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Benefits of sea buckthorn (*Hippophae rhamnoides*) pulp oil based-mouthwash on oral health

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Abbreviated running headline

Sea buckthorn based-mouthwash benefits on oral health

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Abstract

Aim: The purpose of this study was to conduct phytochemical analysis of sea buckthorn pulp oil and to evaluate the antimicrobial, anti-biofilm and antioxidant activities of its mouthwash form.

Methods and Results: Fatty acid composition of the sea buckthorn pulp oil was determined by GC-MS analysis, which revealed that, mono-unsaturated fatty acid, palmitoleic acid and saturated fatty acid, palmitic acid, were the major constituents. The antimicrobial and the anti-biofilm capacities of sea buckthorn pulp oil mouthwash form were evaluated against *Streptococcus gordonii*, *Porphyromonas gingivalis*, *Actinomyces viscosus* and *Candida albicans*, according to the European Norms, and the Biofilm Ring Test®, respectively. These activities were then compared with those of chlorhexidine and herbal mouthwashes. The sea buckthorn-based mouthwash was bactericidal against *S. gordonii* and *P. gingivalis*, bacteriostatic against *A. viscosus* and showed no antifungal effect. Regardless of the strains used, complete inhibition of biofilm formation was achieved. The antioxidant activity of this experimental mouthwash was also assessed by DPPH and NBT assays.

Conclusion: Sea buckthorn mouthwash showed anti-biofilm activities against select single and multiple oral bacterial species.

Significance and Impact of Study:

In this study, a mouthwash derived from sea buckthorn (*Hippophae rhamnoides*) pulp oil has been experimented, for the first time, in order to overcome the problem of a large number of available synthetic mouthwashes which have side effects on teeth, gums, and mucous membranes. This mouthwash seemed to be a suitable alternative for a preventive agent for periodontal inflammation.

Keywords: Sea buckthorn, pulp oil, mouthwash, anti-biofilm, antioxidant.

Introduction

Sea buckthorn plant (*Hippophae rhamnoides*) belongs to the Hippophaë genus and to the Elaeagnaceae family. Being most widespread in China and Russia, the sea buckthorn berries, seeds and leaves extracts, are used in medicinal preparations. It is known to contain several chemical constituents including vitamins, phenolic compounds, lipids, tocopherols, carotenoids and phytosterols (Desbois *et al.*, 2010). Seed and berry oils were shown to be effective for prevention and treatment of gastric ulcers (Xu *et al.*, 2007), cardiovascular diseases (Eccleston *et al.*, 2002), atopic dermatitis (Yang *et al.* 2000), dry mouth of Sjögren Syndrome patients (Le Bell *et al.*, 2002) and antidepressant (Tian *et al.*, 2015). Sea buckthorn and its derivative products, for instance juice and oils, have been shown to possess a wide range of beneficial effects that include promoting regeneration of skin and mucous membranes, improving immune functions, reducing oxidation, and strengthening cardiovascular health (Erkkola *et al.*, 2003).

The sea buckthorn was found to have antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus pneumoniae* (Chaman *et al.*, 2011).

This antibacterial effect may be due to its multiple lipophilic bioactive compounds such as the fatty acids found in its oil and fruits (Erkkola *et al.*, 2003). In fact, amongst the various and vigorous biological activities of fatty acids (FAs) is the capacity to kill the bacteria or inhibit its growth. The antibacterial properties of FAs are used by many organisms to defend themselves against parasitic or pathogenic bacteria where the cell membrane constitutes the prime target of FAs (Desbois *et al.*, 2010).

Generally, long-chain fatty acids were reported to have higher antimicrobial activities against Gram-positive bacteria than Gram-negative ones (Agoramoorthy *et al.*, 2007).

The outer membrane impermeability of the Gram-negative bacteria may cause these differential efficiencies of the fatty acids between the two Gram types. This membrane represents an effective barrier against hydrophobic substances (Agoramoorthy *et al.*, 2007).

The broad spectrum of activity of FAs makes them attractive antibacterial and antioxidant agents for various applications in medicine, agriculture and food preservation, especially when the use of conventional antibiotics and antioxidants is undesirable or prohibited. Due to its richness in bioactive compounds like FAs, the sea buckthorn was used traditionally against various diseases (Kumar *et al.* 2011; Stobdan *et al.*, 2013; Wani *et al.*, 2016).

Although sea buckthorn is known to be effective against several diseases, only few studies have been conducted concerning its efficiency in dental medicine (Wang, 1992; Le Bell *et al.*, 2002; Pentelescu *et al.*, 2012).

Periodontal diseases are bacterial infections caused by a complex interplay between oral bacterial biofilms and host response. The microorganisms are present on the tooth surface in the form of biofilms. In daily practice, the target of prevention and treatment of periodontal

diseases is the removal of the formed biofilms, the inhibition of new biofilm formation and the maintenance of the microbiota responsible for health. Failure in plaque control due to improper techniques of brushing the teeth, lack of motivation for dental hygiene, as well as the role of plaque in the etiology of periodontal diseases are the reasons for incorporating mouthwashes in daily oral care (Barnett, 2006).

Anti-dental plaque agents used nowadays are mainly chemical substances like chlorhexidine, mixtures of fluorides, delmopinol chloride, cetylpyridinium chloride and other agents (Ciancio, 2013; Moharamzadeh, 2017). Since patients are more informed and concerned about the possible side effects of the long term use of those chemical products, a growing interest for natural products has appeared. These latter offer benefits like long-term use with fewer side effects regarding the lowest quantities of preservatives or other additives in their composition (Ekor, 2013).

In the same way, we investigated periodontal disease prevention by sea buckthorn pulp oil-based mouthwash experimentally developed in our laboratory by evaluating the cytotoxic, antioxidant and antibiofilm properties. Tests were carried out against the early colonizers *Streptococcus gordonii* and *Actinomyces viscosus* (Al Ahmad *et al.* 2007) and *Porphyromonas gingivalis* as an important member of the periodontopathic microbiota (Holt *et al.*, 1999). Its antifungal activity was also assessed against *Candida albicans* present in the supragingival plaque (Zijngel *et al.*, 2010), being the most common species of the human oral mycobiome (Ghannoum *et al.*, 2010).

For the best of our knowledge, this study presents, for the first time, the antioxidant and cytotoxic activities of the sea buckthorn mouthwash solution and its ability to inhibit dental biofilm. The effectiveness of the experimentally developed product was compared with those of herbal mouthrinse and a chlorhexidine-based mouthwash, used as “the golden standard”.

The valuable sea buckthorn oils and their biological properties make this plant interesting in terms of oral health improvement.

Materials and Methods

Plant material and mouthwash preparation

The sea buckthorn fruits were collected from Cluj-Napoca, Romania. Fruit samples were air-dried after washing. Then, its pulp oil was obtained by cold-pressed extraction method and was stored in glass flasks at 4°C, shielded from light, until use.

An experimental mouthwash based on sea buckthorn pulp oil was developed. The pure oil was supplemented with cocamidopropyl betaine and xylitol in order to help improve saliva flow, as recommended in commercial mouthwashes. The mouthwash was diluted in the ratio of 1:4 in water prior to use.

The commercial available chlorhexidine-based mouthwash (Expanscience, France) and the herbal solution, Homeodent mouthwash (Boiron, France) were used as references. The latter contains aqua, alcohol, plant extracts from Spanish chamomile (*Anacylus pyrethrum*), vanilla (*Vanilla planifolia*), star anise (*Illicium verum*), guajacum (*Guajacum officinale*), cloves (*Eugenia caryophyllus*), cinnamol, eugenol and linalool. According to the utilization instructions, a diluted solution in the ratio of 1:10 in pure water is recommended.

Chlorhexidine mouthwash contains 0.12% chlorhexidine digluconate, alcohol, polysorbate glycerol, levomenthol and mint aroma.

Chemical analysis

Identification of sea buckthorn fatty acids by gas chromatography-mass spectrometry

(GC-MS)

The identification of fatty acids composition of the sea buckthorn pulp oil was performed by GC-MS. Fatty acid methyl esters (FAMES) were prepared from 10 mg of sea buckthorn pulp oil using 14% Boron trifluoride in methanol (Association of Official Analytical Chemists (AOAC) official method 969.33). FAMES formed from sea buckthorn pulp oil were filtered through 0.45- μm filters (AS021345, JASCO FRANCE), and stored at -80°C until analysis. A system which was composed of a 7020A gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) connected to a mass spectrometry (MS) 5975N detector (Agilent) was used. Data were collected with enhanced ChemStation G1701DA software (Agilent). Sea buckthorn oil samples (1 μL), prepared as described above, were directly injected into the gas chromatograph equipped with a HP-5MS- capillary column (30m x 0.25 mm x 0.25 μm ; Agilent) using H_2 as the gas carrier, with a constant flow rate of 1.5 $\text{ml}\cdot\text{min}^{-1}$. The temperature of the injector was kept at 220°C , and the split ratio was 50:1 $\text{ml}\cdot\text{min}^{-1}$. Chromatographic conditions were as follows: The oven temperature was set in the following order: 50°C for 3 min, $3^{\circ}\text{C}\cdot\text{min}^{-1}$ up to 150°C , 150°C for 10 min, $5^{\circ}\text{C}\cdot\text{min}^{-1}$ up to 220°C , and finally, 220°C for 20 min to clean the column. The column was directly connected to the MS detector, and the electron impact energy was set to 70 eV. The data collected were in the range of 25 to 500 atomic mass units (at $3.25\text{ scans}\cdot\text{s}^{-1}$). Fatty acids peaks were identified by comparing their mass spectra with those held in the NIST MS Data Center 2008 and 2HP-Wiley 138 library (Agilent).

Antioxidant assays

The antioxidant activity of the sea buckthorn based-pulp oil mouthwash was evaluated using the 1,1'-Diphenyl-2-Picrylhydrazyl free radical (DPPH) and superoxide anion scavenging (NBT) assays performed in 96-well microplates.

DPPH assay

The scavenging activity of the mouthwash on DPPH was measured as previously described by Lohézic *et al.* (2013). Briefly, in a 96-well plate, a reaction mixture containing 100 μL of methanolic DPPH (0.5 mM) and 10 μL of the sea buckthorn pulp oil mouthwash methanolic solution was prepared to give a final concentration ranging from 9.37 $\mu\text{g.mL}^{-1}$ to 600 $\mu\text{g.mL}^{-1}$ per well. Gallic acid and quercetin were used as positive controls. The percentage of inhibition at the steady state of each concentration was used to graphically determine the inhibitory concentrations, IC_{50} , defined as the concentration of the oil ($\mu\text{g.mL}^{-1}$) required to obtain 50% of the DPPH reduction. The experiment was done in triplicate. The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = (\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}) / (\text{absorbance}_{\text{control}}) * 100$$

NBT assay

The sea buckthorn pulp oil mouthwash was tested for its free radical scavenging activity using the non-enzymatic phenazine-methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system, which generates superoxide radicals that reduce NBT to a purple-colored formazan. The test was performed according to a modified Hazra *et al.* (2008) method. Briefly, the reaction mixture was prepared in a 96-well plate and consisted of 78 $\mu\text{mol.L}^{-1}$ NADH, 50 $\mu\text{mol.L}^{-1}$ NBT, 10 $\mu\text{mol.L}^{-1}$ PMS and 10 μL of the mouthwash in order to obtain a final concentration ranging from 9.37 to 600 $\mu\text{g.mL}^{-1}$ per well. The reagents were

dissolved in 16 mmol.L^{-1} Tris–hydrochloride buffer at pH 8. After 5 min of incubation at room temperature, the measurement was performed at 560 nm against an appropriate blank to determine the quantity of formazan generated. The percentage of inhibition at the steady state for each concentration was used to calculate the IC_{50} value. This value defined as the amount of the antioxidant required (measured as the concentration of the stock solution added to the reaction mixture) to scavenge 50% of O_2^{\bullet} : the lower value has the better scavenging efficiency of O_2^{\bullet} . The test was performed in triplicate. Ascorbic acid was used as reference.

Biological analysis

Cytotoxicity assay

The viability of human gingival epithelial cells in the presence of sea buckthorn pulp oil mouthwash was measured by MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich)]. The carcinoma cell line, Ca9-22 (Health Science Research Resources Bank, Osaka, Japan) was grown in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, France) supplemented with 2 mmol.L^{-1} Glutamine, 10% heat-inactivated fetal bovine serum (FBS, Lonza, France), 100 mg.mL^{-1} penicillin and $50 \text{ }\mu\text{g.mL}^{-1}$ streptomycin (Sigma Aldrich) in a humidified 5% CO_2 atmosphere at 37°C . In 96-well plates (Sterile, Flat bottom, with lid, Greiner Bio-one, Germany), 1.6×10^4 Ca9-22 cells were seeded per well after their trypsination and enumeration. After incubating the plates for 24 h, the contents of the wells were removed and the mouthwash compound (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and $0.39 \text{ }\mu\text{g.mL}^{-1}$) or only medium (negative control) were added. Triton X-100 (0.1%), a non-ionic detergent, was used as a positive control. Finally, the plates were incubated for 24 h and then treated according to the MTT assay (Mosmann, 1983). The OD was read after 10 min at 595 nm. The experiments were done two times in triplicates and the

results were presented as percentage of cell viability where the untreated control cells were considered to have 100%.

Microbial strains and inoculum preparation

The microbial strains used in this study were *Streptococcus gordonii* DL1, *Actinomyces viscosus* ATCC 43146, *Porphyromonas gingivalis* ATCC 33277 and *Candida albicans* ATCC 26555.

S. gordonii, *P. gingivalis* and *A. viscosus* were cultured in Brain Heart Infusion (BHI) (AES Biomerieux, France) supplemented with 10^{-2} g.L⁻¹ menadione (Sigma), 5×10^{-3} g.L⁻¹ hemin (Sigma) and 5g.L⁻¹ yeast extract (Dutsher). Bacterial cultures were incubated at 37°C in an anaerobic chamber (Macs-VA500, Don Whitley) filled with 80% N₂, 10% H₂ and 10% CO₂ for *A. viscosus* and *P. gingivalis* and in aerobic condition for *S. gordonii*. All bacterial strains were grown on Columbia 3 agar plates supplemented with 5% (v/v) defibrinated horse blood (Eurobio), 25 mg.L⁻¹ of hemin and 10 mg.L⁻¹ of menadione.

C. albicans was grown aerobically at 37°C in liquid and/or on solid Sabouraud medium.

Antimicrobial activity

Antibacterial and antifungal potentials were evaluated according to prEN1040:2006 and prEN1725:1997, respectively. At the exponential phase, the culture of microbial strains was adjusted to 5×10^8 CFU.mL⁻¹ and put in contact with mouthwash solutions for 5 and 10 min as the conventional time points. Subsequently, the potential antimicrobial activity was stopped by a neutralizing solution (3 g.L⁻¹ lecithin, 30 g.L⁻¹ Tween80, 5 g.L⁻¹ sodium thiosulfate, 1 g.L⁻¹ L-histidine and 30 g.L⁻¹ saponin in 0.0025 mol.L⁻¹ phosphate buffer solution) after 5 and 10 min at 15°C. A suspension without mouthwash was used as a negative control. Aliquots (100µl) of each tested mixture and its serial dilutions were plated on Columbia 3 agar and

incubated at 37°C under aerobic or anaerobic conditions according to the strain for microbial enumeration. Each experiment was repeated three times and the results were expressed as means of \log_{10} CFU.mL⁻¹ reductions.

The microbial reduction of 4-5 \log_{10} CFU.mL⁻¹ was considered as antibacterial or antifungal according to European norms, prEN1040 (AFNOR: NF EN 1040, 2006) and prEN 1725 (AFNOR: NF EN 1725, 1997), respectively.

Anti-biofilm formation assay

The potential of each mouthwash to inhibit mono- or poly-species biofilm formation of *S. gordonii*, *P. gingivalis*, *A. viscosus* and *C. albicans* was assessed by the Biofilm Ring Test[®] (BioFilm Control[®] - Saint Beauzire, France). The principle of this methodology was described by Chavant *et al.* (2007). Briefly, in order to determine the capacity to inhibit biofilm formation, the bottom of the wells was pretreated with the mouthwash for 5 min at room temperature in a sterile environment. The microbial suspension ($\approx 10^8$ CFU.mL⁻¹) containing 1% paramagnetic microbeads was inoculated in the pretreated wells. All biofilms tested were incubated at 37°C in anaerobic condition after 3 h except *P. gingivalis* mono-species biofilm that was maintained for 7 h. The plates were then scanned before and after magnetization using the dedicated Scan Plate Reader (BioFilm Control, France) operated with the Biofilm Control Software (BioFilm Control, France). The Complex biofilm was made by mixed pure cultures of *S. gordonii*, *P. gingivalis* and *A. viscosus*. The biofilm formation was expressed as a Biofilm Formation Index (BFI). It is based on the attracted beads forming a spot in the bottom of the wells which is further detected by the Scan Biofilm Reader. The software compares the images of each well before and after magnetization and, through a mathematical algorithm, calculates a corresponding BFI value. The resulting BFIs are inversely proportional to the attached cell number. A BFI value ≤ 2 shows a complete

immobilization of beads due to the sessile cells and corresponds to a complete formed biofilm, whereas a BFI value ≥ 7 indicates high bead mobility under magnetic action that corresponds to the absence of biofilm formation (i.e., control wells). The results are expressed as mean values of BFI from three independent experiments.

Detachment effects on formed biofilm

In order to determine the ability of sea buckthorn mouthwash to detach the oral biofilms, *S. gordonii* and *P. gingivalis* single and/or mixed species biofilms formed in the wells were treated for 5 min in the conditions already mentioned. This property was then compared to the effect of the commercially available chlorhexidine mouthwash. The results were expressed as means of the BFI values from three independent experiments done in triplicates.

To confirm the presence of any microbial strains in all biofilms tested, the adherent cells on Lab tek slide surfaces (Thermo scientific, Menzel, Braunschweig, Germany) were washed twice with PBS and fixed with 95% ethanol.

The Gram-stained biofilms were examined under the highest magnification X1250 (100X objective lens x 1.25 immersion oil) of the light microscope (Olympus BX45, Japan).

Statistical analysis

The ANOVA analysis of variance was used to compare mean values where \log_{10} CFU.mL⁻¹ reduction, BFI, IC₅₀ and viability percent were considered significant for $P < 0.05$.

Results

Analysis of fatty acids composition by GC-MS

The fatty acids profile of sea buckthorn pulp oil was determined using GC-MS.

The results obtained indicate that mono- and poly-unsaturated fatty acids in pulp oil (81.29%) are higher than saturated fatty acids (18.71%)(Table1). Sea buckthorn pulp oil contained

palmitic acid (16:0, 17.99%), oleic acid (18:1n - 9, 7.26%), myristic acid (14:0, 0.72%) and linoleic acid (18:2n - 6, 0.42%). The predominant fatty acid with high quantity was palmitoleic acid (16:1n - 7, 73.61%).

Antioxidant activity

In the study of the antioxidant property of sea buckthorn mouthwash using DPPH assay, it was observed that quercetine and gallic acid, used as positive controls, registered an $IC_{50} = 9 \pm 1 \mu\text{g.mL}^{-1}$ and $6.2 \pm 0.5 \mu\text{g.mL}^{-1}$, respectively. The studied mouthwash with $IC_{50} = 25 \pm 3.10 \mu\text{g.mL}^{-1}$ was shown to have an antioxidant activity lower than that of the positive controls. This result was confirmed by NBT assay where the positive control was vitamin C with an $IC_{50} = 7 \pm 1 \mu\text{g.mL}^{-1}$. The antioxidant activity of the latter was higher than that of the experimented mouthwash which displayed an $IC_{50} = 18.7 \pm 3.04 \mu\text{g.mL}^{-1}$.

Cytotoxicity effect

The cytotoxicity effect of sea buckthorn pulp oil-based mouthwash was evaluated against Ca9-22 cells by MTT assay. It was noted that as the concentration of our compound was increased, the cytotoxicity also increased (Figure 1). The low concentrations (0.39 - $3.13 \mu\text{g.mL}^{-1}$) were significantly non-toxic and the lowest one registered an interesting effect, promoting increased cellular growth by 54% of cell viability in comparison to the non-treated cells, corresponding likely to a hormetic effect. The remaining two concentrations, 6.25 and $12.5 \mu\text{g.mL}^{-1}$, were found to have only 9 and 17% of cell death, respectively. However, the positive control presented by 0.1% Triton X-100 showed 60% of cell death.

Evaluation of the antimicrobial activities

This study revealed a significant bactericidal effect ($P < 0.05$) of the sea buckthorn-based mouthwash compared to the chlorhexidine product against *S. gordonii* after 5 and 10 min of contact with a reduction factor ≥ 4 to $7 \log_{10} \text{CFU.mL}^{-1}$ (Figures 2 and 3). However, the herbal product showed no activity against this strain. (Figures 2 and 3).

With respect to *P. gingivalis*, the sea buckthorn and chlorhexidine mouthwashes showed a non-significant bactericidal effect with a reduction factor $> 7 \log_{10} \text{CFU.mL}^{-1}$ in both times tested ($p > 0.05$). However, the herbal mouthwash had a time-dependent and statistically significant bactericidal effect with a reduction factor $> 5 \log_{10} \text{CFU.mL}^{-1}$ ($p < 0.05$) after 10 min. But, this was less efficient than the sea buckthorn and/or chlorhexidine mouthwashes (Figure 2 and 3).

The sea buckthorn-based mouthwash achieved the anti-*A. viscosus* effect with a reduction factors $\geq 2 \log_{10} \text{CFU.mL}^{-1}$ and $\geq 3 \log_{10} \text{CFU.mL}^{-1}$ after 5 and 10 min of contact, respectively. In the same conditions, the chlorhexidine product completely inhibited the growth of this strain in 5 min (reduction factor $> 7 \log_{10} \text{CFU.mL}^{-1}$). The herbal solution failed to reveal any anti-*A. viscosus* effect.

Regarding *C. albicans*, the chlorhexidine demonstrated a strong antifungal effect with a reduction factor $> 7 \log_{10} \text{CFU.mL}^{-1}$ already after 5 min of contact. But, the sea buckthorn-based product and the herbal mouthwash revealed no antifungal properties ($p < 0.05$).

Anti-biofilm formation activity

The potential of sea buckthorn mouthwash to inhibit mono- or multi-strain biofilms formation was evaluated by the Biofilm Ring Test assay and compared with that of chlorhexidine and herbal commercial mouthwashes.

After 5 min of treatment of the wells surface with the sea buckthorn mouthwash, it showed a high anti-biofilm formation effect similar to that of chlorhexidine (Figure 4A and B). Effectively, in our experimental conditions, neither single- nor multi-strain biofilms were formed at the surface of the wells pretreated with sea buckthorn mouthwash or chlorhexidine product. The BFI scores were revealed to be between 8.7 ± 0.21 and 14.75 ± 0.15 , and 8.15 ± 0.07 and 14.9 ± 0.14 , respectively (Figure 4A). The herbal mouthwash showed no anti-biofilm potential with BFI values between 1.55 ± 0.05 and 2.03 ± 0.08 on the biofilm models used. The values obtained by treatment with chlorhexidine or sea buckthorn mouthwashes were statistically significant ($p < 0.05$).

It is important to note that *C. albicans* did not form mono-species biofilm even when cultured in different media (BHI, Saboureaud) and/or under different conditions (aerobic or anaerobic) (unpublished results). Therefore, it was mixed with the early colonizers, *A. viscosus* and *S. gordonii* in order to be incorporated in multi-strain biofilms.

These biofilms were seen to be the most resistant to the activity of all mouthwashes tested (Figure 4A).

Detachment effect on formed biofilms

The ability of sea buckthorn mouthwash to detach *S. gordonii* and *P. gingivalis* mono- or duo-species biofilms was also assessed and compared to chlorhexidine, used as a strong anti-biofilm formation reference product. The means of the BFI values after 5 min of treatment are expressed in Table 2.

We have obtained only a restrained detachment effect of sea buckthorn mouthwash on *P. gingivalis* single-strain biofilm, with a BFI score = 4.93 ± 0.61 (Figure 5). However, the biofilms established by *S. gordonii* alone and/or in the presence of *P. gingivalis* were resistant to both mouthwashes, showing non-detachment ability.

Discussion

Due to the increased microbial resistance against antibiotics and antiseptics as well as the high treatment costs, there have been adequate motives to search for alternative remedies to overcome this issue (Valle *et al.* 2015; Gupta and Birdi, 2017).

Hence, medicinal plants have been studied as alternative treatments. Studies carried out with *Hippophae rhamnoides* pulp have proved its numerous health benefits. Indeed, in addition to the antioxidant activity (Olas, 2016), its oil has provided antibacterial and antiviral properties (Erkkola *et al.* 2003; Gupta *et al.* 2011; Chaman *et al.* 2011; Zielińska and Nowak, 2017). Besides, Olas (2016) reported that the leaves, fruits and oils of this plant are sources of many bioactive substances including vitamins (A, C and E), unsaturated fatty acids, phenolic compounds, especially flavonoids, and phytosterols, which confer positive effects to the cardiovascular system. In his review, Olas also summarized the current knowledge of the biological roles of sea buckthorn in cardiovascular diseases, in addition to the antioxidant and antibacterial roles of its pulp oil. However, few studies were conducted concerning biological properties of sea buckthorn pulp oil against oral bacteria. This study therefore described the possibility of using this oil as an antioxidant and anti-biofilm mouthwash, for the first time.

Free radicals like superoxide anion can cause extensive gingival tissues damage leading to various pathophysiological diseases like periodontitis (Kundalić *et al.* 2016).

DPPH and NBT assays were used to confer free radical scavenging activity of sea buckthorn, which in turn has great importance in understanding the role of this plant in minimizing the oxidative stress and damage linked to periodontitis.

As shown by the results of this study, the superoxide radical scavenging activities of the sea buckthorn pulp oil mouthwash and the reference compound are increased markedly with increasing concentrations. The results suggest that the mouthwash ($IC_{50} = 18.7 \pm 3.04 \mu\text{g.mL}^{-1}$)

is a potent scavenger of superoxide radical but not more than the standard ascorbic acid ($IC_{50} = 7.0 \pm 1.0 \mu\text{g.mL}^{-1}$).

The IC_{50} , expressing the antiradical activity by DPPH assay obtained without purification of the oil compounds, appeared to be interesting ($25 \pm 3.10 \mu\text{g.mL}^{-1}$), compared to the powerful antioxidant controls, gallic acid and quercetin, with IC_{50} s of $6.2 \pm 0.5 \mu\text{g.mL}^{-1}$ and $9.0 \pm 1.0 \mu\text{g.mL}^{-1}$, respectively.

Many natural antioxidants, mainly phenolic and flavonoids compounds, isolated from different plant materials have been reported to be effective scavengers of superoxide anions (Chaman *et al.* 2011; Yogendra Kumar *et al.* 2013).

Erkkola *et al.* (2003) have suggested that the carotenoids and vitamin E in the pulp oil were responsible for the antioxidant activity and the clear tissue-regenerative effect observed in case of esophagitis caused by irradiation therapy. The authors have also added that the pulp oil is rich in tocopherols, tocotrienols, and plant sterols (Erkkola *et al.* 2003) that could be responsible for the observed antioxidant activity.

The present study has shown that the sea buckthorn-based mouthwash has an important antibacterial activity against *S. gordonii*, being significantly more effective than herbal solution and sometimes greater than chlorhexidine (Figure 2). An important anti-*P. gingivalis* activity was observed for sea buckthorn and chlorhexidine products with a similar non-significant effectiveness after 5 min of contact. Nevertheless, among the tested products, the strongest antimicrobial effect was that of chlorhexidine, being significantly more effective on *A. viscosus* and *C. albicans* than the two other products (Figure 2). Regarding the potential biological roles of sea buckthorn pulp oil, Agoramorthy *et al.* (2007) indicated that some fatty acids such as lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic have potential antibacterial, antifungal and antioxidant activities. According to Kitahara *et al.*

(2004), lauric acid which is the most effective among the saturated fatty acids, showed antimicrobial activity against six strains of *S. aureus* that are Gram positive bacteria.

The GC-MS analysis conducted in this study has shown that the pulp oil contains high quantities of palmitoleic, palmitic and oleic acids. Likewise, Dulf (2012) found out that the dominating fatty acids in pulp/peel and whole berry oils were palmitic (23-40%), oleic (20-53%) and palmitoleic (11-27%) acids. In addition, Erkkola *et al.* (2003) have found that the high level of palmitoleic acid, which up to 50%, differentiates sea buckthorn pulp oil from most other oils of plant origin. The present study confirms that the pulp oil contains higher relative percentage of the above mentioned fatty acids, having potential antibacterial and antifungal effects and making them suitable for clinical application (McGaw *et al.* 2002; Karimi *et al.* 2015).

Jeong *et al.* (2010) found out that polyphenolic monomers epicatechin (EC), epicatechin-gallate (ECg), epigallocatechin (EGC), epigallocatechingallate (EGCg) from sea buckthorn fruits had strong antibacterial effect against *F. nucleatum*. Other studies that assessed the antimicrobial activity of plant extracts demonstrated that green tea polyphenol EGCg completely inhibited the growth of three *P. gingivalis* strains (Sakanaka *et al.* 1996) and was found to be bactericidal after 8 h killing 99.9% of *streptococcus* spp. cells (Yu *et al.* 2004). So, it might be hypothesized that the antibacterial activity of sea buckthorn berry-based mouthwash against *P. gingivalis* and *S. gordonii* is due to the high content of oligomers and polymers of (epi) gallo catechin (EGC) found in berries (Määttä-Riihinen *et al.* 2004) and pomace (Rosch *et al.* 2004).

The antimicrobial efficiency of the pulp oil is not only limited to the antibacterial effect, but it may also have clinical implications in biofilm development, reducing the risks for both teeth decay and gingivitis, as mentioned by Widén *et al.* 2015. Concerning the anti-biofilm potential, the results of this study have shown that treating the surface of the wells with the

sea buckthorn based-mouthwash or chlorhexidine product completely prevented adhesion of *S. gordonii*, *A. viscosus* and *P. gingivalis* by inhibiting single-strain biofilm formation (Figure 4B). In the same way, formation of thicker biofilms with a more complex structure composed of two microbial strains, *S. gordonii* + *P. gingivalis*, *C. albicans* + *S. gordonii* and *C. albicans* + *A. viscosus* and/or of four microbial strains, *S. gordonii* + *A. viscosus* + *P. gingivalis* + *C. albicans*, was fully inhibited by these surface treatments. The inhibition of biofilm formation on pretreated surfaces appeared to be independent of microbial species and the number of strains included in biofilms. However, the herbal mouthrinse was unable to inhibit initiation and formation of new biofilms in the same experimental conditions. With respect to chlorhexidine, the results of this study were consistent with other studies using biofilms formed in a constant depth film fermentor, showing that chlorhexidine is more effective when the surface is pretreated with this product and then subsequently pulsed on the biofilms (Pratten *et al.* 2011). Guggenheim *et al.* (2011) have also demonstrated the potential of chlorhexidine to completely inhibit the bacterial viability of biofilms attached to precoated hydroxiapatite beads.

The results of this study that concern the sea buckthorn oil-based mouthwash have shown interesting potential of preventing biofilm formation of simple and complex biofilms. This is consistent with other studies that reported phytoactive compounds of other plants with inhibitory potential against biofilm formation. Indeed, the non-dialyzable fraction of cranberries and its proanthocyanidins were shown to be effective against *S. mutans* (Steinberg *et al.* 2004) and *P. gingivalis* (La *et al.* 2010). In the same way, it was shown that green tea polyphenols ECg and EGCg inhibited *S. mutans* adherence and biofilm formation (Sakanaka *et al.* 1996). Takarada *et al.* (2004) also mentioned that polyphenols of manuka, tea tree, eucalyptus, lavandula and rosmarinus were able to inhibit the bacterial growth and adherence of *S. mutans* and *P. gingivalis*. Based on these results, polyphenols of sea

buckthorn mouthwash berries might be the responsible compounds for the anti-biofilm effects of this plant. Treating the installed biofilms with this mouthwash did not show great benefit, so, this confirms what has been demonstrated by other authors (Eley, 1999; Moran, 2000); that rinsing with anti-plaque agents cannot replace the mechanical removal methods, but it is an important method for prevention of dental plaque and associated diseases.

Cytotoxicity test performed on human gingival epithelial cells indicated very low cytotoxicity (17% cell death at $12.5 \mu\text{g.mL}^{-1}$) further confirming sea buckthorn oil use as a safe mouthwash. The extract stimulated cellular development for the two lower concentrations ($0.39 \mu\text{g.mL}^{-1}$) which may be due to a hormetic effect. This suggests that sea buckthorn oil could contain nutrients that the cell uses for its growth. This result is consistent with that of Tabatabaei *et al.* (2015) who indicated that low concentration of miswak extract improved gingival ulcers by increasing human dental pulp stem cells viability.

In conclusion, sea buckthorn mouthwash, experimentally developed for the first time, appears to have bactericidal effect against some periodontal pathogens and has the ability to inhibit single- and multi-strain biofilms formation. Besides, the antioxidant effect and the very low cytotoxicity could allow the long-term use of this mouthwash without inducing changes in the oral ecosystem. Further studies are necessary to determine its clinical efficacy and potential to be included in daily home care products.

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Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

None declared.

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Table1. Identified fatty acids in sea buckthorn pulp oil.

Fatty acid	Fatty acid	Quantity %	Fatty acid number	Oméga (ω)
Common name	Systematic name			
Saturated fatty acids				
Palmitic acid	Hexadecanoic acid	17.99	16:0	-
Myristic acid	Tetradecanoic acid	0.72	14:0	-
Total %		18.71		
Unsaturated fatty acids				
Palmitoleic acid	(Z)-9-hexadecenoic acid	73.61	16:1	7
Oleic acid	(Z)-9-octadecenoic acid	7.26	18:1	9
Linoleic acid	(Z,Z)-9,12-octadecadienoic acid	0.42	18:2	6
Total %		81.29		

Table 2. Evaluation of *S. gordonii* and *P. gingivalis* mono- or duo-species biofilms detachment properties of sea buckthorn mouthwash and chlorhexidine product.

Bacterial strain	BFI	
	Sea Buckthorn	Chlorhexidine
<i>S. gordonii</i>	1.53 ± 0.17	1.75 ± 0.75
<i>P. gingivalis</i>	4.93 ± 0.61	1.76 ± 0.04
<i>S. gordonii</i> + <i>P. gingivalis</i>	1.66 ± 0.05	1.89 ± 0.11

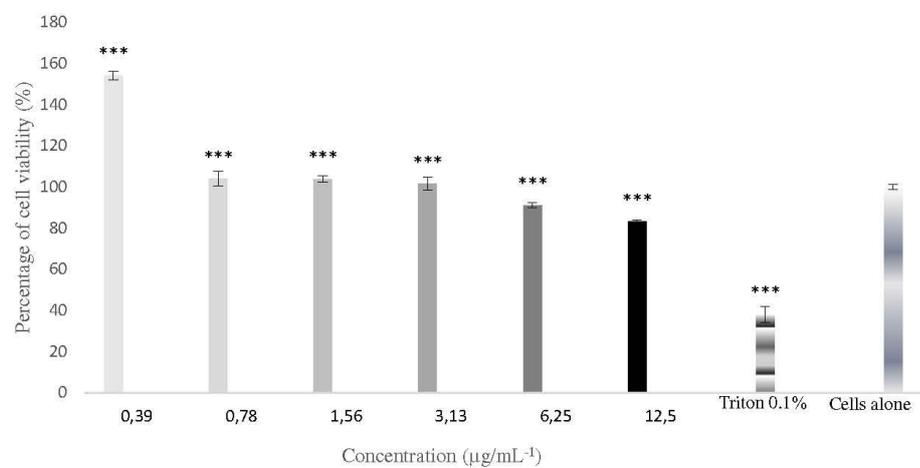


Figure 1. Evaluation of sea buckthorn pulp oil different concentrations (0,39; 0,78; 1,56; 3,13; 6,25 and 12,5 ($\mu\text{g}\cdot\text{mL}^{-1}$), respectively) cytotoxicity on Ca9-22 cells by MTT assay compared to cells treated with Triton 0.1% and cell only. ***P < 0.001 for comparison with 100 % cell.

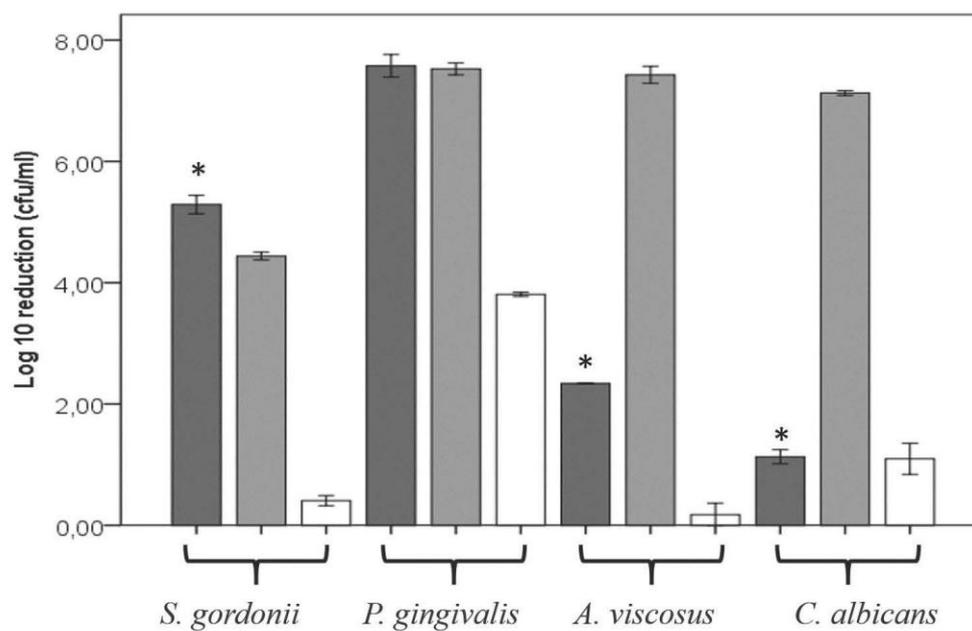


Figure 2. Antimicrobial activity of sea buckthorn, chlorhexidine and herbal mouthwashes against *S. gordonii*, *P. gingivalis*, *A. viscosus* and *C. albicans* after a 5 min exposure. * Statistically significant difference ($p < 0.05$) for antibacterial effect of sea buckthorn mouthwash when compared to chlorhexidine and herbal mouthwashes.

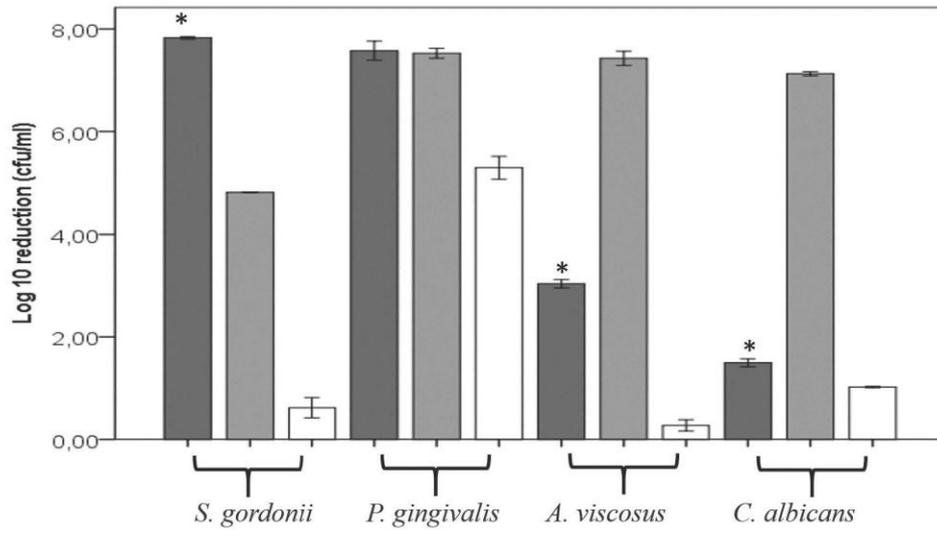
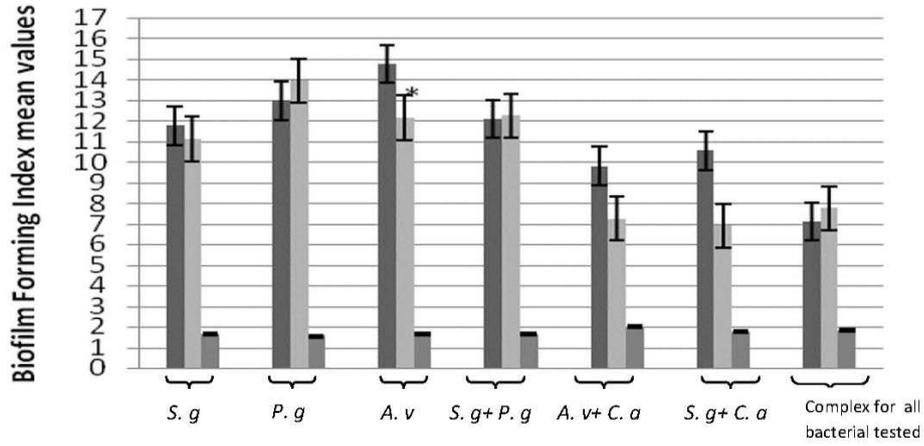


Figure 3. Antimicrobial activity of ■ sea buckthorn, ■ chlorhexidine and □ herbal mouthwashes against *S. gordonii*, *P. gingivalis*, *A. viscosus* and *C. albicans* after a 10 min exposure. * Statistically significant difference ($p < 0.05$) for antibacterial effect of sea buckthorn mouthwash when compared to chlorhexidine and herbal mouthwashes.

A)



B)

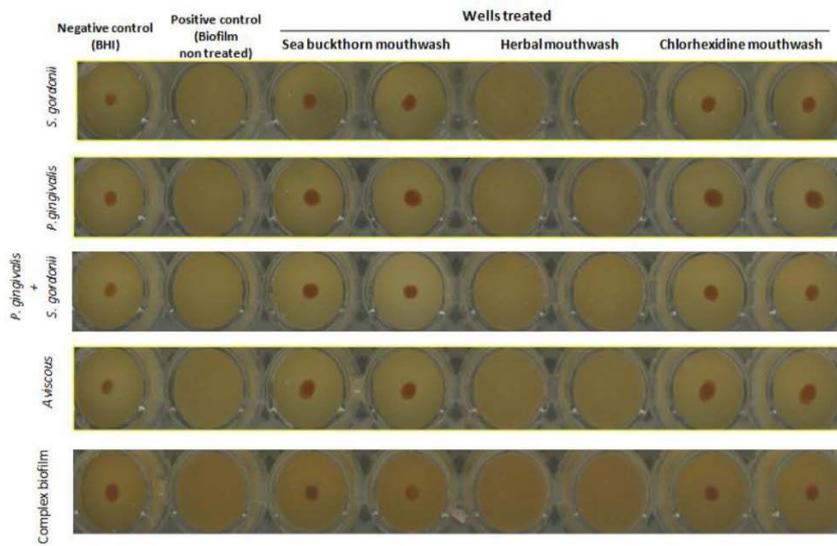


Figure 4. Biofilm formation of the monoculture of *S. gordonii*, *P. gingivalis* and *A. viscosus*, the coculture of *S. gordonii* + *P. gingivalis* and the complex biofilm treated with ■ sea buckthorn, ■ chlorhexidine and ■ herbal mouthwashes. A: BFI (biofilm formation index) values for the different biofilm types in absence or presence of sea buckthorn, chlorhexidine and herbal mouthwashes. * shows a statistical significance after comparing sea

buckthorn mouthwash with chlorhexidine mouthwash ($p < 0.05$) **B**: Biofilm formation of *S. gordonii*, *P. gingivalis* and *A. viscosus*, *S. gordonii* + *P. gingivalis* and complex biofilm in the presence of sea buckthorn, chlorhexidine and herbal mouthwashes. Well 1 (BHI, negative control), well 2 (non-treated single- or multi-strain biofilm, positive growth control), wells 3 to 5 (biofilm treated by sea buckthorn) and wells 6 to 8 (biofilm treated by chlorhexidine).

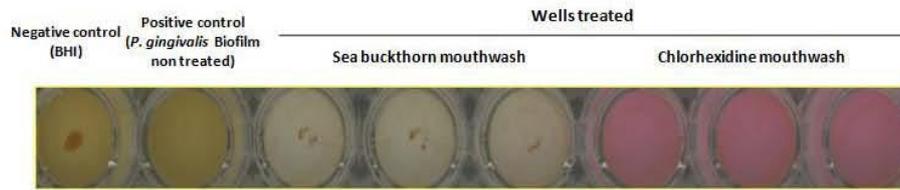


Figure 5. Biofilm detachment effect of the sea buckthorn, chlorhexidine and herbal mouthwashes on the *P. gingivalis* biofilm. Well 1 (BHI, negative control), well 2 (non treated single- or multi-strain biofilm, positive growth control), wells 3 to 5 (biofilm treated by sea buckthorn) and wells 6 to 8 (biofilm treated by chlorhexidine).