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Comparative Evaluation of the Antioxidant Properties of Two Commercially Available Mouthrinses: A Clinico-Biochemical Study

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ABSTRACT

Background: Several mouth rinses are assessed in the literature for the treatment of periodontal and gingival diseases. The study's objective was to evaluate the clinical and biochemical effects of AMFRESH™ mouth rinse with REXIDINE™ and placebo mouth rinse**.**

Materials and Methods: This single-blind, randomized, placebo-controlled clinical trial involved forty-five subjects with gingivitis, randomly divided into three groups: negative control (placebo), positive control (0.2% CHX), and test group (AMFRESH mouth rinse - 1%CHX, 0.1% Resveratrol, 0.1% Chitosan and 0.005% Curcuma longa). Mouth rinsing (adjunctive therapy was continued for 4weeks, clinical parameters (plaque index, gingival index), and biochemical measurements (reactive oxygen metabolites and total antioxidant capacity) were evaluated at baseline and four weeks.

Results: Intragroup comparison, mean values of PI, GI demonstrated significant reduction from baseline to 4 weeks, whereas ROM, TAC levels showed statistical significance in CHX and AMFRESH groups. A statistically significant difference was seen on intergroup comparison for PI, GI, ROM for all the three groups. However, significance was noted in TAC levels of control and CHX and control and AMFRESH groups, but no significance was noted between CHX and AMFRESH groups.

Conclusion: AMFRESH mouth rinse can be considered an alternative to CHX because of its anti-inflammatory and antioxidant properties.

Keywords: Dental plaque, reactive oxygen species, lipid peroxides, antioxidants.

INTRODUCTION

Periodontitis is an inflammatory disease process initiated by plaque biofilm leading to attachment loss, bone loss, and subsequent tooth loss. The inflammatory and immune responses of bacteria and viruses colonize the periodontal. Its associated tissues involve systemic circulation and, ultimately, the body's peripheral systems, leading to a complex series of host-microbial interactions. A loss of homeostatic balance between proteolytic enzymes and their inhibitors, reactive oxygen species (ROS), and antioxidant defense systems are believed to be responsible for tissue destruction. The basis of such dysregulation is in part genetic and environmental factors[1]. ROS comprises free radicals such as superoxide radicals (O_2 •−), hydroxyl radicals (OH^-), and non-free radical species such as hydrogen

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peroxide (H_2O_2) and singlet oxygen ($^1O_2$)[2]. These are continuously produced during regular physiologic events and can initiate membrane lipids' peroxidation, damage crucial biomolecules that includes nucleic acids, lipids, proteins, and carbohydrates. They may also cause DNA damage and lead to mutations. Thus ROS has been implicated in more than 100 diseases[3].

All aerobic organisms have antioxidant defenses, which protect the human body from ROS effects. Hence a growing interest in natural and safer antioxidants has given impetus to explore sources of natural antioxidants. Curcumin (CMN), a natural yellow-colored rhizome, has been used in traditional medicine in Ayurveda and Unani medicine for more than 2000 years. The oxygen radical scavenging activity of CMN was implicated in its anti-inflammatory effects[4]. It inhibits arachidonic acid activities' metabolism and releases steroidal hormones, causing inhibition of pro-inflammatory cytokines and leukotriene synthesis[5].

In recent years, significant attention devoted to chitosan (CHT) for pharmacological and biomedical applications. CHT, a natural polysaccharide obtained by de-acetylation of chitin, found in shells of crustaceans, insects, and the cell walls of fungi, has received much more attention for its anti-microbial activity[6]. Antioxidant properties of CHT derivatives have been studied by Lin & Chou[7]; Xing et al.[8] CHT showed antioxidant activities of 58.3 – 70.2% at 1mg/ml and reducing powers of 0.32 – 0.44 at 10mg/ml. Hence the scavenging ability on hydroxyl radicals and chelating capabilities on ferrous ions proved chitosan a source of antioxidants and a potential candidate as a stimulant for regenerating oral soft tissues[9].

Resveratrol a polyphenolic compound naturally occurring in the fruits and leaves of edible plants. Its common recognition as a natural antioxidant clarified by Zini et al.[10,] who suggested three antioxidant mechanisms: (i) competition with coenzyme Q and, to decrease the oxidative chain complex, the site of ROS generation, (ii) scavenging O_2^- radicals formed in the mitochondria and (iii) inhibition of lipid peroxidation induced by Fenton reaction products. It was demonstrated that Resveratrol inhibits the progression of periodontitis through its anti-inflammatory and stimulatory effects on osteoblastic cells and promote osteogenesis[11].

Chlorhexidine (CHX) is considered the gold standard, used for more than three decades to prevent periodontal diseases due to its antibacterial action. However, the patient's compliance with CHX is diminished by its disagreeable taste and propensity to stain the teeth[12]. Alternatively, higher concentrations of CHX in mouthwash solutions are associated with ulceration of the oral mucosa[13].

Hence, the study aimed to evaluate AMFRESHTM mouth rinse's antioxidant properties consisting of 1%CHX, 0.1% Resveratrol, 0.1% CHT, and 0.005% CMN and REXIDINE™ mouth rinse composed of 0.2% CHX using spectrophotometry.

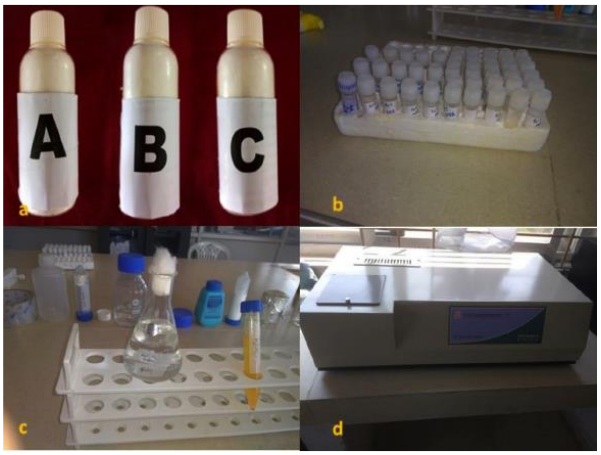

Fig 1: a) Three mouthrinses in different bottles. b) Saliva samples collected for spectrophotometrical analysis. c) dNTPH buffer d) Spectrophotometer (Systronics, Gujarat).

MATERIALS AND METHODS

Forty-five dental professionals attending the department of Periodontics and Oral Implantology were assigned in this randomized controlled clinical trial. Institutional ethical committee and review board-approved research protocol. All examinations and procedures associated with this study followed the Helsinki Declaration of 1975, as revised in 2000. The study was registered and approved by clinical trials registry-India (CTRI/2019/10/021803). All the patients understood and signed an informed consent form for being enrolled in this trial.

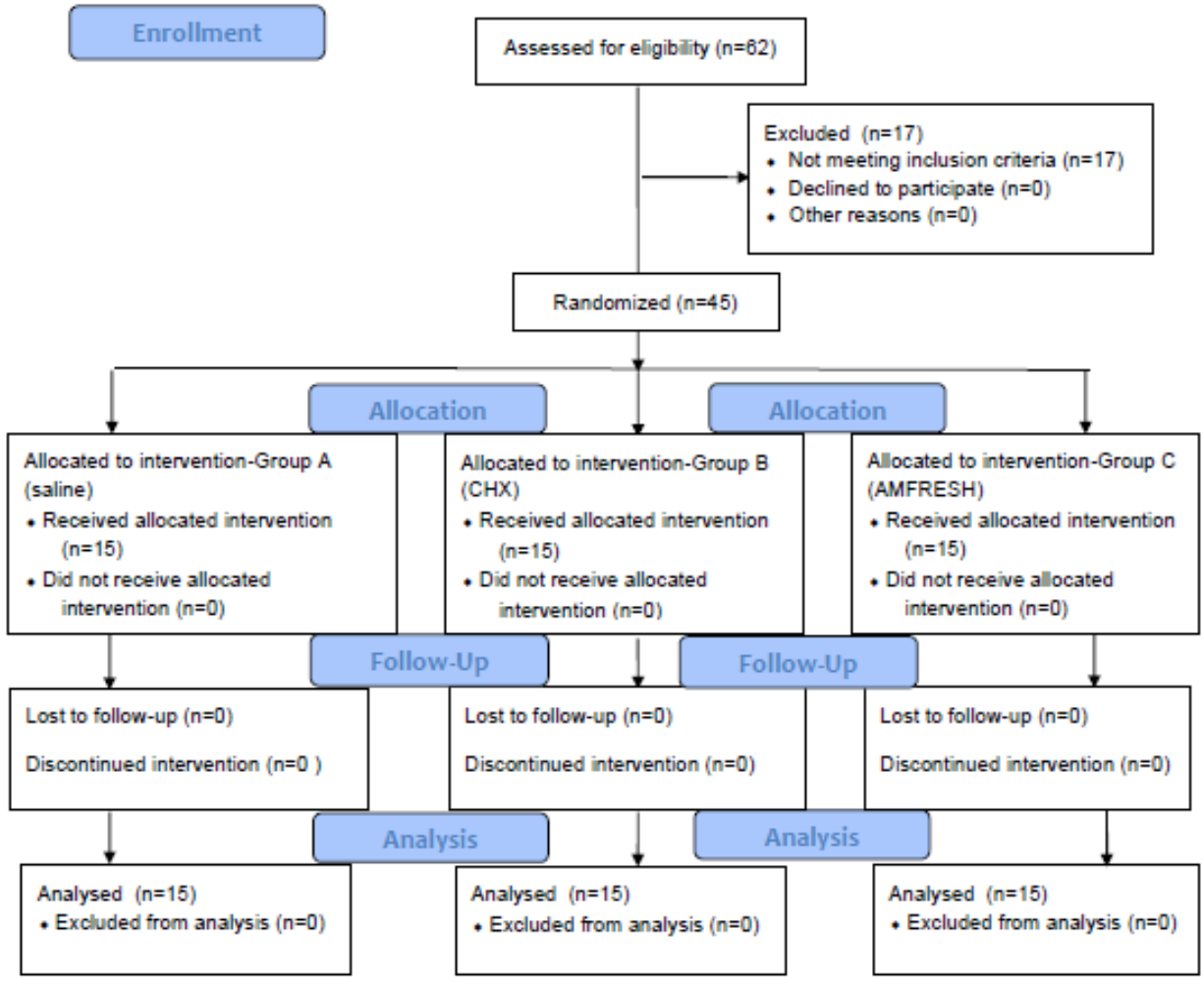

Fig 2: Consort Flow Diagram of The Study.

Table 1: Baseline demographical data of study population

Parameter	Saline	CHX	AMFRESH	Total
Male/female	9/6	7/8	4/11	20/25
Mean age±SD	21±1.31	20.73±1.49	20.47±1.55	20.73±1.44
% Male/female	60/40	46.67/53.33	26.67/73.33	100

CHX – Chlorhexidine mouth rinse group; SD – Standard deviation.

Table: 2 Values (mean±standard deviation in mm) of clinical parameters from baseline to 4 weeks.

Group	Variables	N	Mean±SD	p
Group A	PI-baseline	15	1.160±0.255	<0.001*
	PI-4 weeks	15	0.953±0.320	
	GI-baseline	15	0.773±0.367	<0.001*
	GI-4 week	15	0.660±0.335	
	ROM-baseline	15	436.66±39.66	<0.06
	ROM-4 weeks	15	425±32.39	
	TAC-baseline	15	0.235±0.226	0.142
	TAC-4weeks	15	0.239±0.162	
Group B	PI-baseline	15	1.313±0.372	<0.001*

	PI-4 weeks	15	0.847±0.424	
	GI-baseline	15	0.933±0.453	<0.001*
	GI-4 weeks	15	0.547±0.346	
	ROM-baseline	15	440±29.2	<0.05*
	ROM-4 weeks	15	419.50±36.59	
	TAC-baseline	15	0.208±0.008	<0.05*
	TAC-4weeks	15	0.254±0.021	
Group C	PI-baseline	15	1.387±0.37	<0.001*
	PI-4 weeks	15	0.9±0.3	
	GI-baseline	15	0.813±0.377	<0.001*
	GI-4 weeks	15	0.56±0.329	
	ROM-baseline	15	448.33±34.67	<0.05*
	ROM-4 weeks	15	419.16±29.45	
	TAC-baseline	15	0.236±0.016	<0.05*
	TAC-4weeks	15	0.311±0.010	

*Paired t-test to compare baseline and 4 weeks mean values - group wise; *Significant. PI – Plaque index; GI – Gingival index; ROM – Reactive oxygen metabolite; TAC – Total antioxidant capacity; SD – Standard deviation; n – Number of samples; p – Significance value.

Table 3: Intergroup comparison of plaque index, gingival index, and reactive oxygen metabolite levels at baseline and at 4 weeks

Parameters	Group	N	Mean± Std. Deviation	Std. Error	F	P
PI DIFF	A	15	0.207±0. 1624	.0419	7.686	<.001*
	B	15	0.467±0.2257	.0583		
	C	15	0.487±0.2560	.0661		
GI DIFF	A	15	0.11±0.099	.026	13.814	<.001*
	B	15	0.39±.192	.050		
	C	15	0.25±0.119	.031		
ROM DIFF	A	15	11.667±55.1918	14.2505	1.110	<.042*
	B	15	20.500±50.2671	12.9789		
	C	15	29.167±43.2153	11.1581		
TAC DIFF	A	15	-.0042±0.01915	.00494	7.608	<.002*
	B	15	-.0467±0.02289	.00591		
	C	15	-.0747±0.02560	.00661		

*One-way ANOVA to compare mean values between groups; * Significant. PI – Plaque index; GI – Gingival index; ROM – Reactive oxygen metabolite; TAC – Total antioxidant capacity; p – Significance value; F – F statistic.

Table 4: Tukey's post hoc tests for multiple comparisons

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	p
PI DIFF	A	B	-.2600*	.0797	<0.05*
		C	-.2800*	.0797	<0.03*
	B	C	-.0200	.0797	<0.05*
GI DIFF	A	B	-.273*	.052	<0.05*
		C	-.140*	.052	<0.02*
	B	C	.133*	.052	<0.03*

ROM DIFF	A	B	-8.8330*	18.1848	<0.02*
		C	-17.5000	18.1848	<0.05*
	B	C	-8.6670*	18.1848	<0.03*
TAC DIFF	A	B	.0425*	.00829	<0.04*
		C	.0705*	.00829	<0.04*
	B	C	.0280	.00829	1.000

* Significant. PI – Plaque index; GI – Gingival index; ROM – Reactive oxygen metabolite; TAC – Total antioxidant capacity; p – Significance value

The inclusion criteria were dental professionals within the age range of 20-35, participants with mild to moderate gingivitis, systemically healthy individuals. Exclusion criteria were participants with a history of any medication from the past six months, a history of oral prophylaxis from the past six months, participants with prosthetic or orthodontic appliances, any allergy to active ingredients, smokers, alcoholics, drug abusers, and current users of any mouth rinse.

The present study was designed as a randomized, single-blind, placebo-controlled clinical trial. Sixty-two individuals were examined for eligibility, out of which 45 individuals satisfying the inclusion criteria were allocated to one of the three groups using a computer-generated random allocation sequence. The sample size was decided by power analysis with 95% power and a significance level of 0.05.

Group A: Saline (negative control).

Group B: REXIDINE mouth rinse (positive control group).

Group C: AMFRESH mouth rinse (test group).

A single clinician blinded to the groups assigned to the individuals recorded all the parameters, i.e., Plaque Index(PI)[14], Gingival Index(GI)[15], Reactive Oxygen Metabolites(ROM), and Total Antioxidant Capacity(TAC) were recorded at baseline and four weeks. The saliva was collected in sterile, autoclavable tubes and centrifuged immediately at 1200rpm for 5 minutes. The supernatant obtained was removed and stored at -80^{0C} for further estimation of ROM and TAC. The clinical measurements were done by a single examiner. All the participants received full mouth ultra-sonic scaling and dental polishing by a periodontist.

According to the assigned groups, patients were asked to rinse with 15 ml of given medication for 1 min, two times a day, for up to four weeks. Mouthwashes were kept in three identical opaque bottles coded with A, B, and C (Figure1). Based on the randomization and allocation list, another clinician distributed the bottles to the patients.

At the time of recall visits, every week patient's compliance was assessed. Patients exchanged old bottles with new bottles of medication/placebo in these visits. Subjects were also evaluated about the self-reported complications. The study design was shown with the help of a consort flowchart in (Figure 2).

Measurement of ROM[16]

The salivary ROM level whole oxidant capacity of saliva against N-N-diethyl paraphenylenediamine in the acidic buffer was performed using a spectrophotometer. About 20 µl salivary sample and 1 ml acetate buffer (pH–4.8) were gently mixed in a cuvette, and 20 µl chromogenic substrate N, N-diethyl-p-phenylenediamine was added. After mixing, the cuvette was immediately incubated in the analyzer's thermostatic block for 5 min at 37°C. The 505 nm absorbance was recorded. The Carratelli unit was used for measurement. It was established that 1 CARRU corresponds to 0.08 mg/dl hydrogen peroxide.

Test principle

The test uses the principle of Fenton's reaction. The newly created transition metal ion (iron or copper) catalyzes the breakdown of hydroperoxyl (ROO+) and alkoxyl (RO+) by mixing a biological sample with an acidic buffer (Reagent R). Adding a chromogen (N, N-diethyl-p-phenylenediamine, Reagent R2) can donate an electron that can change its color when oxidized. Using photometric reading available with the FRAS 4 dedicated analytical equipment, it is possible to quantify hydroperoxides available in the sample.

Measurement of TAC[17]

Patients were asked to expel the unstimulated saliva into a 5 ml sterile container, stored at -80 °C until use. Total antioxidant capacity of saliva was detected by biochemical analysis using FRAP TEST with the help of a spectrophotometer (SYSTRONICS, GUJARAT). The whole antioxidants evaluation method is based on saliva's capacity to reduce Fe^{+3} to Fe^{+2} in the presence of 2,4,6,-tripyridyl-s-triazine (TPTZ). Fe^{+3} -TPTZ complex is reduced under acidic conditions by a reducing agent to form Fe^{+2} -TPTZ. Fe^{+2} -TPTZ is easily detectable and measurable because of its intense blue color with maximum absorption at wavelength 593 nm. Therefore, the determining factor in evaluating the speed of Fe^{2} -TPTZ complex formation and reducing ability of samples was determined by the production of blue color; by mixing 3.1 grams of sodium acetate in 16 mL of acetic acid, acetate buffers were prepared. Then, by adding distilled water, raises the volume to 1 liter; and the solution's PH was set to 3.6. The primary reactive solution was a mixture of 2.5 mL of TPTZ, 2.5 mL of iron chloride, plus 25 mL of acetate buffer. The reaction started by adding 30 microliters of gathered saliva. Absorption was measured at the beginning of the response.

Statistical analysis:

Kolmogorov-Smirnov revealed that the variables followed a normal distribution. Therefore, to analyze the data, parametric methods were applied. To compare the mean values between groups, one-way ANOVA was used, followed by Tukey's post hoc tests for multiple pair-wise comparisons. A paired t-test was applied to compare mean values between baseline and four weeks. Statistical software was used (SPSS Statistics for Windows, Version 22.0) to analyze the data, significance level fixed as 5% ($\alpha = 0.05$).

RESULTS

The patient's demographical data were reported in Table 1. For all the three rinses, on the intragroup comparison, mean values of PI, GI demonstrated significant ($P < 0.05$) reduction from baseline to 4 weeks, whereas ROM, TAC levels showed statistical significance in CHX and AMFRESH groups, but no significance was noted in the control group. [Table 2].

In the intergroup comparison of PI, GI, ROM, and TAC levels at baseline and four weeks by one-way ANOVA (Table 3), significance was evident at the end of 4 weeks for all four parameters. Table 4 shows Tukey's post hoc tests for multiple comparisons. A statistical significance was noted for PI, GI, ROM between all the three groups. However, significance was noted in TAC levels of control and CHX and control and AMFRESH groups, but no significance was noted between CHX and AMFRESH groups.

DISCUSSION

Loe et al., in their study, demonstrated accumulation of microbial plaque results in the development of gingivitis, its removal, and control results in the resolution of the disease[18]. Mechanical oral hygiene practices, along with chemical plaque control regularly, can prevent the disease.

Compounds with an antioxidant and anti-microbial activity that can reduce inflammation and restore oral health are successful treatment modalities, with Resveratrol, CMN, CHT, Azadirachta indica being some such compounds. The purpose of recruiting dental professionals in the present study is to have better administration of the two mouth rinses and follow-up.

Ample evidence was present in the literature regarding the positive outcomes of CHX as an adjunctive treatment for gingival health. Hence CHX mouth rinse was taken as a positive control, and the placebo group was included as the negative control in the study. These two well-design controls curtailed the effects of variables other than the independent variables. To the best of our knowledge, this is the first randomized study done to investigate a combination of a mouth rinse containing CHX, CMN, Resveratrol, CHT, and CHX mouth rinse alone on antioxidant properties clinically and biochemically. Therefore direct verification of results was not possible.

On intragroup comparison from baseline to 4 weeks, PI and GI were reduced significantly in all the three groups. A significant reduction in PI and GI scores was observed among all three groups on the inter-group comparison. This observation was in accordance with previous studies conducted by

Aspalli et al., Arunachalam LT et al., and Chatterjee A et al., wherein a comparison of CMN mouth rinse with 0.2% CHX mouthwash in gingivitis patients was performed[19, 20, 21]. Another study by Muglikar S et al. observed a reduction in PI and GI by comparing CMN mouth rinse with CHX along with SRP[22]. In contrast to this, Singh et al. reported a significant decrease of PI and GI in the CHX group than in the CMN group, and this could be due to the formulation used, turmeric gel, which might have influenced the results[23].

In a cross-over study by Sano H et al., CHT rinsing reduced plaque scores and counts of salivary Streptococcus mutants after a 14-day rinsing period. The possible mechanism for reduction of plaque scores and mutans streptococci in saliva might be a modification of the electrostatic interaction between the bacterial cell surface in saliva and tooth pellicle surface, thereby permitting less favorable exchange, suggesting that CHT may be an alternative chemical agent for managing patients who show side effects associated with chlorhexidine[24]. In a study by Mhaske et al., a comparison of 2%CHT, 0.2%CHX, and 2%CHT+0.2%CHX mouth rinse combination showed a reduction of plaque gingival scores in all the three groups. However, a combination of both provided significant results[25]. In contrast to this, a study by Uraz et al. reported chitosan showed little antibacterial activity against the metabolism of plaque bacteria; these results may differ because of various formulations of chitosan used in the study[26]. Bagis et al. evaluated the staining quality of CHT for 3-weeks and found natural staining of CHT on teeth[27].

So far, a few studies reported the effects of Resveratrol and its combinations in the management of gingival & periodontal diseases. CMN, along with Resveratrol, can reduce alveolar bone loss in an animal model of periodontitis[28]. The effect of Resveratrol on perio-pathogens studied by Minagawa T et al., where Resveratrol suppressed the inflammatory responses by inhibiting nuclear factor kappa B ligand, suggesting that it can be used as an adjunct to treat periodontal disease[29]. An in-vitro study by O'Connor et al. reported promising effects of Resveratrol against anaerobic bacteria but did not demonstrate encouraging results when aerobic microorganisms were evaluated. According to the authors, the antioxidant properties of Resveratrol may inhibit the metabolic pathways of the bacteria, which are mandatory for anaerobic survival[30]. On the contrary, Cirano et al. demonstrated that Resveratrol did not promote microbiological outcomes in an experimental model of periodontitis, which could be attributed to the type of resveratrol administration employed[31].

PMNS, the primary mediators of host response against periodontal pathogens, generate increased levels of ROS. An increased ROM level in saliva, GCF, and plasma indicates significant oxidative stress in gingival and periodontal structures[32]. Therefore, Antioxidants are considered emerging prophylactic and therapeutic agents that counteract free radicals' overproduction during periodontal disease.

On intragroup comparison, a significant reduction of ROM Levels was seen in CHX and AMFRESH groups from baseline to 4 weeks; the saline group did not show a significant decrease from baseline to 4 weeks, which could be explained by the re-occurrence of supragingival microbial flora to pretreatment levels, as stated in the review by Greenstein G, 1992[33].

On intergroup comparison, a statistically significant reduction was observed among all three groups. A study by Arunachalam et al., where they compared CHX with CMN, reported a significant decrease in ROM levels in the CMN group suggesting additional benefits can be attributed to the anti-inflammatory effect of CMN[20].

In the present study, the salivary TAC was evaluated by ferric ion reducing antioxidant power assay (FRAP). Various assays were designed to measure TAC as an indicator of the host's total capacity to withstand free radical stress; the FRAP takes advantage of an electron transfer reaction, highly reproducible over a wide concentration range, and reagents are inexpensive. This test's limitation is that it does not measure the antioxidants containing thiol groups and only measures the reducing capability based upon the ferric ion.

In the FRAP assay, reducible oxidant, Fe III, is used in excess. Thus, reducing the Fe III-TPTZ complex by antioxidant, blue-colored Fe II-TPTZ is formed, measured at 595 nm spectrophotometrically. In this essay, the yellow color of the test solution

changes to various green and blue shades depending on the antioxidant samples' reducing power. The compounds reducing capacity may serve as a significant indicator of its potential antioxidant activity[34].

The results showed that the TAC score was significant in Group B and Group C, which could be attributed to the prophylaxis rendered at baseline and reinforcement of oral hygiene instructions at recall intervals. Also, the combination of CHX and other ingredients like Resveratrol, chitosan, and CMN could have contributed to the improved antioxidant levels within the saliva, which can reduce superoxide ions and prevent the formation of hydroxyl radical. A study by Tambe LV et al. compared 0.2% CHX with polyphenolic antioxidant mouthwash in gingivitis patients with fixed orthodontic appliances. The study concluded that polyphenolic mouthwash has similar clinical effectiveness with 0.2% CHX[35].

Thus while evaluating the efficacy of any mouth rinse, TAC upholds as a beneficial parameter in protecting host tissues that are being damaged from the by-standard effect of ROS and should be considered while evaluating the adjunctive benefit of any mouth rinse. The present study's limitations are that gingivitis patients were included demonstrating mild to moderate inflammatory state and relatively a short observation period. However, it is interesting to note that 60% of subjects felt disagreeable taste upon using AMFRESH mouth rinse, which might be due to the combination of Resveratrol and chitosan present in the mouth rinse. Furthermore, larger sample size and observation in periodontitis conditions might help us to evaluate the significant role of CHX, Resveratrol, chitosan, and CMN combinations as anti-inflammatory agents.

CONCLUSION

Within the study's limitations, AMFRESH mouth rinse was found to have an adequate antioxidant capacity compared to CHX mouth rinse but failed to show good bio-compatibility and acceptability by the subjects.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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