MDJ

Evaluation of Anti-bacterial Effect of Rhamnus Prinoides Mouthwash on Streptococcus Mutans Count in a group of Orthodontic Patients with Gingivitis

Karrar N. Al-Mujamaii[*] B.D.S, M.Sc[*]
Athraa M. Al Waheb[**] B.D.S, M.Sc, Ph.D [**]

[*]Ph.D student at College of Dentistry, Baghdad University, Department of Pedodontic and Preventive Dentistry, Iraq, B.D.S, M.Sc. in Preventive Dentistry, E-mail: Dr.karrar82@gmail.com, karrar.nihad@al-yarmok.edu.iq , Phone: 009647716200559.

[**]Prof. Dr. at College of Dentistry, Baghdad University, Department of Pedodontic and Preventive Dentistry , Iraq, B.D.S., M.Sc., Ph.D. in Preventive Dentistry, E-mail: drathraamustafa@codental.uobaghdad.edu.iq ,Phone: 009647905995268.

Abstract

Aim of the study: This study was conducted to evaluate the antibacterial effect of Rhamnus prinoides mouthwash against Streptococcus mutans in a group of orthodontic patients with gingivitis, and to compare the gingival index before and 3 weeks after using Rhamnus Prinoides mouthwash and placebo 2 times daily for study and control group respectively.

Materials and methods: Rhamnus Prinoides mouthwash was extracted from plant leaves, filtered, then it was mixed with ethanol after being dried. The anti-bacterial effect was evaluated by measuring the streptococcus mutans count before and after 3 weeks of using Rhamnus Prinoides mouthwash and placebo in study and control group respectively.

Results: Rhamnus Prinoides mouthwash demonstrated an anti-bacterial effect by significantly reducing the mean streptococcus mutans count from $14.23*10^{-5}$±7.67 (CFU/ml) to $0.45*10^{-5}$±0.28 (CFU/ml) in study group. While in control group mean streptococcus mutans count significantly increased from $13.79*10^{-5}$±11.11 (CFU/ml) to $30.22*10^{-5}$±33.57 (CFU/ml). The mean gingival index found to change significantly from the moderate type at the base line (gingival index = 1.53± 0.26) to mild type on the third week (gingival index= 0.97±0.007) in study group, while in control group it remained with moderate type from the base line visit (gingival index= 1.44±0.16) to the third week (gingival index= 1.46±0.16).

Conclusion: Rhamnus Prinoides mouthwash exhibited a significant anti-bacterial activity by reducing the streptococcus mutans count, and thus it can be considered as a promising oral health care product in the future.

Keywords: Rhamnus Prinoides mouthwash, gingivitis, *streptococcus mutans.*

INTRODUCTION

Because of their wide biological activity, their low cost, as well as their high safety margin, the herbal products are more preferred over the conventional medications that may cause various undesirable side effects in addition to increased bacterial resistance to antibiotic due to prolonged and continuous intake, thus, the herbal products may be used increasingly as a dietary

supplements to fight and/ or prevent a common diseases [(1)].

A variety of plant materials (barks, flowers, fruits, leaves, roots, seeds, stems, and so on) were used for the preparation of herbal medicines. They may usually contain most of the biologically active compounds and may be used primarily for a variety of mild to chronic diseases treatment. Numerous plants may contain alkaloids, polyphenols, as well as volatile oils as an active constituents and are utilized as a popular folk medicines, while others might gain popularity in form of phytomedicines[(2)]. Phenolic compounds are used in preparation of antiseptics, disinfectants, as well as mouth-rinses because of their low toxicity in addition to anti-bacterial properties[(3)].

There may be a growing interest in the utilization of tannins for periodontal disease treatment. Tannins are a family of polyphenols that may precipitate a proteins. Polyphenols may exhibit an in vitro anti-bacterial activity against periodontal pathogens by increasing the oral fluids antioxidant ability[(4)]. Tannins may precipitate the microbial proteins and prevent the microorganisms development[(5)].

The increased resistance of biofilm pathogens to antibiotics as well as the side effects of these therapies made the medicinal plant to be screened as a potential source of antimicrobial and anti-inflammatory agents. Rhamnus prinoides (Rhamnaceae) is one of these medicinal plants, and depending up on the qualitative phytochemical screening study, its methanol fraction might be positive for the flavonoids, saponins, alkaloids, terpenoids, tannins, as well as polyphenols presence, these metabolites are responsible for its antibacterial, anti-inflammatory, and antioxidant activity[(6)]. Additionally, it was demonstrated that the Rhamnus Prinoides exhibited an inhibitory effects on Streptococcus mutans biofilm formation[(7)].

MATERIALS AND METHODS

Ethical Approval

This research was approved from a Research Ethics Committee/ University of Baghdad/ College of Dentistry according to a decision report (Ref. number: 373/Date 30-9-2021).

The sample collection

This study was done to assess anti-bacterial activity of Rhamnus prinoids mouthwash that was extracted from the plant leaves with the aid of Soxhlet apparatus. According to statistical analysis by using G power 3.1.9.7, the present study included 40 patients with skeletal CL I malocclusion (mild to moderate crowding) according to Angles classification in 1900 with moderate gingivitis those patients were randomly allocated to either study or control group to ensure accessibility and isolation[(8)]. The anti-bacterial activity was assessed for those patients before and after three weeks of using prepared mouthwash and placebo(flavored distilled water) by study (20 patients) and control (20 patients) groups respectively, they were instructed to wash their mouth two times daily. Anti-bacterial activity against streptococcus mutans was assessed by measuring the bacterial count (colony forming unit/ ml) in dental plaque sample that was taken from the labial surface of maxillary right lateral incisor by the use of Gracey curette which was performed with one single vertical stroke[(9)]. The plaque sample was inserted immediately in a sterile test tube that contain 1ml Phosphate buffer saline (PBS) to keep the viability of specimen. After that the colony forming units were counted. This plaque sampling was done before and 3 weeks after using Rhamnus Prinoides mouthwash and placebo in study and control group respectively.

Gingival index measurement

Gingivitis was measured according to the previously described criteria[(10)] for the entire dentition at baseline visit (before using

mouthwash) and three times at one week interval (after using mouthwash). With naked eye and under good lighting, a mouth mirror and periodontal probe were used for examination. All surfaces (distal, facial, mesial and lingual\ palatal) of entire dentition were typically examined starting from the upper right second molar and continued over the midline of the jaw to the upper left second molar. Concerning the teeth that are located on the right side of the jaw midline, the sequence of examination included distal, facial and mesial, and the opposite was true for the teeth that are located on the left side of midline where the sequence of examination included mesial, facial, and distal. Concerning the palatal surface of the entire dentition, they were assessed starting from the upper left second molar through the midline of the jaw to the upper right second molar. Moreover, in the lower jaw the entire dentition was examined starting from the lower left second molar and preceding through the midline to the lower right second molar. Concerning the sequence of surfaces examination it was opposite to that of upper jaw where the sequence was distal, facial, and mesial for the teeth that are located on the right side, and mesial, facial, and distal for the teeth that are located on the left side of the jaw midline. Additionally, the lingual surfaces of lower dentition were scored starting from the lower left second molar and preceding through the midline to the lower right second molar [(11)] .

The plant material collection and authentication

The Rhamnus Prinoides leaves were collected from the same tree in orchard in Al-Khalis city/ Diyala Governorate/ Iraq in September 2021after making sure that it was not exposed to pesticides. This plant identification was done by a taxonomist at the Biology Science Department/ College of Basic Science/ Diyala University/ Iraq.

Plant extraction

It was carried out according to a procedure described by previous study[(12)] by taking 20g of Rhamnus Prinoides leaves then dried, powdered, and placed in a cylindrical container called thimble, next put it in the place assigned to the Soxhlet apparatus, and followed by adding Hexan to remove fat and chlorophyll. Extraction was performed for 12h at a temperature (40-60°C), then the powder of plant was transferred to methanol 70% for 3h. After that the extract sprayed with a filter paper, then incubated for two days. The methanol extract was treated with HCl (1%) and filtered again with a filter paper (Whattman 1). The next step was addition of diethyl ether and left for 24 hours and finally the mixture separated into two layers, the upper layer was diethyl ether layer which was removed and the lower layer was the aqueous layer which was collected and adjusted to pH 8.

Cytotoxic effect of Rhamnus Prinoides crude extract

It is well known that the cytotoxic assay is a screening test to evaluate the biocompatibility of the examined substances. In this study Mosmann's Tetrazolium Toxicity assay (MTT) was used to evaluate the cytotoxic effect of Rhamnus Prinoides crude extract on normal cell line. This assay was conducted at the Biology department\ College of Basic Science\ Diyala University on Normal Mice Embryonic Fibroblast (MEF). Table 1 shows the concentrations of tested herb leaves extract in (µg/ml) and the normal cell inhibition percentage for each concentration.

Regarding the cytocompatibility of Rhamnus Prinoides leaf extract, the half lethal concentration (LC50) that is required to kill 50% of fibroblast was obtained by using the equation of simple linear regression graph (Y= 0.0068x+14.535) and by assuming that the Y equal to 50 as shown in Figure 1.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination of crude extract

All concentrations of Rhamnus Prinoides extract that revealed a bacterial growth inhibition were individually mixed with an agar (Brain Heart Infusion) in order to get 25ml of agar, then poured into a petri dishes, after that they were inoculated with 0.1 ml from an activated streptococcus mutans isolates after being harden. This was followed by an incubation (24 hours at 37°C) of these petri dishes including the control plates (negative control that contained Brain Heart Infusion agar and microbial inoculums but without adding the extract, as well as the positive control plates that contained Brain Heart Infusion agar and different extract concentrations but without microbial inoculums). After that each of these petri dish was examined for the microbial growth, in that the MIC was determined as the lowest extract concentration that may inhibit the bacterial (streptococcus mutans) growth as shown in Figure 2. The MIC and MBC are shown in Table 2.

RESULTS

The mean Streptococcus mutans count before using the Rhamnus Prinoides mouthwash and placebo in study ($14.23*10^{-5}$ ±7.67) and control ($13.79*10^{-5}$ ±11.11) group respectively was not significant between groups, while it was lower in study group ($0.45*10^{-5}$ ±0.28) than that in control one ($30.22*10^{-5}$ ± 33.57) with significant difference after using Rhamnus Prinoides mouthwash and placebo in study and control group respectively (Table 3).

Regarding the change in the mean Streptococcus mutans count, it significantly decreases in study group from $14.23*10^{-5}$ ±7.67 (before using Rhamnus Prinoides mouthwash) to $0.45*10^{-5}$±0.28 (after using Rhamnus Prinoides mouthwash), while in control group it significantly increased from $13.79*10^{-5}$ ±11.11 (before using placebo) to $30.22*10^{-5}$ ± 33.57 (after using placebo) as shown in Figure 3.

Table 4 illustrates that there is no statistically significant difference of Gingival index between groups in the baseline, while in each other visits, Gingival index found to be lower in study group than that in control one with significant difference between groups. Regarding changes in gingival index during visits, the mean gingival index significantly decreases from 1.53± 0.26 at the base line (before using Rhamnus Prinoides mouthwash) to 0.97±0.00 at the third week (after using Rhamnus Prinoides mouthwash) in study group while it significantly inclined from 1.44±0.16 at the baseline visit (before using placebo) to 1.46±0.16 at the third week (after using placebo).

Regarding changes in severity of gingival inflammation, the mean gingival index found to change from the moderate type at the base line (gingival index = 1.53± 0.26) to mild type at the third week (gingival index= 0.97±0.00) in study group, while in control group it remained with moderate type from the base line visit (gingival index= 1.44±0.16 to the third week (gingival index= 1.46±0.16 (Figure 4). This figure also demonstrates that there is no great difference between different times for control.

Concerning the correlation between gingival index and streptococcus mutans count, this study showed a positive significant correlation between them as shown in Table 5.

Conflicts of Interest

The authors reported that they have no conflicts of interest.

DISCUSSION

The present study found that the RP mouth wash is safe to be used as antibacterial oral health care product.

Regarding the change in mean Streptococcus mutans count, it significantly decreases in study group, while in control group it significantly increases, these findings may be attributed to antibacterial effect of Rhamnus Prinoides leaf extract, this finding is in agreement with a study which showed that the antibacterial effect may be attributed to the additive or synergistic effect of the plant

secondary metabolites[13], in addition to other studies which showed that the Rhamnus Prinoides possess Triterpenoids that that may have antimicrobial and anti-inflammatory activity, as well as Tannins that have antimicrobial and anti-oxidant activity[14]. Additionally it may be due to certain compounds presence in Rhamnus Prinoides leaf extract which might exert their effect either on the cell membrane of streptococci mutans or on the enzymes that are necessary for the growth of this bacteria and this assumption need further studies for its confirmation.

The mean gingival index showed no significant difference between study and control groups at baseline visit. Concerning the changes in mean gingival index was found to be decreased significantly from the baseline to 1st, 2nd, and 3rd visit (following the use of mouthwash) in study group and increased significantly in control group from the baseline to 1st, 2nd, and 3rd visit after using the placebo. Additionally the severity of gingival inflammation shift from moderate inflammation at base line visit to mild type at the 2nd, and 3rd visit in study group, these findings may be attributed to anti-inflammatory effect of Rhamnus Prinoides leaf extract which is in agreement with a study which demonstrated that the flavonoids as well as their glycosides not only representing this plant major ingredients, but are also responsible for its strong anti-inflammatory as well as antioxidant activities [15].

The current study demonstrated the antibacterial effect against streptococcus mutans only, so further studies needed to be conducted to evaluate and confirm the antibacterial effect against other plaque bacterial species in addition to its antifungal effect. A bigger sample size is recommended for generalization of the findings.

Conclusion

It was concluded that Rhamnus Prinoides (RP) leaf extract might reduce the mean streptococcus mutans count after being used as a mouthwash by orthodontic patients with a moderate gingivitis and improving the gingival conditions to a mild type, and thus it may be considered as a promising oral health care product.

References

1. Ramesh A., Varghese S.S., Doraiswamy J.N. and Malaiappan S. 2016. Herbs as an antioxidant arsenal for periodontal diseases. Journal of intercultural ethnopharmacology; 5(1): 92.
2. Bussmann, R. W., Swartzinsky, P., Worede, A. and Evangelista P. 2011. Plant use in OdoBulu and Demaro, Bale region, Ethiopia. Journal of ethnobiology and ethnomedicine; 7(1): 28.
3. Sekino S. and Ramberg P. 2005. The effect of a mouth rinse containing phenolic compounds on plaque formation and developing gingivitis. J Clin Periodontol; 32: 1083-8.
4. Petti S. and Scully C. 2009. Polyphenols, oral health and disease: A review. J Dent; 37: 413-23.
5. Evans CE., Banso A., Samuel OA. 2002. Efficacy of some nupe medicinal plants against Salmonella typhi: An in vitro study. J Ethnopharmacol; 80: 21-4.
6. Molla Y., Nedi T., Tadesse G., Alemayehu H., and Shibeshi W. 2016. Evaluation of the in vitro antibacterial activity of the solvent fractions of the leaves of Rhamnus prinoides L'Herit (Rhamnaceae) against pathogenic bacteria. BMC Complementary and Alternative Medicine; 16: 287.
7. Fathi R. 2018. Rhamnus prinoides (gesho) extract Inhibits Streptococcus mutans and Candida albicans Polymicrobial Biofilm Formation. A Thesis Submitted to the College of Arts and Sciences/ Georgia State University in Partial Fulfillment of

the Requirements for the Degree of Master of Science/ Department of Biology.
8. Saloom HF, Papageorgiou SN, Carpenter GH, Cobourne MT. 2017. Impact of Obesity on Orthodontic Tooth Movement in Adolescents: A Prospective Clinical Cohort Study. Journal of Dental Research; 96(5): 547-554.
9. Loomer PM. (2004). Microbiological diagnostic testing in the treatment of periodontal diseases. Periodontol 2000; 34: 49-56.
10. Loe H. and Silnese J. 1963. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odont Scand; 21: 533-551.
11. Wei, S. and Lang, N. 1982. Periodontal epidemiological indices for children and adolescents: II. Evaluation of oral hygiene. The American Academy of Pedodontics; 4(1), 65.
12. Harborne, J. B.; Mabary, T. J. and Mabary, H. 1975. Physiological and Functional of Flavonoids. New York, Sanfrancisco.
13. Cowan MM.1999. Plant products as antimicrobial agents. Clin Microbiol Rev.;12: 564–82.
14. Brenzan M., Nakamura C., Dais-Filho BP., Ueda-Nakamura T., Young M. 2007. Anti leishmanial activity of crude extract and coumarin from Calophyllum brasiliense leaves against Leishmania amazonensis. Parasitol Res; 101: 715-722.
15. Chen G.; Mutie F.; Xu Y.; Saleri F.; Hu G.; & Guo M. (2020). Antioxidant, Anti-inflammatory Activities and Polyphenol Profile of Rhamnus prinoides. Pharmaceuticals (Basel, Switzerland); 13(4): 55

Table (1): Descriptive and statistical test of Cytotoxicity among groups using Analysis Of Variance (One Way ANOVA).

Concentration (µg/ml)	Inhibition% ± SD	F	P value
15.62	0		
31.25	0		
62.5	0		
125	0		
250	12.53±1.40	437.5	<0.01*
500	17.53±0.33		
1000	23.82±3.18		
2000	31.71±0.48		
4000	40.15±1.99		
8000	68.86±0.86		

*=significant at p<0.05

Table (2) : MIC and MBC of Rhamnus Prinoides leaves extract

Concentration In mg/ml	0.25	0.5	1	2	4	6	8
Growth	+	+	+	+	-	-	-

Table (3): Descriptive and statistical test of Streptococcus Mutans count among groups and phases.

Phases	Study		control		T	df	P-value
	Mean*10^{-5}	±SD	Mean*10^{-5}	± SD			
Before	14.23	7.67	13.79	11.11	0.14	38	0.88
After	0.45	0.28	30.22	33.57	3.96	38	<0.01*
Mean ± SD	13.77±7.47		-16.42±32.59				
Paired T test	8.24		2.25				
Df	19		19				
P value	<0.01*		0.03*				
Effect size	1.84		0.50 medium				

*=significant at p<0.05.

Table (4): Descriptive and statistical test of gingival index among groups and phases

Groups		Base line	1 week	2 weeks	3 weeks	F	P value	ES
Study	Minimum	1.25	1.01	0.98	0.97	96.36	<0.01*	0.88
	Maximum	1.93	1.26	1.03	0.99			
	Mean	1.53	1.10	0.99	0.97			
	±SD	0.26	0.10	0.01	0.00			
Control	Minimum	1.27	1.25	1.26	1.28	22.47	<0.01*	0.65
	Maximum	1.84	1.81	1.83	1.85			
	Mean	1.44	1.45	1.45	1.46			
	±SD	0.16	0.16	0.16	0.16			
F		1.37	62.14	144.58	175.66			
P value		0.24	<0.01*	<0.01*	<0.01*			
ES		0.03	0.62	0.79	0.82			

*=significant at p<0.05, F= output of repeated measure one way ANOVA, ES= Effect Size

Table (5): Correlation of gingival index with Streptococcus mutans by groups

Groups		Streptococcus Mutans	
		r	p
Study	Gingival Index	0.46	**0.03***
Control	Gingival Index	0.47	**0.03***

*=significant at p<0.05.

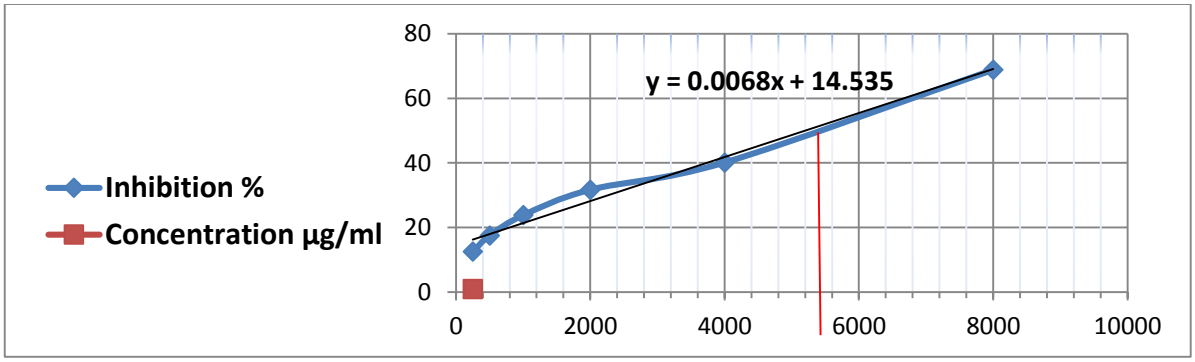

Figure (1): LC50 determination by simple linear regression graph

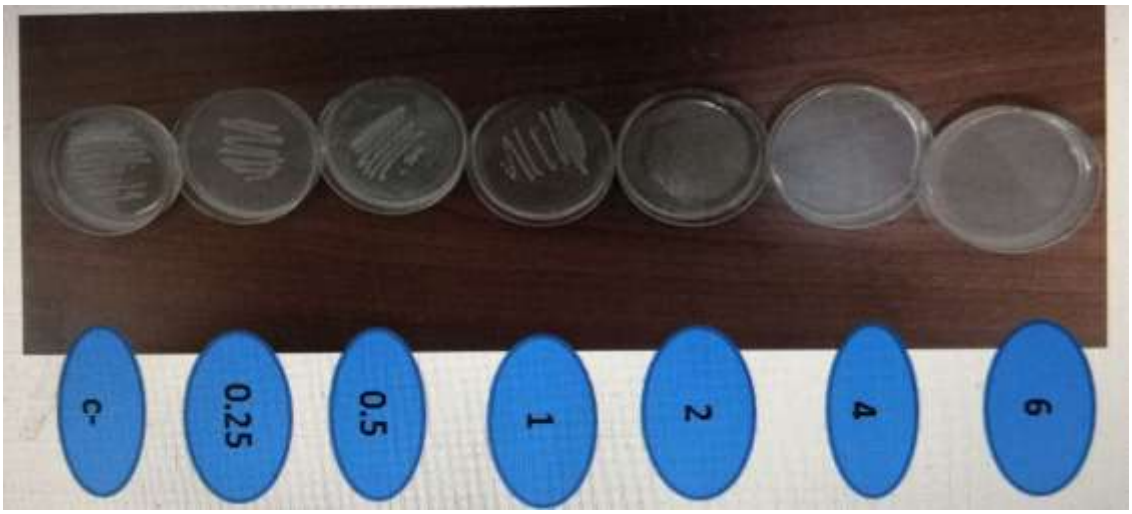

Figure (2): Growth of streptococcus mutans in petri dish.

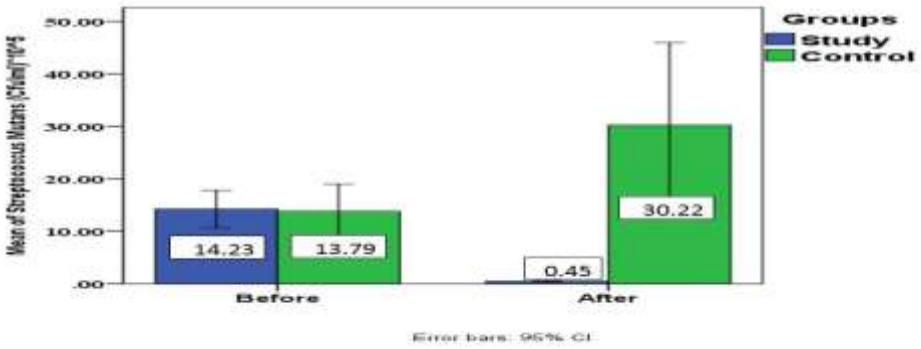

Figure 3: Mean Streptococcus Mutans count before and after mouthwash between groups

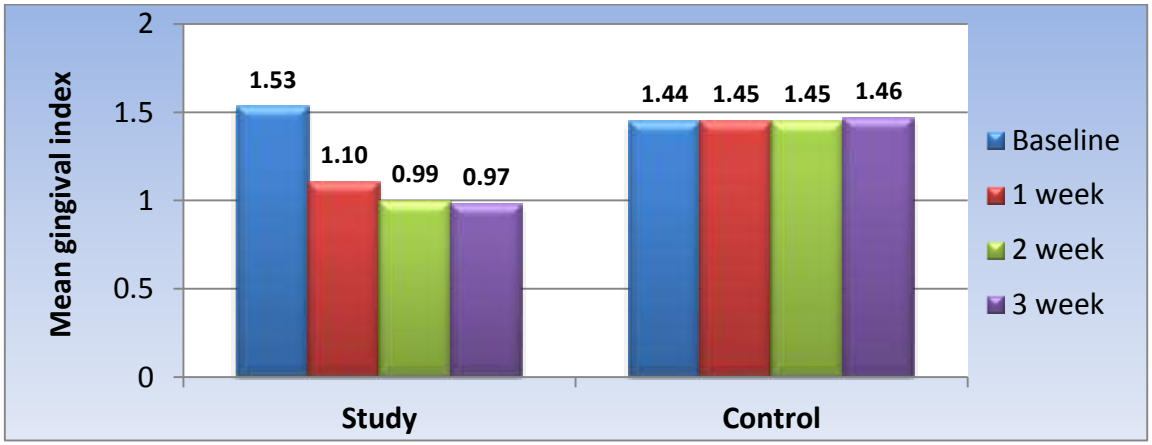

Figure 4: Mean gingival index of groups between visits