These blood alterations may be

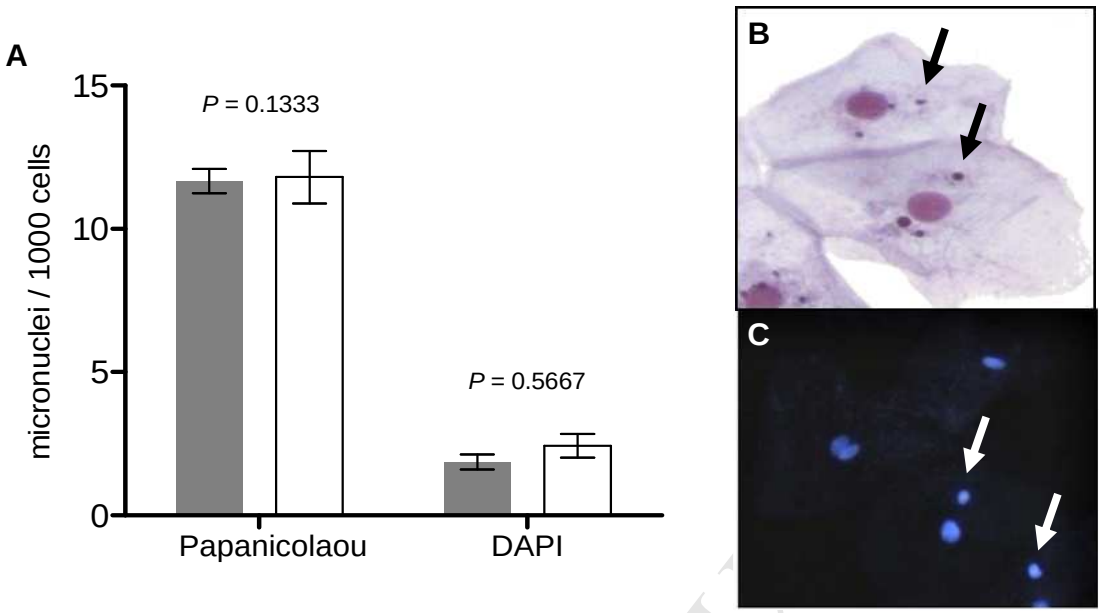
considered random with no correlation to FITOPROT use since the formulation acts as a topical mucoadhesive alternative and was expelled from mouth soon after each use. Otherwise, curcuminoids have low bioavailability and poor aqueous solubility and papers report curcuminoids exert hypoglycemic effect [36,37].

Fig. 3. Participant rinsing her mouth with FITOPROT in day 1 (A); after 10 days of formulation use (B); and fourth clinical evaluation (C), five days after exposure had concluded. No clinical change in soft tissue or dental structures was observed ($p>0.05$).

All other chemistry laboratory and clinical parameters analyzed including; liver and renal functions, burning, edema, erythema, pain, desquamation, odor, difficulty in brushing, appetite loss, headache, vomiting, ulceration, and changes in salivary flow were not changed or mentioned for alteration during FITOPROT use.

3.2. Genotoxic effects upon oral mucosal epithelial cells

In Fig. 4 it can be seen that micronuclei formation scored by Papanicolaou stain were significantly (~9.5-fold) higher than in DAPI for both FITOPROT concentrations. On the contrary, no significant effects were observed between FITOPROT A and B with the different staining procedures ($p=0.1333$ for DAPI and $p=0.5667$ for Papanicolaou stain). Also,



370 demographics and clinical features, such as gender, age, alcohol consumption, daily brushing and
371 flossing frequency did not significantly affect the micronuclei frequencies in any staining
372 methods and these variables had also no effect on the differences between participants who used
373 FITOPROT A or FITOPROT B.

374
375
376 **Fig. 4.** Micronucleus assay by Papanicolaou and DAPI staining from participants submitted to
377 FITOPROT A (shaded columns) and FITOPROT B (open columns), both n=10. From each participant, at
378 least 1000 cells were scored for micronuclei formation. In A, Columns as mean; bars, 95% CI (paired T-
379 test). B represents oral epithelial cells in smears stained by Papanicolaou method and C, DAPI staining.
380 Arrows show micronuclei (magnification, 1000 times).
381

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383 Likewise, in a cytology analysis, FITOPROT in both concentrations did not induce
384 significant changes in morphology and/or cell size, nor induced cytoplasmic granules, broken
385 eggs, apoptosis or cellular binucleation.

386

387 **3.3. Biochemical Analyses**

388 The results of biochemical analyses in total saliva samples are outlined in Table 2.
389 Salivary concentrations of MPO were ~14% higher in participants who used FITOPROT B
390 (0.07±0.02 UMPO/mL saliva) than in participants who used FITOPROT A (0.06±0.02
391 UMPO/mL saliva), and values did not reach statistical significance (p=0.2268).

392 Mean salivary concentrations of MDA was similar among participants who used
393 FITOPROT A versus FITOPROT B, respectively 0.11±0.03 nmol/mL and 0.13±0.05 nmol/mL,
394 and no statistical significance was observed (p=0.1188).

395 For salivary NO concentration, the mean value was ~11% higher in the group of
396 participants who used FITOPROT B (0.35±0.22 µmol/mL) than in the group who used

Table 2. Biochemical detailed data from saliva samples.

Total saliva	FITOPROT A (*n*=10)	FITOPROT B (*n*=10)	*P*
MPO (U/mL)	0.06 ± 0.02	0.07 ± 0.02	0.2268
MDA (nmol/mL)	0.11 ± 0.03	0.13 ± 0.05	0.1188
NO (µmol/mL)	0.31 ± 0.57	0.35 ± 0.22	0.5709

397 FITOPROT A (0.31±0.57 µmol/mL). However, these differences were not statistically
398 significant (p=0.5709).

MPO, myeloperoxidase; MDA, malondialdehyde; NO, nitric oxide.
All data presented as mean ± standard deviation. (Kruskall-Wallis Test for MPO and NO; Mann-Whitney Test for MDA).

3.4. Cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF levels

As noted in Table 3, no significant differences were observed in IL-1β, IL-6, IL-8, IL-12p70 and TNF salivary cytokine concentrations between participants who used FITOPROT A versus FITOPROT B. However, IL-10 level was higher in FITOPROT B than in FITOPROT A. Still, the secretion of all measured cytokines did not differ among the four clinical evaluations nor were they affected by demographics and clinical features. Additionally, salivary cytokine levels found were under physiological standards [38-40].

Table 3. Comparison of salivary cytokine concentrations between participants who used FITOPROT A versus FITOPROT B.

Cytokines	Group	Mean	SD	Median	Min	Max	*n*	p-value
IL-1β (pg/mL)	FITOPROT A	117.57	110.46	82.16	11.74	465.84	10	0.3808
	FITOPROT B	134.61	233.18	54.69	13.12	1121.99	10	
IL-6 (pg/mL)	FITOPROT A	12.82	7.91	11.77	3.66	31.5	10	0.1033
	FITOPROT B	32.82	50.53	15.12	4.46	202.96	10	
IL-8 (pg/mL)	FITOPROT A	1696.09	1384.11	1348.28	135.96	5224.32	10	0.3481
	FITOPROT B	1244.55	985.66	1002.47	299.02	4446.88	10	
IL-10 (pg/mL)	FITOPROT A	2.77	0.8	2.92	0.93	3.84	10	0.0368*
	FITOPROT B	3.49	1.34	3.35	0.22	7.66	10	
IL-12p70 (pg/mL)	FITOPROT A	2.53	1.24	2.46	0	5.38	10	0.0549

	FITOPROT B	3.7	2.43	3.24	0	11.72	10	
TNF (pg/mL)	FITOPROT A	2.24	1.65	2.12	0	5.26	10	0.4892
	FITOPROT B	2.88	2.69	2.64	0	13.7	10	

SD, standard deviation; Min, minimum; Max, maximum; IL, interleukin; TNF, tumor necrosis factor. Statistical significance *P<0.05. Mann-Whitney Test.

4. Discussion

The study presented here provides the first report of safety in FITOPROT use by humans, and is likely to be of value in a phase II trial against OM induced by anticancer treatments. The topical use (mouthwash) of the mucoadhesive formulation containing curcuminoids and *Bidens pilosa* L. extract by healthy individuals was biochemical, cytological and clinically safe in both doses tested. The main side effects observed with FITOPROT use were temporary mucosa/dental surface pigmentation and tooth sensitivity. The pigmentation besides low intensity, was temporarily and disappeared after the use of formulation ceased. Moreover, soft tissue pigmentation is predicted since it reflects the desired mucoadhesive potential of FITOPROT. Three of the four participants who reported tooth sensitivity had already used orthodontic braces

and had exposed root dentin. Additionally, this discomfort was not aggravated by FITOPROT use.

Investigations on medicinal properties of curcuminoids from *Curcuma longa* Linn have provided many *in vivo* effects, such as anti-inflammatory [14], antioxidant [41], and antibacterial [42]. Likewise, *Bidens pilosa* L. extract and/or compounds are reported to have antioxidant [43], anti-inflammatory [44], immunomodulatory [45] and anti-ulcerative properties [46].

OM induced by anticancer treatments is a painful and debilitating side effect, and there is a need for the development of strategies to prevent and manage this complication. A variety of studies has been conducted in the last years proposing mouthwashes for the treatment and/or prevention of OM. Nevertheless, none approach reached gold standard efficacy. Clinical studies conducted by SARVIZADEH et al. [47] observed that morphine mouthwashes were effective in reducing pain and OM induced by head and neck cancer treatments for some hours after use. Likewise, mouthwashes containing granulocyte-macrophage colony-stimulating factor and sucralfate [48], *Aloe vera* [49] and green tea extract [50] have shown improve in mucositis already established symptoms. Povidone-iodine, NaCl 0.9%, water salt soda solution and chamomile mouthwash were also recommended. However, the effectiveness is still unclear [51].

ELAD et al. [52] assessed the tolerability of a curcumin mouthwash for the prevention of OM in pediatric patients, and no adverse events were documented. FRANCIS & WILLIAMS [53] published a study in which curcuminoids with honey was effective as complementary therapy on treatment-induced OM. In another pilot study involving a proposed mouthwash to treat OM, DIAZ-SANCHEZ et al. [54] concluded that the use of bioadhesive chlorhexidine gel 0.2% does not reduce the frequency of mucositis in the oral cavity or concomitant pain.

In our study, it was seen the pharmaceutical technology applied in FITOPROT development was succeeded in improving extracts low bioavailability and poor aqueous solubility. Moreover, for the treatment of oral lesions such as OM, a stable and tolerable formulation, which interacts with mucin that covers mucosa and increases medicines local bioavailability is very promising.

The extensive studies in cell and animal models developed by our group have strengthened a solid basis for evaluating the safety and efficacy of FITOPROT components in treating mucositis. ÁVILA et al. [19] found that a mucoadhesive formulation of *Bidens pilosa* L. significantly reduced intestinal inflammatory infiltrate and cellular vacuolization, and modulated the expression of Bax, Bcl-2 and p53 in the intestinal tissue. SANTOS FILHO et al. [21] evaluated the effects of a mucoadhesive formulation containing curcuminoids, and noticed that the formulation protected intestinal mucosa from villi shortening and crypts deepening, decreased MPO activity and MDA formation, suggestively acting as anti-inflammatory and antioxidant on the pathogenesis of 5-FU-induced intestinal mucositis. BASTOS et al. [20] observed that a mucoadhesive formulation containing curcuminoids and *Bidens pilosa* L. extract significantly attenuated body weight loss, duodenal damage and restored intestinal proliferative activity by modulating inflammatory response and intestinal oxidative stress caused by 5-FU. And, SANTOS FILHO et al. [22] found that FITOPROT protected HaCaT cells against 5-FU-triggered toxicity through antioxidant and anti-inflammatory mechanisms, and restored cellular proliferative capacity.

Curcuminoids safety, tolerability, and nontoxicity have been well-established. Human clinical trials proved there was no treatment-related toxicity with doses up to 8 g/day [55]. In the same way, *in vivo* toxicological studies reported that *Bidens pilosa* L. extract does not produce

acute oral toxicity up to a dose of 10.000 mg/kg [56]. In this phase I study, the maximum daily concentration of curcuminoids extract used was 900 mg, and 18.000 mg for *Bidens pilosa* L. based on NOAEL 1.000mg/kg of extracts per kg of body. Thus, based on *in vitro*, pre-clinical and toxicological studies of the extracts addressed here, the two FITOPROT concentrations were established for this phase I trial in order to determine safety and tolerability, as well as define the optimal concentration to be used in the phase II trial.

In humans, oral genotoxicity caused by anticancer therapies has been investigated by several studies [57,58]. Radiotherapy and chemotherapeutic drugs can affect the DNA from healthy cells resulting in toxicogenetic and toxicogenomic effects such as single and double-strand breaks, DNA-protein crosslinks and oxidative damage [58,59]. One of the most sensitive and used endpoints to detect genotoxicity in oral mucosa cells is the measurement of micronuclei frequency [60]. Micronuclei are non-incorporated chromosomes or chromosome fragments present in the main nucleus during mitosis [58]. Micronucleus test is highly relevant due it represents true mutagenic events undergoing a nuclear division [60].

Data from possible genotoxic effects in exfoliated oral mucosal cells show that micronuclei results depend greatly on the staining methods. With Papanicolaou, which is a nonspecific stain, micronuclei frequencies were almost ten times higher than in DAPI, a DNA-specific stain. According to NERSESYAN et al. [26], this difference can be explained because nonspecific stains reflect the levels of cell keratinization, and it is known that cells from the oral cavity have elevated levels of karyorrhexis and keratinization. Although this variation was observed, values found are considered normal, due oral mucosal cells are constantly exposed to a variety of substances, microorganisms, mechanical trauma, among other factors [27]. It was also interesting that there was no statistical significance between FITOPROT A and FITOPROT B in

the staining procedures, and cytology analysis did not show induced changes in morphology and/or cell size, nor induced broken eggs, cytoplasmic granules, apoptosis or cellular binucleation, evidencing that the formulation was safe in both concentrations.

Saliva is used in many tests due to its components that rapidly reflect the nature and amplitude of the host response to various oral mucosal damage, such as inflammatory diseases, trauma, etc [61]. Total saliva collection is a simple and non-invasive process, and salivary biomarkers have proved high-sensitivity for some diagnostic purposes by reflecting closely the intra-oral condition [62]. Thus, we decided to analyze salivary MPO, MDA and NO levels to verify if FITOPROT could cause inflammation or tissue damage in the oral mucosa.

Results from biochemical parameters analyzed in saliva revealed higher activity of all three markers in participants who used FITOPROT B than those who used FITOPROT A. Nonetheless, as expected, MPO, MDA and NO levels were within normal standards, demonstrating that both concentrations of the formulation did not cause inflammation or any other oral mucosal damage. Our data are in accordance with some studies that applied such biochemical analysis for evaluating drugs' efficacy and toxicology, related to all MPO [63], MDA [64], NO [65], curcuminoids [66] and *Bidens pilosa* L. [44,67].

There is plentiful evidence that cytokines such as IL-1ß, IL-6, IL-8, IL-12 and TNF are increased during inflammatory processes [68]. These pro-inflammatory cytokines are mainly produced by macrophages, monocytes and neutrophils, and are involved in the up-regulation of inflammatory reactions like second phase of mucositis [1,3,11,69]. In the present study no significant difference were found in the concentration of pro-inflammatory measured cytokines in the participants saliva samples. As for IL-10 (an anti-inflammatory cytokine) significant change was observed ($p=0.0368$). Data suggest that standard concentrations of pro-inflammatory

cytokines do not differ from the use of FITOPROT A versus FITOPROT B. On the other side, participants who used FITOPROT B presented higher levels of IL-10, which might support an anti-inflammatory effect by the formulation in a phase II trial. Additionally, when compared to literature, all salivary cytokine levels found were under physiological standards [38-40,70].

Since many different agents have been reported in the literature for preventing and treating OM, no therapy has shown clear advantages to support their use [71]. In this phase I study, FITOPROT demonstrated to be safe and tolerable in both tested doses, with no systemic or local adverse events. Correlations among evaluated parameters in the studied groups also lead us to propose that FITOPROT A (mucoadhesive formulation containing 10 mg/mL of curcuminoids extract + 20% v/v of *Bidens pilosa* L. extract) is suitable for evaluation in a phase II trial as treatment against OM.

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6. Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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Highlights

No participant experienced toxicity or discomfort during FITOPROT use.

FITOPROT demonstrated to be safe and tolerable in both tested doses.

FITOPROT did not induce genotoxicity, systemic or local adverse events.

FITOPROT is promising as treatment against oral mucositis in a phase II trial.