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Original papers

The *in vitro* activity of selected mouthrinses on the reference strains of *Trichomonas tenax* and *Entamoeba gingivalis*

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ABSTRACT. Protozoa, such as *Trichomonas tenax*, *Entamoeba gingivalis* and *Leishmania braziliensis*, may be present in the mouth but their role in the pathophysiology of oral diseases is not clear yet. The use of various types of mouthrinses plays an important role in maintaining proper oral hygiene and in removing some of the microbial components from the oral cavity. The purpose of this study was to investigate the effects of selected mouthrinses on the reference strains of *Trichomonas tenax* and *Entamoeba gingivalis* which can be a part of the oral cavity microbiota. Two standard strains *Trichomonas tenax* (ATCC 30207) and *Entamoeba gingivalis* (ATCC 30927) were used and metronidazole as a drug used in the treatment of infections caused by protozoa as well as fourteen agents used as mouthwashes were tested, with two pure compounds acting as mouthrinse ingredients, i.e. 20% benzocaine and 0.2% chlorhexidine, as well as 12 commercially-available formulas: Azulan, Colgate Plax Complete Care Sensitive, Corsodyl 0.2%, Curasept ADS 205, Dentosept, Dentosept A, Eludril Classic, Listerine Total Care, Octenidol, Oral-B Pro-Expert Clinic Line, Sylveco and Tinctura salviae. The protozoonicidal activity of the preparations was evaluated on the basis of the ratio of dead to living ratios after incubation in an incubator (37°C) for 1, 10 and 30 min. Protozoa were counted in the Bürker chamber in each case up to 100 cells in an optical microscope (over 400×). The criterion for the death of protozoa was the lack of movement and changes in the shape and characteristics of cell disintegration. The curves of activity were obtained after experiments conducted for 5–7 different solutions of each preparation. On the basis of the curves, the solution killing 50% of the population (CL_{50}) was calculated. All mouthrinses tested in this work in their undiluted form acted lethally on both protozoa. Benzocaine, used as a local anesthetic, has etiotropic properties which can be useful for supporting antiprotozoal treatment. Chlorhexidine confirmed its high efficiency in the eradication of potentially pathogenic protozoa. The use of mouthrinses is an important complement for other procedures intended to maintain correct oral hygiene.

Keywords: reference strains, *Trichomonas tenax*, *Entamoeba gingivalis*, mouthrinses, antiprotozoal activity

Introduction

The oral cavity creates a specific microbiota (all organisms which inhibit human body) whose qualitative and quantitative status changes during human ontogenesis from birth to the elderly. In interdental spaces, plaque and numerous mucous membrane diverticula, there are hundreds of different microorganisms (viruses, bacteria, fungi and protozoa), each having a particular role, strongly interacting with each other and with the host, in sickness or in health. The oral microbiome has an important role in the maintenance of oral cavity not only during health, but in pathophysiology of its diseases as well [1–4].

Among viruses which are causing different changes in the oral cavity the most common are Coxsackie A1-6, A8, A10, A12, A16, A22 and HHV-1, 2, 3, 4, 5, HPV 1, 2, 4, 6, 11, 13, 32. Approximately 772 prokaryotic species from six broad phyla: Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Bacteroidetes and Spirochaetes constitute 96% of total oral bacteria. The study of mycobiota in the oral cavity of healthy individuals showed the presence of 101 species belonging to 85 (75 cultivable) types of fungi, most commonly represented by *Candida*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, *Cryptococcus* as well as *Geotrichum*, *Penicillium*, *Scopulariopsis*, *Malassezia* and *Epi-*

coccum. Protozoa, such as *Trichomonas tenax*, *Entamoeba gingivalis* and *Leishmania braziliensis*, may also be present in the mouth as parasites [5–11]. So the second most abundant microbiota after the gastrointestinal tract is human oral cavity where there are good conditions for the development of different microorganisms, which can create a biofilm playing an important role in the development of different diseases; in fact the importance of a great number of them is not so clear. Anyway, the involvement of the entire oral communities suggests that some microorganisms play role in the pathophysiology of periodontitis [12].

Actions aimed at maintaining proper oral hygiene should be performed from the moment of eruption of the first milk teeth (6–12 months old) to the end of our lives. Among different methods, various types of rinsing are useful [13,14], which is also used to treat local lesions (gingivitis, periodontitis, surgical treatment) or general (immune disorder, chemotherapy) [15]. The use of various types of mouthrinses plays an important role in maintaining proper oral hygiene and in removing some of the microbial components from the oral cavity [16]. As recommended by the American Dental Association (ADA), mouthwashes should not adversely affect the individual components of a normal microbiota, but only eliminate pathogens [17].

In the previous paper, we have shown the effects of selected mouthrinses on the eight reference strains of fungi: *C. albicans* (CBS 2312), *C. albicans* (L 45), *C. albicans* (ATCC 24433), *C. dubliniensis* (CBS 7987), *C. glabrata* (CBS 862), *C. krusei* (CBS 573), *C. parapsilosis* (CBS 10947) and *C. tropicalis* (CBS 2424) [18]. The purpose of this study was to investigate the effects of selected mouthrinses on the reference strains of *Trichomonas tenax* and *Entamoeba gingivalis* which can be a part of the oral cavity microbiota.

Materials and Methods

The research was carried out on two standard strains *Trichomonas tenax* (ATCC 30207) and *Entamoeba gingivalis* (ATCC 30927). Protozoa were multiplied on ATCC Medium 2692 and ATCC Medium 1171, respectively. The volume of 0.2 cm^3 of 24 h protozoan culture containing approximately 100,000 individuals was placed in agglutination tubes. Fourteen agents used as mouthwashes were tested, with two pure compounds acting as mouthrinse ingredients, i.e. 20% benzocaine and 0.2% chlorhexidine, as well as 12 commercially-available formulas: Azulan, Colgate Plax Complete Care Sensitive, Corsodyl 0.2%, Curasept ADS 205, Dentosept, Dentosept A, Eludril Classic, Listerine Total Care, Octenidol, Oral-B Pro-Expert Clinic Line, Sylveco and Tinctura salviae. The same volume (0.2 cm^3) of the appropriate dilution was added from the investigated rinses, as well as metronidazole as a drug used in the treatment of infections caused by protozoa. The preparations were dissolved immediately before use in distilled water; protozoonicidal properties were examined in a series of solutions in geometrical progression. Each experiment was repeated 3 times, assessing protozoon mortality after 1, 10 and 30 minutes.

The protozoonicidal activity of the preparations was evaluated on the basis of the ratio of dead to living ratios after incubation in an incubator (37°C) for 1, 10 and 30 min. Protozoa were counted in the Bürker chamber in each case up to 100 cells in an optical microscope (over 400×). The criterion for

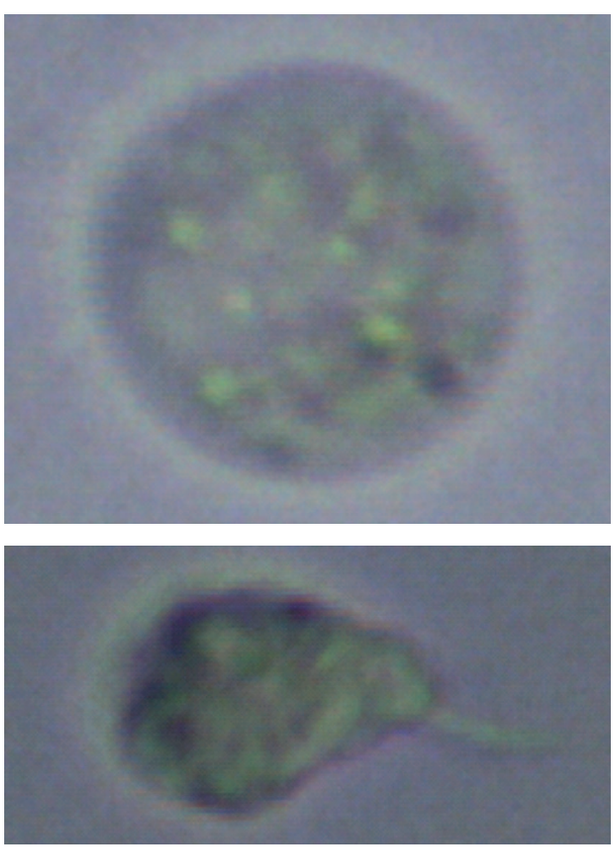


Fig. 1 a,b. Changes of *Trichomonas tenax* under protozoonicidal solutions – no movement, rounded shape, loss of flagella

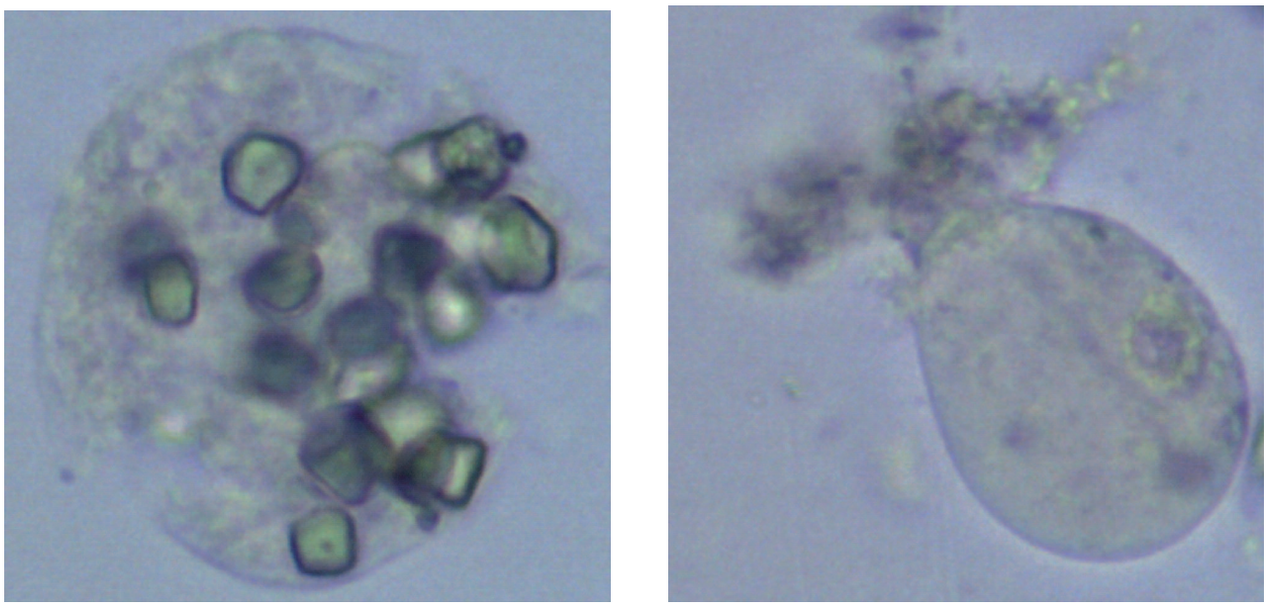


Fig. 2 a,b. Changes of *Entamoeba gingivalis* under protozoonicidal solutions – the cell membrane breaks and the contents leak out

the death of protozoa was the lack of movement and changes in the shape and characteristics of cell disintegration (Figs. 1,2).

The curves of activity were obtained after experiments conducted for 5 to 7 different solutions of each preparation [19].

On the basis of the curves, the solution killing 50% of the population (CL_{50}) was calculated using the Kadłubowski's formula:

$$CL_{50} = \frac{C_2 - C_1}{\bar{N}_2 - \bar{N}_1}(50 - \bar{N}_1) + \bar{C}_1$$

where $\bar{N}_1$ and $\bar{N}_2$ are arithmetic means of mortality rates of protozoa, $\bar{C}_1$ and $\bar{C}_2$ are the average of the respective concentrations of the test preparation [20].

Results and Discussion

All mouthrinses tested in this work in their undiluted form acted lethally on both *Entamoeba gingivalis* (Gros) Brumpt (ATCC 30927) and *Trichomonas tenax* (Muller) Dobell (ATCC 30207), after 1, 10 and 30 minutes, respectively. The detailed data is gathered in Tables 1 and 2. Even in the lowest dilutions of Dentosept A assessed (0.0078), the mortality of *Trichomonas tenax* was 50% after 10 minutes and 70% after 30 minutes, while *Entamoeba gingivalis* demonstrated 10% and 20% mortality, for 10 and 30 minutes, respectively. This confirms the high activity of tested rinses against protozoa.

After plotting the activity curves, examples of which are shown in Figs 4 and 5, the concentration/lethal dilution for 50% of subjects (CL_{50}) was calculated. The CL_{50} values and the smallest dilutions resulting in mortality of 50% of *T. tenax* and *E. gingivalis* protozoa are included in Table 3.

As can be seen from the table above, the CL_{50} values for chlorhexidine, benzocaine and metronidazole were lower for *T. tenax* compared to *E. gingivalis,* while for rinses, the smallest dilutions resulting in mortality of 50% of protozoa, were lower with respect to *T. tenax*; for both of the

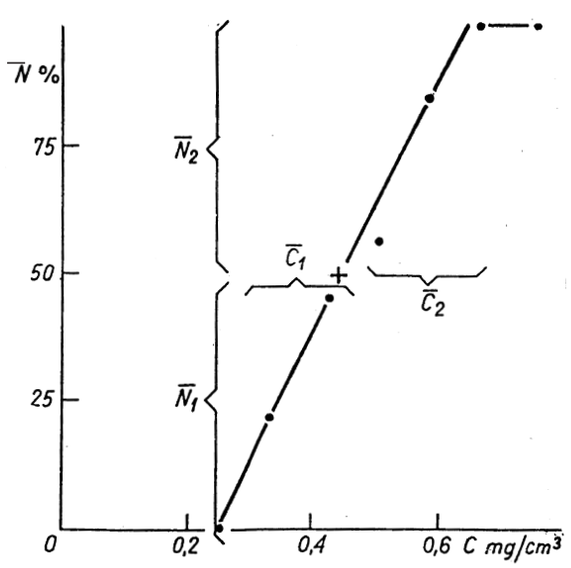


Fig. 3. The curve of activity used for CL_{50} calculation

Table 1. Mortality (%) of *Trichomonas tenax* under different solutions of specimens

Rinse	No*	Dilution							
		0**	1:1	1:4	1:8	1:16	1:32	1:64	1:128
Chlorhexidine 0.2%	1.	100	80	27	0	0	0	0	0
	2.	100	90	80	60	40	20	0	0
	3.	100	98	90	70	50	20	0	0
Listerine	1.	95	65	23	0	0	0	0	0
	2.	100	90	69	40	20	10	0	0
	3.	100	98	90	70	40	20	0	0
Oral-B	1.	100	28	0	0	0	0	0	0
	2.	100	95	90	70	40	20	0	0
	3.	100	100	93	90	70	40	0	0
Colgate	1.	100	80	32	0	0	0	0	0
	2.	100	100	92	80	50	30	0	0
	3.	100	100	100	90	70	50	0	0
Sylveco	1.	92	65	30	0	0	0	0	0
	2.	100	100	90	69	30	10	0	0
	3.	100	100	92	80	43	20	0	0
Corsodyl	1.	100	70	28	0	0	0	0	0
	2.	100	98	80	40	20	2	0	0
	3.	100	100	90	70	42	20	0	0
Curasept	1.	90	32	0	0	0	0	0	0
	2.	100	100	80	39	20	2	0	0
	3.	100	100	92	60	30	10	0	0
Octenidol	1.	100	92	35	0	0	0	0	0
	2.	100	100	92	80	50	20	0	0
	3.	100	100	98	90	70	40	0	0
Azulan	1.	100	75	20	0	0	0	0	0
	2.	100	100	90	70	30	10	0	0
	3.	100	100	97	78	50	27	0	0
Tinctura Salviae	1.	100	25	0	0	0	0	0	0
	2.	100	100	100	92	80	50	0	0
	3.	100	100	100	97	90	80	0	0
Dentosept	1.	100	30	0	0	0	0	0	0
	2.	100	100	100	97	90	60	45	30
	3.	100	100	100	100	95	80	70	45
Eludril	1.	100	90	25	0	0	0	0	0
	2.	100	100	87	70	35	12	0	0
	3.	100	100	92	78	50	26	0	0
Dentosept A	1.	100	23	0	0	0	0	0	0
	2.	100	100	100	100	92	80	65	50
	3.	100	100	100	100	100	100	85	70
Benzocaine 2%	1.	100	85	60	35	0	0	0	0
	2.	100	100	72	50	28	8	0	0
	3.	100	100	80	62	31	14	0	0
Metronidazole	1.	85	40	10	0	0	0	0	0
	2.	70	50	20	0	0	0	0	0
	3.	80	55	35	0	0	0	0	0

* examination 1 – after 1 min; 2- after 10 min; 3 – after 30 min. ** 0 – without dilution

Table 2. Mortality (%) of *Entamoeba gingivalis* under different solutions of specimens

Rinse	No*	Dilution							
		0**	1:1	1:4	1:8	1:16	1:32	1:64	1:128
Chlorhexidine 0.2%	1.	100	95	75	32	0	0	0	0
	2.	100	100	86	69	52	28	0	0
	3.	100	100	88	71	52	31	0	0
Listerine	1.	100	67	25	0	0	0	0	0
	2.	100	100	65	49	29	9	0	0
	3.	100	100	19	62	49	31	0	0
Oral-B	1.	100	70	32	0	0	0	0	0
	2.	100	100	91	70	29	7	0	0
	3.	100	100	90	72	58	28	0	0
Colgate	1.	92	58	24	0	0	0	0	0
	2.	100	100	71	48	19	5	0	0
	3.	100	100	74	55	20	7	0	0
Sylveco	1.	95	70	25	0	0	0	0	0
	2.	100	100	70	39	19	4	0	0
	3.	100	100	72	40	24	5	0	0
Corsodyl	1.	100	92	70	32	0	0	0	0
	2.	100	100	64	42	23	11	0	0
	3.	100	100	71	52	40	25	0	0
Curasept	1.	100	80	31	0	0	0	0	0
	2.	100	100	90	64	39	15	0	0
	3.	100	100	90	70	40	16	0	0
Octenidol	1.	100	72	28	0	0	0	0	0
	2.	100	100	89	68	39	18	0	0
	3.	100	100	90	70	40	20	0	0
Azulan	1.	100	90	60	30	0	0	0	0
	2.	100	100	79	46	24	6	0	0
	3.	100	100	80	50	28	5	0	0
Tinctura Salviae	1.	90	68	25	0	0	0	0	0
	2.	100	100	89	76	49	16	0	0
	3.	100	100	90	79	50	20	0	0
Dentosept	1.	100	100	82	70	28	0	0	0
	2.	100	100	100	82	37	6	0	0
	3.	100	100	100	85	40	7	0	0
Eludril	1.	100	90	62	27	0	0	0	0
	2.	100	100	88	65	26	1	0	0
	3.	100	100	90	68	27	5	0	0
Dentosept A	1.	100	100	85	70	31	0	0	0
	2.	100	100	95	80	63	33	25	10
	3.	100	100	99	90	74	60	40	20
Benzocaine 2%	1.	100	100	90	80	35	0	0	0
	2.	100	100	88	73	59	36	0	0
	3.	100	100	88	77	60	42	0	0
Metronidazole	1.	100	90	45	15	0	0	0	0
	2.	94	80	50	30	10	0	0	0
	3.	96	90	70	50	30	0	0	0

* examination 1 – after 1 min; 2 – after 10 min; 3 – after 30 min. ** 0 – without dilution

Table 3. The values of CL_{50} and the smallest dilutions causing mortality of 50% of protozoa for individual rinses after 1, 10 and 30 minutes

Rinse	*Trichomonas tenax*			*Entamoeba gingivalis*		
	examination after					
	1 min	10 min	30 min	1 min	10 min	30 min
Chlorhexidine 0.2%	0.3279	0.0938	0.0625	0.1401	0.0604	0.0595
Listerine	0.3922	0.1681	0.0833	0.3915	0.1250	0.0673
Oral-B	0.5069	0.0833	0.0416	0.3927	0.0945	0.0542
Colgate	0.3941	0.0625	0.0312	0.3905	0.1333	0.1161
Sylveco	0.3539	0.0946	0.0743	0.3931	0.1714	0.1696
Corsodyl	0.3921	0.1563	0.0804	0.1927	0.1705	0.1146
Curasept	0.5086	0.1585	0.1042	0.3941	0.0900	0.0833
Octenidol	0.5549	0.0625	0.0416	0.3924	0.0858	0.0833
Azulan	0,3918	0.0938	0.0625	0.1914	0.1402	0.1250
Tinct. Salviae	0.5067	0.0312	0.0130	0.3933	0.0648	0.0625
Dentosept	0.5071	0.0208	0.0094	0.1560	0.0801	0.0764
Eludril	0.3946	0.0078	0.0052	0.1913	0.0487	0.0234
Dentosept A	0.5071	0.0893	0.0625	0.1561	0.1010	0.0976
Benzocaine 2%	0.2183	0.1250	0.1008	0.0821	0.0502	0.0451
Metronidazole	0.6354	0.5000	0.4375	0.3602	0.2500	0.1250

protozoa the strongest effect was seen in the case of Dentosept A.

Trichomonas tenax may play a role in the pathophysiology of periodontal diseases. If we compare the prevalence of *T. tenax* in diseased periodontium and among young people without changes in periodontium it ranges from 0 to 94.1% and from 0–4%, retrospectively. Protozoa prevalence is higher in patients with Down's Syndrome and periodontitis (18.8%) than in healthy individuals (3%). More than half of patients are included in the third category of periodontal therapeutic needs, which indicates a significant disease advancement. *T. tenax* prevalence is 3 times higher in patients with profound than peripheral periodontitis [21–24]. Benabdelkader et al. [25], by combining a polyphasic approach that associates culture and qPCR, found a statistically significant correlation between the periodontitis severity and the presence of different strains of *T. tenax*, but it was not possible to determine whether they are a cause or a consequence of the disease.

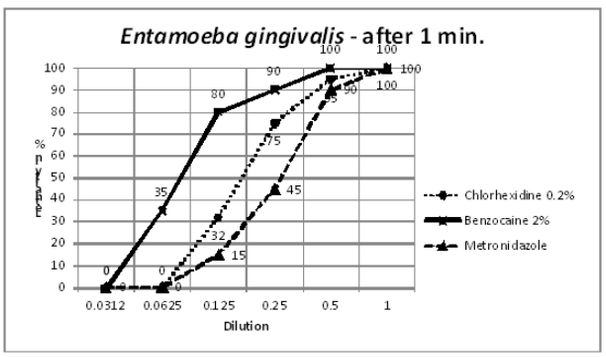
T. tenax displays all pathogenic traits like cell adhesion, synthesizes and secretes proteinases, hemolysins and cellular damages, characteristic of *T. vaginalis*, and therefore it must be considered as a pathogenic species [26–28].

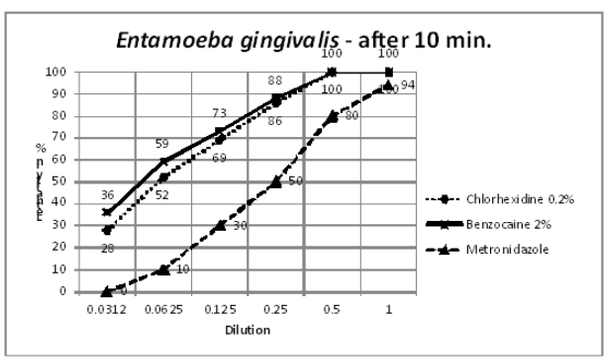
Mature dental plaque is an oral structure favoring the growth and survival of *T. tenax* in the physiologic conditions in the human mouth and in some circumstances it spreads from the dental plaque into the diseased endodontium [29].

T. tenax can contribute to the development of dental cavity caries [30]. It can be responsible not only for oral diseases, but for tonsils, salivary glands, lymph nodes or respiratory tract infections as well [31–36].

The main symptoms of trichomonosis are bleeding gums when taking meals and brushing teeth, mouth pain; inflammation of the gums with bright red tinges and deepened gingival pockets [26,28].

Entamoeba gingivalis is detected mainly in patients with periodontium lesions (up to 83.3%), which arise, e.g. under the influence of proteolytic enzymes produced by this protozoan. It should be underlined that periodontitis can be present in different age, also among children [37,38]. The relationship between *E. gingivalis* and inflammation of periodontitis, plaque accumulation, and the occurrence of necrotic and ulcer gingivitis has been indicated. In gingival pockets with a depth of 1 to 3





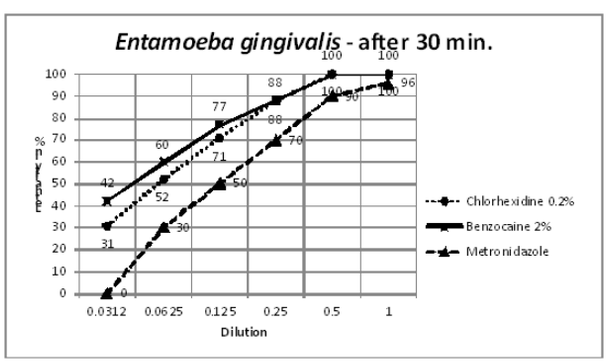
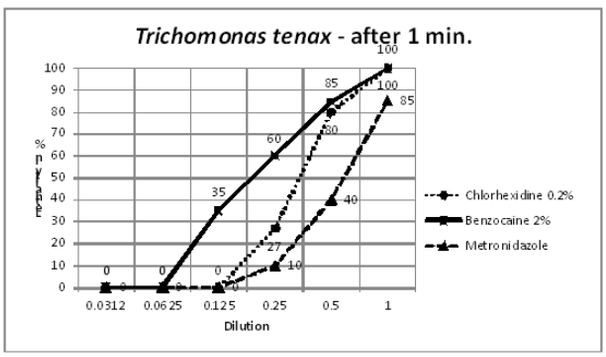
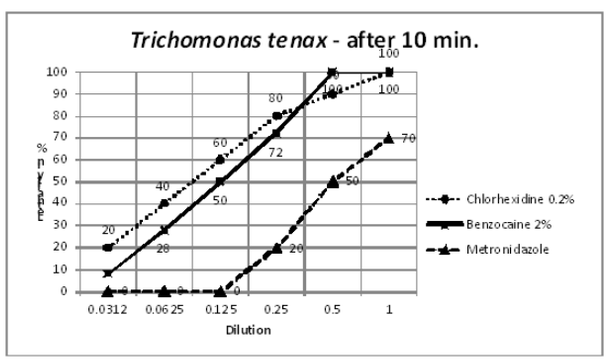


Fig. 4. The curves of 0.2% chlorhexidine, 2% benzocaine and metronidazole activity after 1 min, 10 min and 30 min on *E. gingivalis*





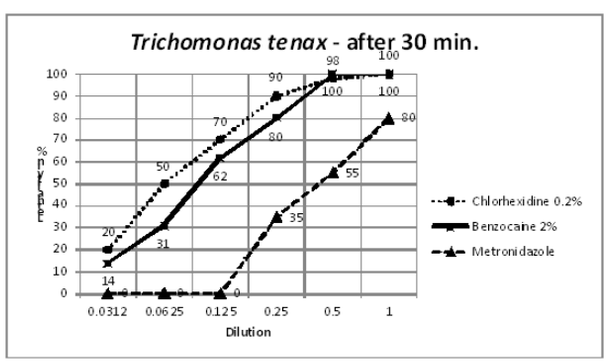


Fig. 5. The curves of 0.2% chlorhexidine, 2% benzocaine and metronidazole activity after 1 min, 10 min and 30 min on *T. tenax*

mm, the frequency of detection of these protozoa is 29%, while in pockets with a depth of 4 to 6 mm it is up to 53% [39-41].

Studies have also shown that *E. gingivalis* can cause not only periodontal diseases and dental cavity caries but also osteomyelitis, and even infections caused by intrauterine contraceptive device (IUD) [24,30].

In the course of oral cavity amoebosis, fatigue, headache, sore throat unpleasant odor from the mouth, and gum itchiness [42] may occur.

According to a study carried out by Ponce de Leon et al. [43] among people who are wearing dentures, protozoa are found in almost 3/4 of them, in one fifth of cases the infections were caused by both protozoa simultaneously.

Mouth washing and tooth brushing are significantly correlated with the prevalence of *E. gingivalis* and *T. tenax,* so attention paid to oral and dental health standards is able to prevent people from infection with those parasites [24,30].

Only effectiveness of mouthrinses against bacteria and fungi was a subject of different studies. In the available literature, no publications were found regarding the action of particular mouthwashes on protozoa present in the oral cavity, so the data obtained in this work cannot be compared with the data of other authors. In the available literature, there is only one article which compares the prevalence of protozoa in the oral cavity among people who use or do not use mouthrinses; the prevalence is statistically higher if

people do not use mouthrinses [24].

In conclusions we can say that: the oral rinses have an antiprotozoal action; Benzocaine, used as a local anesthetic, has etiotropic properties which can be useful for supporting antiprotozoal treatment; Chlorhexidine confirmed its high efficiency in the eradication of potentially pathogenic protozoa; the use of mouthrinses is an important complement for other procedures intended to maintain correct oral hygiene.

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