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RESEARCH ARTICLE

Antimicrobial effect of the extracts from *Hypericum perforatum* against oral bacteria and biofilm formation

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ABSTRACT

Context: One traditional medicines, *Hypericum perforatum* L. (Hypericaceae), possesses several beneficial effects against depression, ulcers, dyspepsia, abdominal pains, burns, bacterial infections, migraine headaches, and sciatica.

Objective: The present study investigates the antimicrobial activity of the ethanol extract (HP-EtOH) of *H. perforatum* and its sub-extracts, namely *n*-hexane (HP-hexane), chloroform (HP-$CHCl_3$), ethyl acetate (HP-EtOAc), *n*-butanol (HP-*n*-BuOH), and water (HP-H_2O) extracts, against *Streptococcus mutans*, *S. sobrinus*, *Lactobacillus plantarum*, and *Enterococcus faecalis*.

Materials and methods: For the evaluation of the antimicrobial activity, flowering aerial parts of *H. perforatum* were extracted with EtOH and then this extract was fractionated to obtain five sub-extracts in different polarities. Antimicrobial activities of HP-EtOH and its sub-extracts against *Streptococcus mutans*, *S. sobrinus*, *L. plantarum*, and *E. faecalis* were assessed by using colorimetric micro-well dilution at concentration ranges of 64–0.5 μg/ml as well as resazurin microplate and modified microtiter-plate assays between the ranges of 100 and 0.78125 μg/ml.

Results: According to the results of the present study, HP-H_2O sub-extract displayed strong antibacterial activity (MIC values 8 μg/mL) against *S. sobrinus* and *L. plantarum,* and exerted moderate activity against *S. mutans* and *E. faecalis* at 32 and 16 μg/mL concentrations, respectively. Other sub-extracts also demonstrated antimicrobial activity against *S. sobrinus* at a concentration of 16 μg/mL. HP-EtOAc and HP-*n*-BuOH showed antimicrobial activity against *L. plantarum* and HP-EtOAc and HP-H_2O were also active against *E. faecalis* at the same concentrations (16 μg/mL).

Conclusion: According to the results, we suggest that *H. perforatum* could be employed as a natural antibacterial agent in oral care products.

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Antimicrobial activity, Hypericaceae, medicinal plants, oral hygiene

Introduction

Dental caries is a bacterial-originated infectious disease occurring in the presence of acidogenic and aciduric bacteria interacting with other micro-organisms (Hamada & Slade 1980; Marsh 2003). Since dental caries is considered as a public health problem, the discovery of agents having antibacterial potential has long been a goal of medical science (Bowen 2002). Dental caries could be prevented by the application of some materials capable of killing or inactivating the causative bacteria. However, the rise of multi-drug resistant organisms causes a challenge in the treatment of infective diseases. To overcome these kinds of problems, researchers have conducted various studies for the discovery of novel pharmaceutical agents (Pfaller et al. 2003; Orhan et al. 2008; Pfaller et al. 2009; Ozçelik et al. 2010; Piras et al. 2013). Indeed, new antibacterial materials are produced by the incorporation of active antibacterial agents into biomaterials (Fang et al. 2006).

Recent studies on dental caries have mainly focused on *Streptococcus mutans* which leads to the initiation of dental caries in humans (Loesche 1986). *Streptococcus sobrinus* was also reported to be associated with the development of formation of caries within teeth (Okada et al. 2005). In the caries process, *Lactobacillus* was found to be the major contributor, although it is a part of the normal oral microflora (Van Houte 1994). *Enterococcus faecalis* is another bacterial pathogen associated with endodontic infections (Seneviratne et al. 2013).

Hypericum perforatum L. (Hypericaceae) is a perennial herb known as St. John's Wort. The plant has been reported to have various biological activities such as antidepressant, wound-healing, anti-inflammatory, antidiabetic, and includes several phytochemicals such as

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naphthodianthrones, flavonoids, prenylated phloroglucinols, tannins, and volatile oil (Suntar et al. 2010). Recent studies have revealed its antimicrobial activity against a number of bacterial and fungal strains. Indeed, *H. perforatum* was reported to be used externally as antiseptic and for wound healing in Turkish folk medicine (Baytop 1999). In the present study, the ethanol extract of *H. perforatum* and its sub-extracts were investigated for their antimicrobial activities by using oral pathogens including *S. mutans*, *S. sobrinus*, *Lactobacillus plantarum*, and *E. faecalis* by using micro-well dilution, resazurin microplate, and modified microtiter-plate assays.

Materials and methods

Plant material

Aerial parts of *H. perforatum* L. were collected from Ankara, Beypazarı, Gürsögüt, in June 2006 and identified by Prof. Dr. Mecit Vural from the Department of Botany, Faculty of Art and Science, Gazi University. A voucher specimen (GUE-2463) is kept in the Herbarium of Faculty of Pharmacy Gazi University.

Preparation of the extract/sub-extracts

The preparation of the extract and fractions was described previously by Suntar et al. (2010). Shade-dried, powdered aerial parts (1000 g) of *H. perforatum* was subjected to extraction with 96% EtOH and evaporated to dryness under reduced pressure and low temperature (40 °C) using a rotary evaporator to give "HP-EtOH" (yield: 31.3%). The residual dried extract was dissolved in 400 ml of aqueous methanol and transferred to a separator funnel and extracted with *n*-hexane. Extracts were evaporated to give 'HP-Hexane' (yield: 13.7%). The remaining methanol phase was evaporated and the residual methanol extract was diluted with distilled water to 400 ml and successively extracted with $CHCl_3$ and EtOAc. Each solvent extract was evaporated to dryness to give 'HP-$CHCl_3$' (yield: 1.4%) and 'HP-EtOAc' (yield: 27.9%). The remaining aqueous extract was further extracted with *n*-butanol and evaporated to dryness to give 'HP-BuOH' (yield: 21.7%). The final aqueous phase was also evaporated to dryness 'HP-FH_2O' (yield: 28.6%).

The prepared extracts were subjected to thin layer chromatography (TLC) analysis. The same amount of extracts was spotted on TLC aluminum plates (Kieselgel 60 F_{254}) and a mixture of ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:26) was used as the solvent system. Polyethylene glycol reagent was used for the detection of the spots. When the plates were examined, greenish brown coloured spots, and orange yellow zones were observed, which represent naphthodianthrone and flavonoid-type compounds, respectively. The other unvisible spots were analyzed under UV_{254} and UV_{366}.

Antibacterial activity assays

Microdilution assay

For the antibacterial activity, tests American Type Culture Collection (ATCC) of *S. mutans* (ATCC21752), *S. sobrinus* (ATCC6715), *L. plantarum* (ATCC80141), and *E. faecalis* (ATCC29912) were used. Culture suspensions, stock solutions, and inoculums were prepared according to the methods of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards (NCCLS)), and all other conditions were applied as described in our earlier studies (Ozçelik et al. 2006, 2008). The strains were diluted in the Todd–Hewitt broth (Oxoid, Basingstoke, UK), subsequently, 0.1 mL quantities obtained from the dilutions were inoculated onto various media. For the streptococci (MS), Mitis Salivarius agar/-broth (Difco Laboratories, Detroit, MI) supplemented with sucrose and bacitracin were used. Rogosa agar/-broth (Merck, Darmstadt, Germany) were used for the Lactobacilli. Mueller Hinton Agar (Merck, Darmstadt, Germany) was used for Enterococci. These inoculums were incubated in microaerofilic atmosphere (Anaerocult C, Merck, Darmstadt, Germany) at 37 °C for 2 d (Rogosa et al. 1951; Gold et al. 1973; Orhan et al. 2008).

Resazurin microplate assay (REMA)

REMA assay was carried out as serial two-fold dilution of each pharmaceutical material, which is prepared directly to achieve the desired concentration by adding 100 µg/mL of the working solution. Initial dilutions of the materials were prepared in dimethyl sulphoxide (DMSO) solution and subsequent two-fold serial dilutions were performed in medium in the microplates. The micro-organisms were added *per* well. Growth control wells contained bacteria without the test material and used for the calculation of the inhibition of viability. A stock solution of the resazurin sodium salt powder was prepared in sterile distilled water in 0.01% concentration. It was filtered, thereafter, appropriate amount of resazurin (5 µL of 1 g/L) was added *per* well. The plates were incubated at 37 °C. The wells were observed after 24 and 48 h for a colour change from blue to pink by

visual examination and MIC value was defined as the lowest concentration of drug that prevented a colour change. The test was carried out in triplicate (Palomino et al. 2002).

Microtiter-plate assay (biofilm production assay)

A modified microtiter-plate test was used to determine the biofilm formation. Briefly, bacteria were grown overnight on Colombia agar plates and subcultured onto trypticase soy agar plus 5% glucose. Aliquots of 100 µL were inoculated in six parallel wells of a 96-well polystyrene plate. After incubation for 24 h at 37 °C, the wells were rinsed with buffer A (0.01M potassium phosphate buffer made isotonic with saline, pH 7.5) to remove detached cells and then fixed with 150 µL absolute methanol for 10 min. Attached bacterial material was then stained by adding 150 µL crystal violet (1% w/v) for 20 min. The plates were rinsed with tap water (to remove excess crystal violet dye) and the amount of attached material was measured by solubilisation of the crystal violet dye in 150 µL of 33% glacial acetic acid. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

Percentage inhibition of viability was defined as [(OD pharmaceutical materials containing wells-mean background OD]/[(OD pharmaceutical materials free wells-mean background OD] × 100. The lowest concentration displaying an inhibition of $\geq$99% was considered as the MIC (Stepanović et al. 2007; Melake et al. 2012). Figures were drawn by using Sigma Plot program (Sigma, St. Louis, MO).

Results and discussion

The present study was conducted to investigate the antimicrobial activity of the HP-EtOH and its sub-extracts namely HP-hexane, HP-$CHCl_3$, HP-EtOAc, HP-*n*-BuOH, and HP-H_2O against some oral micro-organisms (*S. mutans*, *S. sobrinus*, *L. plantarum*, and *E. faecalis*) by using colorimetric micro-well dilution, resazurin microplate, and modified microtiter-plate assays. Table 1 provides the antimicrobial results obtained from the visual and fluorimetric Resazurin Reduction Assay. The aqueous extract was found to be more efficient than the other sub-extracts against all oral pathogens at MIC values of 8–32 µg/mL and *S. sobrinus* and *L. plantarum* were the most sensitive. The activities of HP-hexane, HP-$CHCl_3$, HP-EtOAc, HP-*n*-BuOH, and HP-H_2O sub-extracts were found to be moderate against *E. faecalis* (MIC values: 16–32 µg/mL). Additionally, the sub-extracts showed similar activity against the biofilm formation with the range from IC_{50} value: 7.23–29.91 µg/mL (Figures 1–4).

Hypericum perforatum is a very valuable plant due to its bioactivity potential and chemical constituents as well as its traditional use for the treatment of infectious diseases (Baytop 1999). Naphthodianthrones, flavonoids, prenylated phloroglucinols, and tannins were reported as the chemical constituents of *H. perofrotum,* which was found to have a wide range of pharmacological effects such as antidepressant, wound-healing, antiviral, and antimicrobial activities (Saddiqe et al. 2010). Several studies were conducted regarding the *in vitro* antibacterial activity of the crude extracts of *H. perforatum* (Brondz et al. 1982; Grauds 1997; Keles et al. 2001; Tolkunova et al. 2002).

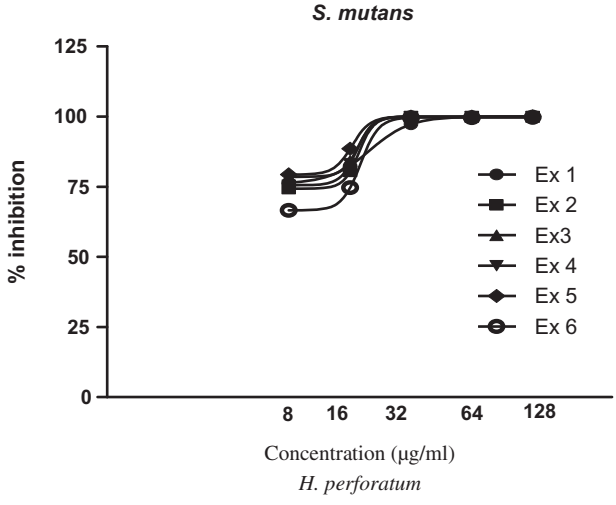

Figure 1. Concentration related curves for % inhibition of *Hypericum perforatum* water (H_2O), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), hexane (Hx), and ethanol (EtOH) extracts against *S. mutans*.

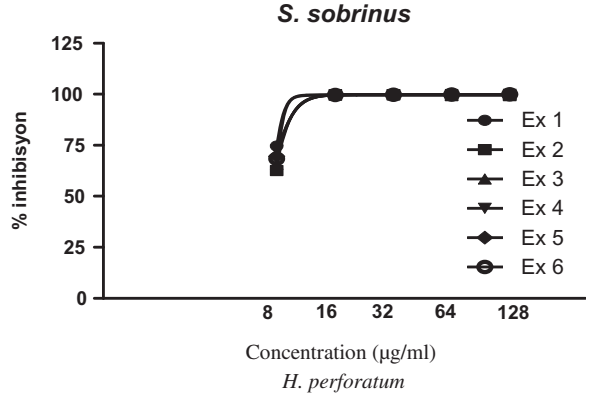

Figure 2. Concentration related curves for % inhibition of *Hypericum perforatum* water (H_2O), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), hexane (Hx), and ethanol (EtOH) extracts against *S. sobrinus*.

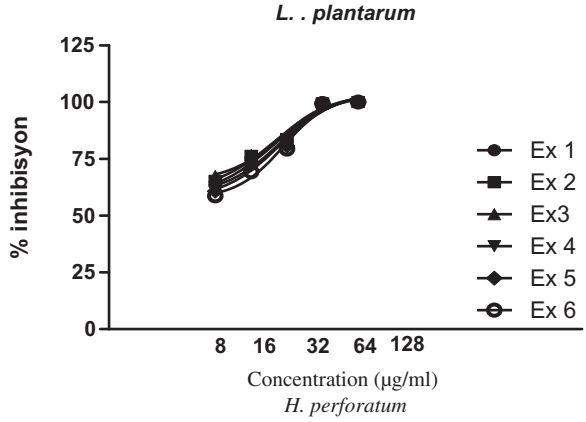

Figure 3. Concentration related curves for % inhibition of *Hypericum perforatum* water (H_2O), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), hexane (Hx), and ethanol (EtOH) extracts against *L. plantarum*.

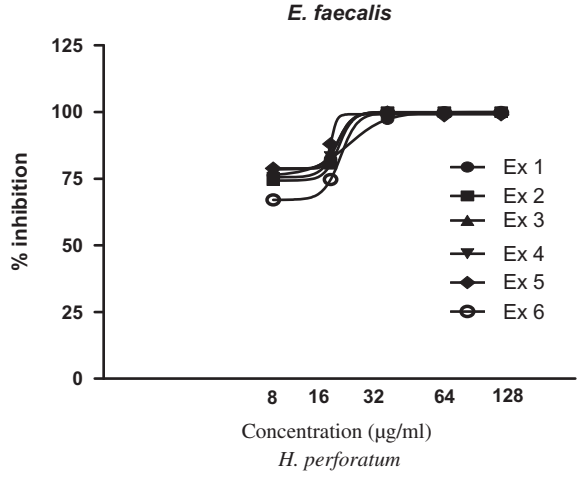

Figure 4. Concentration related curves for % inhibition of *Hypericum perforatum* water (H_2O), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), hexane (Hx), and ethanol (EtOH) extracts against *E. faecalis*.

In a previous study by Molochko et al. (1990), the anti-staphylococcal effects of *H. perforatum* extracts in different solvent combinations were evaluated against reference strains of *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. The water/alcohol/glycerol extracts were found to be the most active. In another study, the hydroalcholic extracts of *H. perforatum* were evaluated for their activity against *Enterococcus faecium*, *Bifidobacterium animalis*, *L. plantarum*, and *E. coli*. Hydrous solutions were found to be active against Gram-positive bacteria, particularly towards methicillin-resistant strains of *S. aureus* (Reichling et al. 2001). Similarly, the study by Dadgar et al. (2006) revealed that ethanol extract showed antibacterial activity against methicillin-resistant and sensitive *S. aureus* strains. Meral and Karabay (2002) reported the antibacterial activity of methanol extracts of *H. perforatum* against both Gram-positive (*S. aureus*, *S. epidermidis*, and *E. faecalis*) and Gram-negative (*P. aeruginosa*, *E. cloacae*, and *E. coli*) bacteria. Although, the antibacterial activity showed variations for different solvent extracts of *H. perforatum* against Gram-positive (Staphylococcus *oxford, S. aureus*, *Streptococcus mutans*, and *Streptococcus sanguis*) and Gram-negative bacteria (*Proteus vulgaris*, *P. aeruginosa*, and *E. coli*) (Barbagallo and Chisari 1987), in general, it was shown to have more potent antibacterial effect against Gram-positive bacteria than Gram-negative bacteria (Reichling et al. 2001; Avato et al. 2004).

Moreover, Yesilada et al. (1999) demonstrated the potential anti-*Helicobacter* effect of the extracts and sub-extracts of *H. perforatum* against *H. pylori*. The study was supported by Reichling et al. (2001).

In our previous study, ointment formulation containing extract of *H. perforatum* aerial parts shortened the healing time of wounds, probably due to the potential antimicrobial activity (Suntar et al. 2010). Indeed, *H. perforatum* oil has been widely used as herbal remedy in the healing of bruises, injuries burns and wounds. In several researches, the essential oil of *H. perforatum* was shown to have potent antibacterial activity against *K. pneumoniae*, *Salmonella enteridis*,

Table 1. Broth dilution minimum inhibitory concentrations (MICs) and IC_{50} values of *Hypericum perforatum* extracts against biofilm formation.

	Micro-organisms							
	S. mutans ATCC 21752		*S. sobrinus* ATCC 6715		*L. plantarum* ATCC 80141		*E. faecalis* ATCC 29912	
Extracts	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
HP-EtOH	64	17.99	16	7.23	32	29.91	32	18.06
HP-Hexane	64	16.35	16	7.29	32	27.61	32	16.11
HP-$CHCl_3$	32	17.20	16	8.03	32	27.58	32	17.20
HP-EtOAc	32	17.66	16	7.52	16	25.08	16	17.66
P-BuOH	32	17.50	16	7.25	16	27.27	32	17.50
HP-H_2O	32	20.08	8	7.60	8	24.90	16	20.08
AMP	<0.12	–	<0.12	–	<0.12	–	0.5	–

AMP, Ampicillin; HP, *Hypericum perforatum*; IC_{50}, inhibition concentration.

S. lutae, *S. aureus*, *E. coli*, and *B. subtilis* (Gudzic et al. 1997; Rancic et al. 2005; Radulovic et al. 2007; Saroglou et al. 2007). Peeva-Naumovska et al. (2010) investigated the antibacterial activity of *H. perforatum* oil against bacterial strains. The results revealed that inhibition of bacterial growth was registered for *Streptococcus pyogenes*, *S. viridans, Micrococcus luteus*, and *Moraxella catarrhalis* strains. In another study, *H. perforatum* oil exerted antimicrobial activity towards *S. aureus*. LC–DAD–MS data revealed the presence of pseudohypericin, hypericin, and biapigenin (Erdogan Orhan et al. 2013).

In a number of studies, hyperforin was isolated as the antibacterial active component from the nonpolar extracts of *H. perforatum* (Brondz et al. 1982; Schempp et al. 1999). However, it was emphasised that the antibacterial effect of hyperforin are only seen at high concentrations (Schempp et al. 1999). Avato et al. (2004) investigated the antimicrobial effects of the different extracts of *H. perforatum* against various micro-organisms. The ethyl acetate sub-extract was found to be the most active and the main constituents of this extract were found to be flavonoids, hypericins and hyperforins. Besides these, xanthones from *H. perforatum* were also shown to impair the pathogen growth (Franklin et al. 2009).

Conclusion

The results of the present study revealed that the aqueous sub-extract of *H. perforatum* was the most active fraction among the other sub-extracts against all tested oral pathogens namely, *S. mutans*, *S. sobrinus*, *L. plantarum*, and *E. faecalis*. Therefore, we suggest that the water-soluble constituents of the sub-extract are responsible from the antibacterial activity.

Declaration of interest

The authors declare no conflicts of interest.

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