

Virtual screening to identify the protein targets in common dental pathogens interacting with menthol

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Abstract

Deducing the molecular pathway underlying the antimicrobial effect of phytochemicals is an inevitable part of drug discovery. Selection of potential targets on the microbial pathogens will eventually lead to eradication of microbes and effective treatment. In this context, the present *in silico* study identifies vital targets in the dental pathogens interacting with menthol. The STITCH tool was used for identifying the protein drug interaction, VICMPred and VirulentPred tools were used for identifying the functional class and virulence nature of proteins. PSORTb was used to locate the sub-cellular location of the virulent proteins. The study results indicate that menthol interacts with virulence factors of *Treponema denticola*. These factors play a crucial role in cell survival and hence can be a good target for further *in vitro* and *in vivo* studies. To conclude, menthol was found to interact with crucial proteins of dental pathogens which can be targeted to achieve promising results.

Keyword: Menthol, common dental pathogen, bacteria, DNA replication, in silico approach.

Introduction

Microbial pathogens in the oral cavity dwell in a poly-microbial environment which enhances their

interaction with other pathogens. Eradication of microbes from the site of infection becomes complex due to several features, both natural and acquired exhibited by the pathogen. Biofilm formation, intrinsic or extrinsic drug resistance has more often hampered the treatment process [1,2]. Herbal medicine and phyto-compounds have revolutionized the treatment strategy and has taken a giant leap in the field of modern medicine, due to its safety, efficacy and cost-effectiveness. Common dental pathogens such as *Streptococcus mutans*, *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* have been studied extensively so as to reveal their virulence properties. Biofilm formation is one of the best characterized features which enable bacterial pathogens to survive selective pressure in the presence of antibiotics [3]. Antimicrobial drug resistance is yet another bewildering property of endodontic pathogens which usually turns treatments futile [4]. Novel antimicrobials are the need of this hour to combat the menace created by these organisms. Virtual screening of bioactive compounds is one method which can provide clues on the molecular targets within a short period of time. This reduces laborious wet lab procedures which are considered to be standard methods to study drug susceptibility or resistance of a pathogen.

In line with the above facts, menthol is a bioactive compound extracted from the plant *Mentha piperita*. Peppermint oil obtained from the plant has a variety of therapeutic applications and has been used in mouthwashes, toothpastes, bath and topical preparations [5]. Topical application of menthol provides an analgesic effect by inhibiting Ca^{2+} currents of neuronal membranes [6]. Essential oils [EO] extracted from *Mentha piperita* exhibited potential antimicrobial effect against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* ATCC 14452, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium*, *Bacillus cereus*, *Candida albicans* and *Candida tropicalis* [7]. A recent study also reported the effect of EO from different plant sources against oral pathogens. EO containing

menthol, thymol and carvacrol were found to be more effective against *S. mutans* and *Lactobacillus* species, individually or in combination with chlorhexidine [8].

Materials and methods

In the present in silico study, the phytochemical menthol was tested against anaerobic oral pathogens viz., *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *Treponema denticola* and *Tannerella forsythia*. STITCH tool was used to reveal the interactions between the compound and the protein repertoire in the pathogen. VICMPred and VirulentPred softwares were subsequently used to check the virulence nature of the proteins targeted by menthol. PSORTb was used to identify the sub cellular location of the virulent protein. The methodology adopted and computational tools employed have been described in detail.

Strains used in the study:

The following strains available in the STITCH database were used for the present study.

Streptococcus mutans UA159, *Enterococcus faecalis* V583, *Porphyromonas gingivalis* ATCC 33277, *Treponema denticola* ATCC 35405, *Tannerella forsythia* ATCC 43037 [9].

Analysing protein interaction network:

STITCH is an exhaustive pipeline which can be used for predicting the interactions between chemicals and proteins. The interactions are of two types [a] direct or physical and [b] indirect or functional associations which arise from data accumulated in the primary databases. The repertoire of proteins which interacts with *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* were used for predicting virulence [9]. The FASTA format of protein sequences were retrieved from the National Centre for Biotechnology Information [NCBI] domain and used for predicting the functional class of proteins and their virulence properties [<https://www.ncbi.nlm.nih.gov/protein/?term=>].

Prediction of functional class of interacting proteins:

VICMpred server aids in the classification of pathogenic microbial proteins into four major classes namely, virulence factors, information and storage processing, cellular process and metabolism. The principal virulence factors such as adhesins, toxins and haemolytic molecules are identified based on the support vector machine [SVM] algorithm which classifies proteins based on their amino acid composition and sequence pattern [10].

Prediction of virulence properties of interacting protein:

The identification of virulent bacterial protein targeted by a drug or phytocompound helps in substantiating the antimicrobial activity of the compound. VirulentPred is a yet another SVM based method, used for automated prediction of virulent proteins based on the sequences [bioinfo.icgeb.res.in/virulent]. The scores with positive predicted values are more often categorised into virulent protein and those with negative predicted values are categorised as avirulent proteins [11].

Prediction of sub cellular localisation of proteins:

The identification of the sub cellular localisation of virulence proteins are of prime importance as the efficiency of the compound lies in target identification. Cell surface proteins are readily targeted, whilst, the cytoplasmic or nuclear proteins need proper drug delivery systems to target the protein of interest. Hence, PSORTb was used for identification of sub-cellular location of virulence proteins [12].

Results and discussion

The *in silico* approach employed identified the potential virulence factors targeted by menthol in *Treponema denticola* among all the pathogens investigated in the present study. Menthol was found

to interact with topoisomerase IV, B subunit, putative, DNA gyrase subunit A, DNA gyrase subunit B of *Treponema denticola*, which were also identified as virulence factors by VirulentPred tool [Table 1]. Interestingly, menthol was found to bind to DNA gyrase and topoisomerase of all the pathogens studied and hence considered as a “topoisomerase poison” [Figure 1]. Apart from this protein, ankyrin repeat protein was the second most common protein target of menthol. Glycosyltransferase, a crucial protein of *S.mutans* was found to be complex with menthol. The subcellular location of the virulence proteins identified in *Treponema denticola* was in the cytoplasm [Table 2].

Endodontic regeneration is highly dependent on eradication of microbial pathogens at the site of infection. Antibiotic dressing is usually preferred to avoid microbial growth [13]. Nevertheless, this process creates a selective pressure on the microbes to adapt to the antibiotic laden environment transforming them into antibiotic resistant strains. Now the process of treatment becomes even more difficult as these strains are recalcitrant to antibiotic therapy. In this state of alert, an enormous number of phytochemicals have been identified and their bioactive principles are revealed which can be utilized as potent antimicrobials. Screening of these compounds using agar well diffusion methods is used as a standard method to evaluate their efficacy against specific pathogens. This procedure is time consuming, expensive and does not provide any clue about the underlying mechanisms leading to the death of the pathogen.

An alternative strategy which is gaining a lot of appreciation is screening bioactive compounds using computational tools. This procedure virtually screens for the best compound and identifies molecular targets on the pathogens which can be utilized as a candidate protein. Numerous studies have substantiated on the mode of action of menthol against bacterial and fungal pathogens.

Bacterial topoisomerase has been regarded as one of the best drug targets [14] owing to its role

replication, transcription, repair and recombination [15]. The present study documents the interaction of menthol with gyrase and topoisomerase of all the bacterial pathogens tested.

Peppermint oil [PO] from *Mentha piperita* was shown to possess potent antimicrobial and antioxidant properties. Both gram positive positive [*S. aureus* ATCC 25923 and *S. pyogenes* ATCC19615], and gram negative bacteria [*Escherchia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 13883] were selected for the antimicrobial assay. The line of sensitivity observed was in the pattern *S. aureus* > *S. pyogenes* > *K. pneumoniae* > *E.coli*. Gram negative pathogens exhibited a minimal degree of resistance to antibacterial, which might be due to the presence of lipopolysaccharides. Interestingly, PO produced a greater zone of inhibition against *S. aureus*, *S. pyogenes* and *K. pneumonia* in comparison to the positive control gentamycin [10 IL of 10 lg/mL concentration] [16]. The inhibition of bacterial pathogens may be attributed to the presence of major components of PO which include menthol [29-48%], menthone [20-31%], menthofuran [6.8%] and menthyl acetate [3-10%] [17].

Korkmaz et al. reported the use of menthol in toothpastes aids in the reduction of *S.mutans* and *Staphylococcus aureus* [18]. Bouyahya and colleagues tested the antimicrobial activity of Mentha pulegium essential oil [MPEO] against several bacterial species. *Bacillus subtilis*, *Proteus mirabilis* and *S.aureus* were found to be inhibited by MPEO [19]. The effect of menthol solution and oral hygiene status of dental students were tested using chlorhexidine as control. Although, menthol did not produce similar results as that of chlorhexidine, menthol mouthwash exhibited a significant reduction in plaque, gingival and bleeding indices in mice model [20]. Several studies based on *in silico* methodology have aided in identifying molecular targets in dental pathogens against non-steroidal anti-inflammatory drugs [21] and phytochemicals [22]. More investigations directed towards the molecular aspects of menthol could enrich its role as a potent antimicrobial agent.

Conclusion

The present study throws light on the molecular targets of menthol which is topoisomerase and gyrase. Thus, it can be used as a potential drug of choice against several oro-dental pathogens. Although the study has a lot of merits, the major limitations of the study are, [a] interaction of compounds may not be the same in a biological environment, [b] there can be homology between the targeted bacterial proteins and host proteins, and [c] the binding observed may be physical and may not affect the functional properties. Hence, *in vitro* and *in vivo* studies are warranted to justify the effect of menthol on dental pathogens.

Disclaimer regarding Consent and Ethical Approval:

As per university standard guideline, participant consent and ethical approval have been collected and preserved by the authors

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Conflict of Interest: The authors declare that they have no conflict of interest.

References:

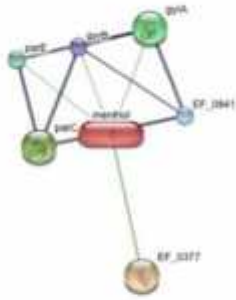
1. Zilm PS, Butnejski V, Rossi-Fedele G, Kidd SP, Edwards S, Vasilev K. D-amino acids reduce *Enterococcus faecalis* biofilms in vitro and in the presence of antimicrobials used for root canal treatment. PLoS One. 2017 Feb 2;12(2):e0170670.
2. Lins RX, Hirata R Junior, Wilson M, O Lewis MA, Fidel RAS, Williams D. Comparison of genotypes, antimicrobial resistance and virulence profiles of oral and non-oral *Enterococcus faecalis* from Brazil, Japan and the United Kingdom. J Dent. 2019;84:49-54.
3. Vijayashree Priyadharsini J, Smiline Girija A.S, Paramasivam A. *In silico* analysis of virulence genes in an emerging dental pathogen *A. baumannii* and related species. Archives of oral biology, 2018b, 94: 93-98.
4. Vijayashree Priyadharsini J, Smiline Girija A.S, Paramasivam A. *Enterococcus faecalis* an Emerging Microbial Menace in Dentistry-An Insight into the In-Silico Detection of Drug

Resistant Genes and Its Protein Diversity. *Journal of Clinical and Diagnostic Research*, 2018; 12(10): GC06-GC10.

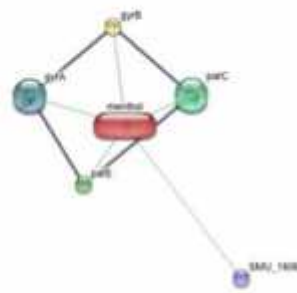
5. Herro E, Jacob SE. *Mentha piperita* (peppermint). *Dermatitis*. 2010;21(6):327-9.
6. Galeotti N, Di Cesare Mannelli L, Mazzanti G, Bartolini A, Ghelardini C. Menthol: a natural analgesic compound. *Neurosci Lett*. 2002;322(3):145-8.
7. Marwa C, Fikri-Benbrahim K, Ou-Yahia D, Farah A. African peppermint (*Mentha piperita*) from Morocco: Chemical composition and antimicrobial properties of essential oil. *J Adv Pharm Technol Res*. 2017;8(3):86-90.
8. Tardugno R, Pellati F, Iseppi R, Bondi M, Bruzzesi G, Benvenuti S. Phytochemical composition and in vitro screening of the antimicrobial activity of essential oils on oral pathogenic bacteria. *Nat Prod Res*. 2018;32(5):544-551.
9. Szklarczyk D, Santos A, von Mering C, et al. STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data. *Nucl Acid Res* 2016;44:D380-4.
10. Saha S, Raghava GPS. VICMpred: SVM-based method for the prediction of functional proteins of gram-negative bacteria using amino acid patterns and composition. *Genom Proteom Bioinform* 2006;4:42-7.
11. Garg A, Gupta D. VirulentPred: a SVM based prediction method for virulent proteins in bacterial pathogens. *BMC Bioinformatics* 2008;9:62.
12. Yu NK, Wagner JR, Laird MR, et al. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 2010;26:1608-1615.
13. Sabrah AH, Yassen GH, Gregory RL. Effectiveness of antibiotic medicaments against biofilm formation of *Enterococcus faecalis* and *Porphyromonas gingivalis*. *J Endod*. 2013;39(11):1385-9.
14. Bradbury, B.J Pucci, M.J. Recent advances in bacterial topoisomerase inhibitors. *Curr. Opin. Pharmacol*. 2008, 8, 574–581.

15. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat. Rev. Mol Cell Biol.* 2002;3:430–440.
16. Singh R, Shushni MAM, Belkheir A. Antibacterial and antioxidant activities of *Mentha piperita* L. *Arabian Journal of Chemistry.* 2015; 8, 322–328.
17. Clark RK, Menory RC. Environmental effects of peppermint (*Mentha piperita*). *Aust. J Plant Physiol.* 1980;7:685–692.
18. Korkmaz FM, Ozel MB, Tuzuner T, Korkmaz B, Yayli N. Antimicrobial activity and volatile constituent analysis of three commercial herbal toothpastes containing *Aloe vera* L. and *Fragaria vesca* L. extracts. *Niger J Clin Pract.* 2019;22(5):718-726.
19. Bouyahya A, Et-Touys A, Bakri Y, Talbau A, Fellah H, Abrini J, Dakka N. Chemical composition of *Mentha pulegium* and *Rosmarinus officinalis* essential oils and their anti-leishmanial, antibacterial and antioxidant activities. *Microb Pathog.* 2017;111:41-49.
20. Ali NA, Abbas MJ and Al-Bayaty FH. Evaluation of Potential Effect of Menthol Solution on Oral Hygiene Status of Dental Students in a University in Iraq. *Tropical Journal of Pharmaceutical Research.* 2015; 14 (4): 687-692.
21. Vijayashree Priyadharsini J. In silico validation of the non-antibiotic drugs acetaminophen and ibuprofen as antibacterial agents against red complex pathogens. *J Periodontol.* 2019;90(12):1441–1448.
22. Ushanthika T, Smiline Girija AS, Paramasivam A, Priyadharsini JV. An in silico approach towards identification of virulence factors in red complex pathogens targeted by reserpine [published online ahead of print, 2019 Jul 17]. *Nat Prod Res.* 2019;1–6.

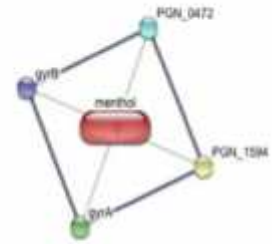
(a) *Enterococcus faecalis*



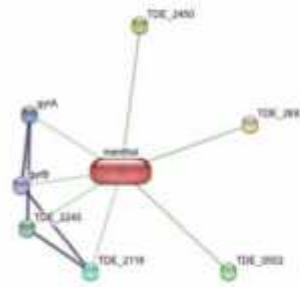
(b) *Streptococcus mutans*



(c) *Porphyromonas gingivalis*



(d) *Treponema denticola*



(e) *Tannerella forsythia*

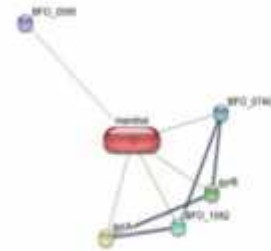


Figure 1: Interaction of menthol with protein repertoire of dental pathogens.

Table 1: Protein interaction network of menthol against common dental pathogens

Organism	Identifier	Proteins which interacts with menthol	VICMPred Functional	VirulentPred	Virulent Pred Score
<i>Enterococcus faecalis</i>	EF_0841	Hypothetical protein	Information and storage	Avirulent	-1.033
	EF_0377	Ankyrin repeat family protein	Cellular Process	Avirulent	-0.332
	parC	DNA topoisomerase IV subunit A	Metabolism	Avirulent	-1.018
	parE	DNA topoisomerase IV subunit B	Metabolism	Avirulent	-1.041
	gyrA	DNA gyrase subunit A	Cellular Process	Avirulent	-1.009
	gyrB	DNA gyrase subunit B	Cellular Process	Avirulent	-1.031
<i>Porphyromonas gingivalis</i>	PGN_0472	DNA topoisomerase IV A subunit	Metabolism	Avirulent	-0.988
	PGN_1594	DNA topoisomerase IV B subunit	Metabolism	Avirulent	-0.929
	gyrA	DNA gyrase A subunit	Cellular Process	Avirulent	-0.995

	gyrB	DNA gyrase B subunit	Cellular Process	Avirulent	-1.020
<i>Streptococcus mutans</i>	SMU_1806	Glycosyltransferase	Cellular Process	Avirulent	-0.633
	parC	DNA topoisomerase IV subunit A	Metabolism	Avirulent	-1.012
	parE	DNA topoisomerase IV subunit B	Metabolism	Avirulent	-1.010
	gyrA	DNA gyrase subunit A	Cellular Process	Avirulent	-1.020
	gyrB	DNA gyrase subunit B	Cellular Process	Avirulent	-1.057
<i>Treponema denticola</i>	TDE_2450	Ankyrin repeat protein	Metabolism	Avirulent	-0.400
	TDE_2693	Ankyrin repeat protein	Cellular Process	Avirulent	-1.043
	TDE_0502	Ankyrin repeat protein	Metabolism	Avirulent	-1.042
	TDE_2118	Topoisomerase IV, A subunit, putative	Metabolism	Avirulent	-1.029
	TDE_2245	Topoisomerase IV, B subunit, putative	Virulence factor	Avirulent	-1.006
	gyrA	DNA gyrase subunit A	Virulence factor	Avirulent	-1.023

	gyrB	DNA gyrase subunit B	Virulence factor	Avirulent	-1.021
<i>Tannerella forsythia</i>	BFO_0740	DNA topoisomerase IV subunit A	Metabolism	Avirulent	-1.004
	BFO_1082	Putative DNA gyrase, B subunit	Metabolism	Avirulent	-0.997
	BFO_0595	Hypothetical protein	Metabolism	Avirulent	-0.986
	gyrA	DNA gyrase subunit A	Cellular Process	Avirulent	-1.008
	gyrB	DNA gyrase subunit A	Metabolism	Avirulent	-0.985

Table 2: Sub-cellular location of virulence proteins identified in *Treponema denticola*

Organism	Virulence factor	Subcellular localisation of
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		the protein
<i>Treponema denticola</i>	Topoisomerase IV, B subunit, putative	Cytoplasm
	DNA Gyrase subunit A	Cytoplasm
	DNA Gyrase subunit B	Cytoplasm