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Original Article

Effect of Bulgarian propolis on the oral microflora in adolescents with plaque-induced gingivitis

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ABSTRACT

We compared the effect of two therapeutic approaches (marketed toothpaste and addition of Bulgarian propolis extract to the toothpaste) on gingival inflammation, plaque formation and oral microbial flora on Bulgarian adolescents with moderate plaque-induced gingivitis. The participants were divided randomly into two groups of 35 students. The first group was instructed to use marketed toothpaste in their routine oral hygiene. The second group was instructed to add 10 drops of Propolin® to the toothpaste before every brushing. The Gingival index and Plaque index were registered and dental plaque samples were collected on the first visit and on the 20th day of the study. After the treatment, the number of students with Gingival index = 1.1–2.0 in the second group was significantly lower than the respective number in the first group. *Neisseria* spp. and *Streptococcus* spp. were present in all samples before and after treatment. The addition of propolis resulted in the complete eradication of *Streptococcus mutans*, *Candida albicans*, *Fusobacterium varium*, *Gram-negative cocci*, *Gram-positive rods*, *Porphyromonas asaccharolyticus*, *Prevotella bivia*, *Prevotella intermedia*, *Prevotella melani* and *Streptococcus intermedius*. The analyses of Propolin® composition revealed it was a black poplar type propolis and is rich in compounds with pronounced antimicrobial activity. In conclusion, the addition of Bulgarian propolis to the toothpaste improved the gingival health in adolescents with moderate plaque-induced gingivitis and resulted in increased activity against potential periodontal and cariogenic pathogens.

Introduction

Over the past few years, interest in natural products as possible antibacterial agents for oral health maintenance formulations has increased (Tatikonda et al., 2014; Ercan et al., 2015). The antibacterial, antifungal, antiviral, antitumor, immunomodulatory and anti-inflammatory properties of propolis have been studied and reported extensively (Draganova-Filipova et al., 2010; Araujo et al., 2012; Machorowska-Pieniązek et al., 2013; Vagish Kumar, 2014). These beneficial activities were shown to be related to the presence of varieties of biologically active compounds, such as rutin, ferulic acid, quercetin, caffeic acid phenethyl ester (CAPE), artepillin C, pinocembrin, chrysin, galangin etc.

The most common periodontal disease in children and adolescents is plaque-induced gingivitis. It is considered to be the second most prevalent oral disease after dental caries affecting over 75% of the world population (Papapanou, 1999; Califano, 2003; Petersen, 2003). Gingivitis is an inflammatory disease, which affects the gingival soft tissues. Peak onset of gingivitis is between 11 and 13 years of age (Novaes Júnior et al., 2004; Cobb, 2008). Poor oral hygiene leads to dental plaque accumulation and subsequently to gingivitis (Gafan et al., 2004). However, the disease can be reversed and mechanical plaque removal is considered to be the most effective method as long as the patient performs it properly (Franco Neto et al., 2008; Tatikonda et al., 2014). Dental plaque as a biofilm is a complex community that consists of bacteria attached to each other and mostly to the tooth surface (Cobb, 2008; Marsh, 2010).

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Importantly, the presence of some microbiota inhibits the growth of pathogenic genera and therefore it is of great importance for oral health. Furthermore, an important requirement for the antimicrobial agents used in products for oral hygiene is their selective activity against pathogenic bacteria (Marsh, 2010).

On the other hand, the extracellular matrix produced by bacteria hinders the effect of antimicrobial agents (Cobb, 2008). Efficient treatment of gingivitis could be influenced also by patient compliance. Not all patients could remove the plaque effectively and recently interest in additional approaches (*e.g.* use of mouthwash) is increasing. However, the adverse effects of some components of the mouthwashes limit their long-term use (Franco Neto et al., 2008; Tatikonda et al., 2014). An alternative approach is an application of natural products with well-known antibacterial properties, for example, propolis. However, there are relatively few reports on Bulgarian propolis and most of them are *in vitro* studies (Boyanova et al., 2006; Gardjeva et al., 2007). To our knowledge, the effects of Bulgarian propolis on oral health and microflora in adolescents have not been studied before. Hence, the aim of the present study was to compare the effect of two therapeutic approaches (marketed toothpaste and addition of 20% hydroalcoholic extract of Bulgarian propolis to the same toothpaste before brushing) on gingival inflammation, plaque formation, and the oral microbial flora of Bulgarian adolescents with moderate plaque-induced gingivitis.

Materials and methods

Products

The propolis extract Propolin® (lot number: 01-07062018) the toothpaste "Astera parodont active"® (lot number: № 3141108-8) and the commercial propolis extract (lot number: 01816/ТД 11/2015) were obtained from the local pharmacy.

Patients

A preliminary screening for plaque-induced gingivitis in 1391 students was performed at the Humanitarian High School "St. St. Cyril and Methodius" in Plovdiv, Bulgaria. Gingival disease was diagnosed in 531 students. During the initial examination oral health was evaluated in accordance with WHO instructions. The Gingival index was used for the evaluation of the gingival status. Oral hygiene was assessed by the Plaque index (Löe, 1967).

Study design

The participants were selected, as follows:

- Inclusion criteria: (1) physically and mentally healthy adolescents (with permanent dentition); (2) age between 12 and 18 years; (3) patients of both genders; (4) diagnosis of moderate plaque-induced gingivitis (GI = 1.1–1.9).
- Exclusion criteria: (1) treatment with an orthodontic appliance; (2) severe deformities of jaws and teeth; (3) severe plaque-induced gingivitis; (4) smokers.
- Adolescents meeting the described requirements were selected and asked for consent. A total of 70 high-school students with moderate gingival inflammation (according to Löe and Silness) were included in the study (Löe, 1967). Carious lesions were treated, poor dental restorations were corrected and calculus was removed, if present.
- The examinations were performed in the school dental office after standardization of the examination to ensure uniform interpretation and criteria. All examinations were carried out with the same lighting, after isolation and drying the teeth. Individual sterile mirrors, probes, and bead probes were used. The results were stored in specific lists, developed for the purpose of examinations of adolescents with plaque-induced gingivitis.

Material collection

At the first visit dental plaque samples were obtained from the dried vestibular tooth surface near the gingival margin of mandibular central incisors using sterile curettes. They were placed in a Stuart transport medium (Himedia, India) and delivered within 1 h to the Department of Microbiology and Immunology, Faculty of Pharmacy at the Medical University of Plovdiv for microbiological examination (Jorgensen et al., 2015). Sample processing began immediately after the arrival of specimens at the Laboratory.

Clinical exam

After the sample collection, the adolescents were motivated to perform regular oral hygiene. The subjects were provided with a medium bristle toothbrush and the toothpaste "Astera parodont active"®. This marketed Bulgarian product contains aqua, sorbitol, hydrated silica, peg-8, aluminum lactate, sodium lauryl sulfate, cellulose gum, aroma, limonene, sodium monofluorophosphate, zinc citrate, titanium dioxide, PVP, sodium saccharin, allantoin, methylparaben, triclosan and CI 12490. The participants were instructed to brush their teeth according to the Bass technique for 2.5–3 min two times daily (morning and evening).

Regarding the teeth brushing technique there is no uniform standard. The horizontal scrub method is recommended in children and the modified Bass technique in adults (Hayasaki et al., 2014). Based on these reports we chose the Bass technique for our study.

The participants were divided randomly into two groups of 35 students. The first group (A) was instructed to use the provided toothpaste. The second group (AP) received additionally a vial of Propolin®. Propolin® is also a marketed product and contains standardized 20% hydroalcoholic extract of Bulgarian propolis. The adolescents in group AP were instructed to add 10 drops of Propolin® (equivalent to 2.5 mg Bulgarian propolis) to the amount of toothpaste used for every brushing.

A control examination for group A and group AP was performed weekly to reinforce the patients' motivation and to evaluate compliance. The used amount of toothpaste and Propolin® was determined. A final examination was performed on the 20th day of the study. The Gingival Index (GI) and Plaque Index (PLI) were registered and dental plaque samples were collected for microbiological analysis.

Microbiological evaluation

Bacterial aerobic isolation was performed by specimen inoculation in Tryptic soy agar plates with 5% defibrinated sheep blood (Liofilchem, Italy) for 24 h at 36 ± 1 °C and for *Candida* isolation in Chromatic™ Candida agar (Lioflichem, Italy) in aerobic conditions for 48 h at 35 °C. Simultaneous anaerobic cultures in ready-made Schaedler agar + 5% sheep blood plates (bioMerieux, France) were inoculated in special anaerobic pouches (Genbag Anaerobic, bioMerieux, France) for 48 h at 35 °C (Egwari et al., 2011).

Microbial identification

Isolates were further Gram stained and identified using conventional methods and API system (bioMerieux-France). Aerobic bacteria recovered from Tryptic soy agar plates with sheep blood 5% were identified by API 20 Strep (bioMerieux, France) after incubation in this system at 36 °C ± 1 °C in aerobic conditions for 4–4½ h to obtain a first reading and for 24 h to obtain a second reading if

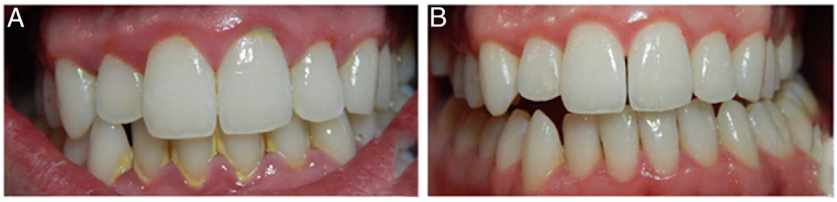

Fig. 1. Dental status of adolescents after 20 days of treatment with marketed toothpaste (A) and Propolin® added to the toothpaste (B).

Table 1
Effect of two clinical approaches on the aerobic microbial flora, isolated from seventy adolescents with plaque-induced gingivitis (GI = 1.1–2.0). Multiple species were isolated from all samples. [a] $p < 0.05$; [b] $p < 0.005$.

Group	Isolated microorganism	Number of positive samples before treatment (%)	Number of positive samples after treatment (%)
A	*S. viridans* group (excl. *S. mutans*)	35 (100)	35 (100)
	S. mutans	9 (25.7)	4 (11.4)
	Neisseria spp.	35 (100)	35 (100)
	C. albicans	5 (14.3)	2 (5.7)
AP	*S. viridans* group (excl. *S. mutans*)	35 (100)	35 (100)
	S. mutans	10 (28.6)	0 (0)[b]
	Neiseria spp.	35 (100)	35 (100)
	C. albicans	6 (17.1)	0 (0)[a]

required. Isolated anaerobic bacteria were identified on the basis of the API 20A identification system after 48 h of incubation. *Candida* spp. were identified on the basis of different color colonies on Chromatic Candida agar, morphology tests for the presence of hyphae (mycelium) or pseudohyphae (pseudomycelium) on RAT Medium (Rice Agar Tween) and using API 20C AUX following the manufacturer's instructions. Identification was obtained with the numerical profile.

Chemical composition of propolis extracts

Individual phenolic compounds in the propolis were determined on an Agilent 1220 HPLC system (Agilent Technology, USA) as described in Slavov et al. (2017).

The polar non-volatile compounds in the propolis extract were determined by gas chromatography–mass spectrometry (GC–MS) according to Bankova et al. (2016). A Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometry system equipped with HP-5ms column (30 m, 0.25 mm ID, 0.5 μm thickness) was used. The temperature was increased from 60 to 300 °C at the rate of 5 °C/min, and a 10 min hold at 300 °C. The injector temperature was 280 °C, and the interface temperature was 300 °C. Helium was used as a carrier gas (flow rate 0.8 ml/min). The split ratio was 1:10 and the ionization voltage 70 eV. The identification of the compounds was made comparing the mass spectra and the retention times with literature and authentic propolis samples (Isidorov, 2015).

The volatile aroma substances were analyzed by GC–MS on an Agilent GC 7890 with mass-selective detector Agilent MD 5975 and column HP-5ms as described by Vasileva et al. (2018).

The analyses were performed in triplicate and the data are given as mean values.

Statistical analysis

Data were analyzed statistically using SPSS v.19.0. The effect of two clinical approaches on the aerobic microbial flora before and after treatment was assessed using the Mc Nemar statistical test. The mean percentage of total ion current of non-volatile polar metabolites in the propolis extract Propolin® and the other commercial propolis extract was compared using Student's *t*-test.

Results

The final examinations revealed a significant decrease in the number of adolescents with PLI = 1.1–2.0 and GI = 1.1–2.0 in both groups before and after treatment. Comparing the number of students with PLI in both groups at the end of the study demonstrated a significant reduction in the number of subjects with PLI = 1.1–2.0 in group AP versus students in group A – 3 (8.5%) vs 9 (25.7%); $p < 0.05$.

Similar results were obtained when the adolescents with GI = 1.1–2.0 were compared. After the treatment, the number of students with GI = 1.1–2.0 in group AP was significantly lower than the respective number in group A – 2 (5.7%) vs 7 (20.0%); $p < 0.05$. No statistically significant difference was registered in either (PLI and GI) scores at the initial examination.

No side effects of the treatment were reported in either group during the study. The combined treatment with toothpaste and propolis was rated as very good by the students in the group (Fig. 1).

There was no statistically significant difference between the groups regarding the isolated microorganisms before the treatment. The microbiological analysis showed that *Neisseria* spp. and *Streptococcus* spp. were present in all samples before and after treatment (Table 1). According to Doern and Burnham (2010), *S. viridans* group consisted of *S. mutans* group, *S. salivarius* group, *S. anginosus* group, *S. mitis* group, *S. sanguinis* group, and *S. bovis*. Given the major role of *S. mutans* in the development of caries, this group is presented separately in the present study. The addition of propolis to the toothpaste (group AP) resulted in a significant decrease in the number of samples positive for *S. mutans*. This therapeutic approach led to a significant reduction in the number of *Candida albicans* positive adolescents. However, complete eradication of *S. mutans* and *C. albicans* was achieved only in the AP group.

The studied therapeutic approaches had a great influence on the anaerobic microflora (Table 2). The marketed toothpaste led to eradication of only three groups: *Fusobacterium varium*, *Gram-negative cocci* and *Prevotella intermedia*. The addition of propolis resulted in complete eradication of *F. varium, Gram-negative cocci*, *Gram-positive rods*, *Porphyromonas asaccharolyticus, Prevotella bivia*, *P. intermedia*, *Prevotella melani*, and *Streptococcus intermedius*.

In the subsequent experiments, the composition of Propolin® was assessed by HPLC and GC–MS. The most common types of substances identified in propolis samples were aromatic acids and their derivatives, cinnamic acid derivatives, flavonoids, terpenes and

Table 2
Effect of two clinical approaches on the anaerobic microbial flora, isolated from seventy adolescents with plaque-induced gingivitis (GI = 1.1–2.0). Multiple species were isolated from all samples. [a] $p < 0.05$.

Group	Isolated microorganism	Number of positive samples before treatment (%)	Number of positive samples after treatment (%)
A	*1. Bifidobacterium* spp.	2 (5.7)	1 (2.9)
	2. Fusobacterium varium	6 (17.1)	0 (0)[a]
	3. Gr (−) cocci	2 (5.7)	0 (0)
	4. Gr (−) rods (excl. *Porphyromonas*)	10 (14.3)	4 (8.6)[a]
	5. Gr (+) rods	1 (2.9)	1 (2.9)
	6. Porphyromonas asaccharolyticus	3 (8.6)	1 (2.9)
	7. Prevotella bivia	1 (2.9)	1 (2.9)
	8. Prevotella intermedia	4 (11.4)	0 (0)
	9. Prevotella melani	5 (14.3)	1 (2.9)
	10. S. intermedius	2 (5.7)	1 (2.9)
AP	*1. Bifidobacterium* spp.	1 (2.9)	1 (2.9)
	2. Fusobacterium varium	7 (20.0)	0 (0)[a]
	3. Gr (−) cocci	3 (8.6)	0 (0)
	4. Gr (−) rods (excl. *Porphyromonas*)	5 (5.7)	1 (2.9)
	5. Gr (+) rods	2 (5.7)	0 (0)
	6. Porphyromonas asaccharolyticus	4 (11.4)	0 (0)
	7. Prevotella bivia	2 (5.7)	0 (0)
	8. Prevotella intermedia	5 (14.3)	0 (0)
	9. Prevotella melani	5 (14.3)	0 (0)
	10. S. intermedius	3 (8.6)	0 (0)

Table 3
Individual phenolic substances in Propolin®. The results are presented as mean concentration (μg/ml) ± SD.

Phenolic substance	Propolin®	Commercial propolis extract
Chlorogenic acid	135.20 ± 3.31	2.99 ± 0.58
Caffeic acid	28.51 ± 2.10	0.81 ± 0.08
p-Coumaric acid	527.62 ± 3.59	256.12 ± 2.45
3,4-Dihydroxy-benzoic acid	–	6.45 ± 1.01
Ferulic acid	731.01 ± 2.18	25.31 ± 1.03
Quercetin	45.68 ± 1.45	359.61 ± 1.36
Quercetin-3-β-glucoside	19.32 ± 1.81	40.62 ± 1.12
Myricetin	44.79 ± 1.43	–
Kaempferol	1235.74 ± 1.31	134.44 ± 1.65
Rutin	3979.05 ± 3.69	2615.47 ± 2.13
Catechin	132.76 ± 2.14	–

fatty acids. The analyses suggested that Propolin® used in the clinical studies exhibited high total polyphenolic and flavonoid content (Table 3) compared to randomly chosen commercial propolis extract. The highest concentration of phenolic acids was observed for ferulic acid – 731.0 ± 2.2 μg/ml, and for the flavonoids – rutin (3979.0 ± 3.7 μg/ml) and kaempferol (1235.7 ± 1.3 μg/ml).

The main volatile compounds determined were terpenoids (Supplementary material). Furthermore, various non-volatile polar metabolites were identified in Propolin® (Table 4) and were compared with randomly chosen commercial propolis extract.

Discussion

The microbial biofilm is considered as the main factor in the pathogenesis of periodontal diseases and caries (Chandki et al., 2011).

A significant reduction in the number of adolescents with GI = 1.1–2.0 in both groups before and after the treatment was registered. Similar results were observed regarding the PLI. The obtained results are consistent with those reported by Machorowska-Pieniążek et al. (2013). The authors performed a clinical study of the effect of 3% ethanol extract of Brazilian propolis on 41 patients with cleft lip palate and mean age 12.37 years. The use of propolis toothpaste for 35 days led to reduced dental plaque and gingival inflammation in comparison to the toothpaste without propolis. A similar study, performed by Tanasiewicz et al. (2012) on adult patients confirmed the beneficial effect of propolis-containing preparations on the periodontium. Gingivitis regression was observed in a clinical study after treatment with Brazilian green propolis gel (Cairo do Amaral et al., 2006), and significant plaque reduction was reported after treatment with propolis-containing marketed product (Bhat et al., 2015).

The addition of propolis to the toothpaste showed a great effect on the microbial flora in the oral cavity. *In vitro* studies reported the antimicrobial activity of propolis against *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *P. melaninogenica*, *A. actinomycetemcomitans*, *C. gingivalis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans* (Gebara et al., 2002; Lu et al., 2005; Siqueira et al., 2015). Boyanova et al. (2006) performed an *in vitro* evaluation of the activity of 30% ethanolic extract of Bulgarian propolis against 94 anaerobic bacteria. *Clostridium*, *Bacteroides*, and *Propionibacterium* species were found susceptible.

In the present study, *Neisseria* spp. and *S. viridans* group (excl. *S. mutans*) were recovered from all samples at baseline. Similar results were reported by Machorowska-Pieniązek et al. (2013). The prevalent bacteria in the plaque differed depending on the stage of biofilm maturation. The presence of *Streptococcus* spp. is a common occurrence at the initial stage of colonization. Anaerobic microbiota (*e.g. Fusobacterium* spp., *Porphyromonas* spp., and *Prevotella* spp.) are recovered more often in more mature biofilm (Cobb, 2008).

Interestingly, not all of these bacteria are considered as pathogens. *Streptococcus*, *Neisseria*, *Bifidobacterium* could be found in a healthy oral cavity. *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Treponema* are often isolated from periodontal pockets (Marsh, 2000; Marsh, 2003).

The obtained results revealed that both therapeutic approaches (toothpaste with and without propolis) had no influence on *Neisseria* spp. and *S. viridans* group (excl. *S. mutans*). Their isolation frequency was 100% in all participants before and after the study. Henceforth, a conclusion could be made that the marketed product and the added propolis have little or no influence on the normal bacterial flora in the oral cavity. At the same time, *S. mutans* which play a major role in caries development was altered (Jeon et al., 2011). The marketed product lessened the number of the samples positive for *S. mutans*, while the addition of propolis resulted in the complete eradication of the bacteria after a 20-day treatment. The antibacterial activity of propolis against *S. mutans* was also reported by Koo et al. (2002) and Oda et al. (2016). According to Koo et al. (2002), the most active compounds in propolis were apigenin and tt-farnesol. Duailibe et al. (2007) conducted a study on 41 volunteers aged 11 to 30 years and found an impaired growth of *S. mutans* in saliva

Table 4
Non-volatile polar metabolites in propolis extract Propolin® and other commercial propolis extract. The results were presented as mean percentage of total ion current ± SD ([a]$p < 0.05$).

Compound	Class	Propolis extractPropolin®	Commercial propolis extract
Glycerol	Alcohol (polyol)	1.50 ± 0.11[a]	0.71 ± 0.12
Benzoic acid	Aromatic acid	0.72 ± 0.08	0.54 ± 0.10
Monosaccharides	Carbohydrates	4.79 ± 0.13	–
Hydroquinone	Phenol	–	0.59 ± 0.08
Cinnamic acid	Organic acid	–	0.38 ± 0.08
p-Coumaric acid	Organic acid	1.04 ± 0.09	–
Caffeic acid	Hydroxycinnamic acid	2.31 ± 0.11	–
iso-Ferulic acid	Hydroxycinnamic acid	0.60 ± 0.10	–
Ferulic acid	Hydroxycinnamic acid	1.94 ± 0.12	2.32 ± 0.11
Dimetoxy-cinnamic acid	Organic acid	0.74 ± 0.11	1.27 ± 0.10
3-Methyl-3-butenyl caffeate	Ester	1.54 ± 0.09	–
2-Methyl-2-butenyl caffeate	Ester	0.72 ± 0.08	–
3-Methyl-2-butenyl caffeate	Ester	2.63 ± 0.12	–
Pentenyl ferulat	Ester	0.72 ± 0.09	–
Pinobanksin chalkone	Flavonoid	1.64 ± 0.08[a]	1.18 ± 0.07
iso-Sakuranetin chalkone	Flavanone	0.64 ± 0.09	0.75 ± 0.10
Pinocembrin chalkone	Flavanone	10.10 ± 0.013[a]	5.17 ± 0.12
Pinobanksin	Flavonoid	4.02 ± 0.14	3.76 ± 0.12
Pinostrobin chalkone	Flavonoid	1.04 ± 0.09[a]	1.86 ± 0.11
Benzyl coumarate	Ester	0.44 ± 0.08	–
Pinocembrin	Flavanone	7.02 ± 0.10[a]	2.91 ± 0.09
Pinobanksin acetate chalkone	Ester	1.84 ± 0.11[a]	0.62 ± 0.07
Galangin	Flavonol	22.09 ± 0.12[a]	7.41 ± 0.14
Pinobanksin propanoate chalkone	Ester	0.54 ± 0.10	–
Benzyl caffeate	Ester	2.12 ± 0.09	–
Pinobanksin pentanoate chalkone	Ester	0.54 ± 0.09	–
3-*O*-methyl-pinobanksin	Flavonoid	1.80 ± 0.11	–
Pinobanksin acetate	Flavonoid	5.22 ± 0.08[a]	1.68 ± 0.12
Chrysin	Flavone	10.01 ± 0.11[a]	12.44 ± 0.13
Phenetyl caffeate (CAPE)	Ester	2.54 ± 0.14	–
Pinobanksin butanoate	Ester	0.43 ± 0.08	–
Pinobanksin propanoate	Ester	0.63 ± 0.09[a]	1.01 ± 0.10
Tectochrysin	Flavone	3.02 ± 0.11[a]	5.66 ± 0.12
Di-hydroxy-metoxy flavone	Flavone	0.33 ± 0.09[a]	2.41 ± 0.12
Pinobanksin pentanoate	Ester	1.21 ± 0.10[a]	2.37 ± 0.11
Kaempferol	Flavonol	1.62 ± 0.10[a]	0.81 ± 0.12
Kaempferol methyl ether (all isomers)	Flavonol	–	4.40 ± 0.14
Quercetin methyl ether (all isomers)	Flavonol	3.63 ± 0.13[a]	0.88 ± 0.09
Triterpenoids	Triterpenoid	1.23 ± 0.11	–

samples after application of propolis. Burdock (1998) found that caffeic acid esters (including CAPE) possess significant antimicrobial activity. Carreño et al. (2017) reported potent antioxidant activity and protective effect against oxidative stress of CAPE. Quercetin, *p*-coumaric and ferulic acids also were attributed to possess, besides a significant antioxidant activity, inhibitory action on microorganisms (Burdock, 1998; Banskota et al., 2001).

Similar results were obtained with *C. albicans*. The results of the present study were in accordance with Herrera et al. (2010). They found an inhibited growth of *Candida* spp. after *in vitro* treatment with Chilean propolis solutions. However, Morawiec et al. (2013) found that a Brazilian propolis containing toothpaste had little influence on the number of samples, positive for *C. albicans*. The different results could be explained by the different compositions of both types of propolis (as discussed in subsection 3.3 and above in section 4) or by the influence of the formulation. Morawiec et al. (2013) included 3% ethanol extract of propolis in the toothpaste formulation. In the present study, the adolescents were instructed to add 20% extract to the toothpaste before each teeth brushing, providing immediate delivery of the extract to the oral cavity.

Oda et al. (2016) evaluated also the effect of Brazilian green propolis on the gingival and periodontal fibroblasts *in vitro* and found low cytotoxicity at concentrations up to 2000 μg/ml. Salomão et al. (2004) compared the antimicrobial activity of Bulgarian and Brazilian propolis. The authors proposed a potential relation between the higher antibacterial activity of Bulgarian propolis and the higher content of flavonoids.

Regarding the adverse reactions, Anauate-Netto et al. (2014) reported a clinical study focused on mouthwash containing propolis. Seven of twenty participants in the propolis-treated group reported breath alteration, burning sensation, yellow teeth, taste alteration and bitter taste during the study period. However, the study confirmed the anti-inflammatory effect of the propolis in gingivitis. In contrast, no adverse reactions were reported during the present study in both groups.

In the present study, the isolation frequency of the anaerobes was also affected. The toothpaste reduced the number of samples, positive for *F. varium*, *Prevotella intermedia*, and *P. melani*. However, the addition of propolis evoked a greater effect on the anaerobic microflora. Complete eradication of *F. varium*, *Gram-negative cocci*, *Gram-positive rods*, *P. asaccharolyticus*, *P. bivia*, *P. intermedia*, *P. melani*, and *S. intermedius* was observed to have taken place after the 20 day treatment period. *Bifidobacterium* spp. remained unaffected while Gram-negative rods (excl. *P. asaccharolyticus*) decreased substantially. These results supported the initial hypothesis which motivated the present study relating to the beneficial effect of propolis addition to the toothpaste. This therapeutic approach affected the pathogenic microflora in greater abundance than the normal commensal microbiota. As mentioned above, the *in vitro* activity of propolis against *P. intermedia*, *P. melaninogenica, Porphyromonas gingivalis*, and *Fusobacterium nucleatum* was reported by Gebara et al. (2002). However, the biofilm bacteria showed increased resistance against antimicrobial agents (Marsh, 2010) and the progression of a periodontal disease depended also

on the host response (Cobb, 2008). Additional tests should be conducted in order to reveal the effect of propolis on the host response.

It is well known that terpenoids possess significant antimicrobial activity (Burdock, 1998; Banskota et al., 2001). The highest concentrations were observed for eucalyptol, pinene, and limonene (12.08 ± 0.21, 12.07 ± 0.12 and 9.25 ± 0.15 μg/ml propolis, respectively). The eucalyptol, pinenes and limonene were found to exhibit potent antimicrobial activity in numerous studies (Hendry et al., 2009; Bevilacqua et al., 2010; Rivas da Silva et al., 2012).

Among the substances identified with significant antioxidant and antimicrobial effects were cinnamic acid derivatives: caffeic acid, *p*-coumaric acid, ferulic acid etc., benzoic acid derivatives: *p*-hydroxybenzoic acid and isomers, 3,4,5-trihydroxybenzoic acid ethyl ester etc., flavonoids: pinocembrin, chrysin, apigenin, galangin etc. (Aygun, 2017). All of them were found in Propolin® in significant amounts (Table 4) and could be attributed to the observed antimicrobial effect of the propolis extract exhibited during the regular tooth brushing.

The results of the analyses were compared with fifteen commercial propolis products and extracts from different regions of Bulgaria, Austria, Italy, France, and Hungary (Gardjeva et al., 2007; Slavov et al., 2013). It was found that Propolin® exhibited high concentration of cinnamic acid derivatives (caffeic acid, CAPE, and *p*-coumaric acid) and flavonoids (pinobanksin, pinocembrin and galangin). The comparison of the non-volatile metabolites content of propolis (Table 4) also suggested that Propolin® possesses significant amounts of biologically active substances (Burdock, 1998; Banskota et al., 2001). The GC–MS analyses results were in accordance with the published data for the composition of the poplar type propolis. The Propolin® extract exhibited the specific markers of the black poplar (*Populus nigra*) – pentenylcaffeates – which were not found for the other commercial propolis extract.

The limitations of this study include the fact that it was a single-centered study conducted at the Medical University-Plovdiv for one Bulgarian town as well as the lack of molecular-biology analysis. The study used conventional microbiological cultures and identification tests but not molecular-biological techniques. PCR-based methods, Microarray, NGS have current applications in medical diagnostics, but they are not yet applied routinely to dentistry. However, PCR-based technology will yield faster, more accurate and easier to use results. Culture-based microbiological studies are important but they limit the diagnostic results giving the fact that oral diseases are of a polymicrobial nature together with nonspecific etiology. As a result the fullest understanding of the effect of different substances on oral microflora may come from an integrated approach including both culture and molecular-biological techniques.

Conclusions

The present study revealed that the addition of Bulgarian propolis, which was found to be rich in biologically active substances (flavonoids and their derivatives, terpenoids, esters etc.), to toothpaste, containing triclosan and zinc improved substantially the gingival health and led to increased activity against potential periodontal and cariogenic pathogens such as *S. mutans*, *C. albicans*, *Prevotella* spp., *Porphyromonas* spp., and Gram(−)cocci. The improved oral health and gingival condition after the treatment with propolis provided a background for its clinical and everyday application. The combined application of Bulgarian propolis with other well-known antimicrobial agents, such as triclosan and zinc citrate, could significantly contribute to the better oral hygiene of patients.

Ethics statement

The study design was approved by the Ethics Committee of the Medical University-Plovdiv, Bulgaria (approval number: 3/12.10.2010). The protocol was conducted in accordance with the Declaration of Helsinki and Tokyo, Good Clinical Practice guidelines, and national laws. All procedures were performed after a written informed consent was signed by the parents and verbal consent was obtained from the subjects.

Authorship

SP contributed to the conception, design, performed preliminary screening, assessed the oral hygiene of the adolescents, treated carious lesions, corrected poor dental restorations, and removed calculus, if present; EA contributed to the conception, design, analysis, and interpretation, drafted, and critically revised the manuscript; PG contributed to the conception, design, microbiological examination, and microbial identification; ZP contributed to the conception, design, and statistical analysis; VK contributed to the conception, design, analysis,data interpretation, and writing the manuscript; AA and AS contributed to GC–MS and HPLC analysis of propolis extracts and interpretation of the results; MM contributed to the conception, design, microbiological examination and microbial identification, and critically revised the manuscript. All authors gave their final approval and agreement to be accountable for all aspects of the work.

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Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.11.001.

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