(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
05 May 2022 (05.05.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/090568 A1

(51) International Patent Classification:
A61K 8/49 (2006.01) *A61K 8/35* (2006.01)
A61K 31/121 (2006.01) *A61K 36/752* (2006.01)
A61Q 11/00 (2006.01) *A61P 31/04* (2006.01)

(21) International Application Number:
PCT/EP2021/080378

(22) International Filing Date:
02 November 2021 (02.11.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
116867 30 October 2020 (30.10.2020) PT

(71) Applicants: **UNIVERSIDADE DO PORTO** [PT/PT]; PRAÇA GOMES TEIXEIRA, S/N, 4099-002 PORTO (PT). **UNIVERSIDADE DO MINHO** [PT/PT]; LARGO DO PAÇO, 4704-553 BRAGA (PT).

(72) Inventors: **AZEVEDO PINA VAZ, Irene Graça**; UNIVERSIDADE DO PORTO - FACULDADE DE MEDICINA DENTÁRIA / CINTESIS € CENTRO DE, INVESTIGAÇÃO, 4099-002 PORTO (PT). **ASCENSÃO LOPES, Maria**; UNIVERSIDADE DO PORTO - FACULDADE DE ENGENHARIA - DEPARTAMENTO DE ENGENHARIA, METALÚRGICA, 4099-002 PORTO (PT). **VALENTE LUCAS FERREIRA, Inês Ribeiro**; UNIVERSIDADE DO PORTO - FACULDADE DE MEDICINA / FACULDADE DE MEDICINA DENTÁRIA /, CINTESIS, 4099-002 PORTO (PT). **RANGEL HENRIQUES, Mariana Contente**; UNIVERSIDADE DO MINHO - DEPARTAMENTO DE ENGENHARIA BIOLÓGICA, 4710-057 BRAGA (PT). **DA COSTA RODRIGUES, Maria Elisa**; UNIVERSIDADE DO MINHO - DEPARTAMENTO DE ENGENHARIA BIOLÓGICA, 4710-057 BRAGA (PT).

(74) Agent: **PATENTREE**; Rua de Salazares, 842, Edf. NET, 4149-002 Porto (PT).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2022/090568 A1

(54) Title: ANTI-BIOFILM FORMULATIONS, AND USES THEREOF

(57) Abstract: An anti-biofilm formulation for use in the treatment of infections comprising: methyl ethyl ketone and an organic co-solvent or an essential oil, wherein the anti-biofilm formulation is a biofilm disruptor.

D E S C R I P T I O N

ANTI-BIOFILM FORMULATIONS, AND USES THEREOF

Technical field

[0001] The present disclosure relates to an anti-biofilm formulation, preferably an endodontic formulation that disrupts endodontic biofilm. In particular, for the treatment of dental infections, for example persistent endodontic *Candida albicans* or *Enterococcus faecalis* biofilms.

Background

[0002] There is a pattern for certain microorganisms to remain after chemo-mechanical treatment of root canals in teeth with apical periodontitis. Endodontic procedures may select for the more resilient organisms, also known as "oral persisters", while the susceptible Gram-negative anaerobes are more easily eliminated. Compared to strict anaerobes, facultative anaerobes are likely to be more resistant to antimicrobial and mechanical endodontic procedures; hence, inefficient treatments may select for the most robust segment of the root canal microbiota. Once established, gram positive microorganisms such as non-mutant streptococci, enterococci and lactobacilli and some yeast appear to survive following root-canal treatment of teeth with clinical and radiographic signs of apical periodontitis. Gram–negative anaerobes were relatively sporadic suggesting that anaerobes normally do not survive in the restricted nutritional environment found in treated root canals. *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (Meto A *et al.* 2019) are frequently observed inside pulp canal system and they are one of the multiple factors responsible for the failure of endodontic therapy. However, for some microorganisms as *Enterococcus faecalis*, as it is rarely isolated in untreated infected pulps, its presence could be a consequence of fluid leakage from the oral environment via gaps at the restoration-tooth interface following coronal leakage. It is known that microorganisms inside the root canal system do not live in a planktonic state, but rather in a biofilm form. The presence of a biofilm was detected in the apical root canals of teeth with primary and secondary infections and has shown its

important role in the etiology of apical periodontitis (Ricucci D *et al.* 2010). Biofilms may remain unaffected in areas of the main canal that were untouched by instruments.

[0003] Non-surgical endodontic retreatment is a conservative option for the management of persistent apical periodontitis associated with root-filled teeth or where new disease has emerged after root canal filling. Antimicrobial and anti-biofilm disruption are the most important goals to achieve success in endodontic retreatment (Neelakantan P *et al.* 2017). Therefore, it is mandatory to regain access to the apical foramen by the complete removal of the potentially infected filling materials (gutta-percha and sealer) in order to create space to irrigating solutions and dressings, and to reseal all portals of entry to prevent recurrence of the infectious disease. The techniques for the removal of obturating materials include rotary files, ultrasonic instruments, heat, hand files and endodontic solvents. Despite all the technology currently available, the complete removal, although identified as a crucial step, is not still possible (Rossi-Fedele G *et al.* 2016). Remnants of gutta-percha and sealer are often detected, adhered to the root canal walls. These filling remnants may cover areas in which residual microbial biofilms remain undisturbed by irrigants and dressings, with an increased risk for maintaining periradicular inflammation and thus be the cause of an endodontic failure (Chavez de la Paz LE *et al.* 2010). The mechanical action of the instruments can be effective in eradicating biofilms, when they are accessible, either by removing them completely (adhesive biofilm failure) or by disrupting the biofilm architecture (cohesive failure), rendering their components more accessible to biocides (Busanello FH *et al.* 2018). Biofilms comprise mainly water, a matrix of extracellular polymeric substances (EPS) and microorganisms. This structural organization determines the susceptibility of biofilms to biocides (Busanello FH *et al.* 2018). Firstly, the presence of highly negatively charged polyelectrolytes in the biofilm matrix offers diffusion resistance to antimicrobials, protecting the biofilm against chemical stresses. Secondly, the viscoelastic properties, as a result of the structural composition, dictate the ability of biofilms to deform and adapt under mechanical stresses, thereby influencing its removal.

[0004] Once structured as a biofilm, microbial agents have enhanced resistance to antibiotics and disinfectants as a result of their complex and heterogenous arrangement as a microbial sessile population embedded into an extracellular minimally permeable

polymeric matrix (Meto A *et al.* 2019). Intracanal irrigant treatments have to be focused towards the eradication of root canal infection and possibly endodontic biofilm disruption, enabling the change of the bacteria to their planktonic form, and thus making them more susceptible to antimicrobial agents.

[0005] Differences in cell membrane integrity, biofilm structure and metabolic activity after exposure to common endodontic irrigants and dressings have been reported:

- Alkali showed the least antimicrobial effect on biofilms of root canal bacteria. The tolerance to alkali compounds (ex: calcium hydroxide) seems to be correlated to the expression of resistant phenotypes in the biofilm communities. (Chavez de Paz LE *et al*. 2010).
- Sodium hypochlorite (NaOCl) has been reported as effective, impacting the membrane integrity of microorganisms and removing cells from biofilms, although recently stressed that it induces a viable but non-cultivable state of bacteria in biofilms and that might contribute to bacterial persistence (Chavez de Paz LE *et al.* 2010).
- Ethylenediaminetetraacetic acid (EDTA), although a non-antibiotic agent, presented some antimicrobial efficacy due to the chelating effect on calcium and iron, breaking up the polymeric matrix structure of biofilms (Chavez de Paz LE et al. 2010). However, concerns about toxicity have been reported.
- Chlorhexidine (2% CHX) – reported with mild antibiofilm efficacy (Chavez de Paz LE *et al*. 2010).
- Phosphoric acid and citric acid – although without direct antibiofilm efficacy, they can have some effect in the removal of accumulated debris.
- Synergy was obtained with antibiotic-nonantibiotic combinations.

[0006] Current endodontic solutions include:

- Cupral ($CaOH_2$ + Cu) - initial evidence on the efficacy of Cupral against preformed microbial biofilms: *E. faecalis*, *P. aeruginosa*, *S. aureus* and *C. albicans* (Meto A et al. 2019).

- Photo-activated disinfection (PAD) can be an adjunct to mechanical agents with further reduction the bacterial load; however, *E. faecalis*, *V. parvula* and *C. albicans* were recovered from root canal samples after removing the root-filling materials, after PAD.
- Laser and ultrasonic activation of NaOCl as adjunctive disinfection procedures (Ordinadola-Zapata R et al. 2014).

[0007] The actual recommended irrigation regimen involves a sequential use of sodium hypochlorite and a decalcifying agent (NaOCl + EDTA) as a final irrigant protocol. However, it was observed that biofilm was resistant, persisting even after endodontic conventional disinfecting procedures (Alshanta OA *et al.* 2019).

[0008] Current solvents used include: Chloroform, eucalyptol, xylene, Endosolv R (Septodont, Cedex, France), Endosolv E (Septodont) (tetrachloroethylene), which have been used for both endodontic filling materials, gutta-percha and sealer. Nevertheless, their effects are by far insufficient, especially for sealer dissolution. Chloroform is still recognized as one of the most effective solvent in endodontics, being classified as group 2B carcinogen by the International Agency for research on Cancer. Endosolv R, specific for epoxy resinous sealer, contains as its main component formamide, a toxic substance in animal testing and human cells. Xylene and eucalyptol raise concerns about toxicity and essential oils, such as orange oil, have been reported to be as less effective, and also has the potential to cause toxicity associated with the percentage of d-limonene present. The role of agitation with these traditional solvents, except for chloroform, show controversial results (Rossi-Fedele G. et al. 2017), being claimed that in the actual state the supplementary enlargement of root canals with NiTi rotary or precurved hand instruments to achieve some reduction of filling remnants during retreatment is still recommended.

[0009] Solvents traditionally used for endodontic retreatment were neither specific for the sealer, with its main aim being gutta-percha's removal, nor designed for targeting biofilms. This can explain their lack of utility with the advancements of new instruments designed for gutta-percha removal. Due to micro-CT studies, it was highlighted that not only were there sealer remnants persisting, but also gutta-percha inside dentinal tubules and isthmuses, namely in teeth with post-treatment persistent apical periodontitis.

Additionally, lowering the infectious burden has been stressed as an important factor in improving the prognosis of endodontic (re)treatment.

[0010] It was previously reported that a non-traditional organic solvent for endodontics, with specificity for epoxy resin sealers' dissolution, the most widely used sealer, methyl ethyl ketone (MEK), can be enhanced by ultrasonic agitation (Ferreira I et al. 2017).

[0011] Document US2009162301 A1 describes an antiseptic composition containing a polar aprotic solvent (e.g., methyl ethyl ketone), an alcohol (e.g., isopropanol) and/or an additional antiseptic agent such as iodine. In certain embodiments, the antiseptic composition may be used as a mouthwash or mouth flush solution. It is mentioned that this invention overcomes limitations in the prior art by providing an improved antiseptic. The inventor has made the surprising discovery that the inclusion of a low concentration of a polar aprotic solvent (e.g., dipolar aprotic solvents, DMSO or DMA at a concentration of less than about 30%) in an antiseptic (e.g., an antiseptic comprising an alcohol and/or an iodophor) results in a dramatic improvement in the antimicrobial properties of the antiseptic.

[0012] Document JP4979971 describes a solvent for softening/dissolution of gutta-percha filling material used in dental treatment, especially in endodontic treatment.

[0013] "Improvement of the efficacy of endodontic solvents by ultrasonic agitation" by Ferreira *et al* 2019 assess the effect of agitation in the improvement of the dissolution of gutta-percha by endodontic solvents. Gutta-percha samples were exposed to tetrachloroethylene, eucalyptol and orange oil, with and without ultrasonic agitation and then compared to chloroform.

[0014] "Limonene inhibits *Candida albicans* growth by inducing apoptosis" by Thakre *et al* 2018 describes the excellent anti-*Candida* activity against planktonic growth (yeast), morphogenesis (hyphae), and biofilm growth of R-limonene

[0015] "New insight into the dissolution of epoxy resin-based sealers" by Ferreira et al 2017 describes the evaluation of methyl ethyl ketone (MEK) as a solvent for the dissolution of endodontic filling materials as an alternative to chloroform, enhanced by ultrasonic

agitation. Antibiofilm properties of methyl ethyl ketone was not described, neither is the presence of any synergistic effects.

[0016] These facts are disclosed in order to illustrate the technical problem addressed by the present disclosure.

General Description

[0017] The present disclosure relates to an anti-biofilm formulation, preferably an endodontic formulation that disrupts endodontic biofilm. In particular, for the treatment of dental infections, for example, persistent endodontic *Candida albicans* or *E. faecalis* biofilms.

[0018] In an embodiment, the anti-biofilm formulations of the present disclosure are able to disrupt persistent endodontic biofilms, for example *Candida albicans* or *E. faecalis* biofilms which are usually resistant to conventional endodontic treatment procedures.

[0019] An aspect of the present disclosure relates to an anti-biofilm formulation for use in the treatment of infections comprising: methyl ethyl ketone and an organic co-solvent or an essential oil, wherein the anti-biofilm formulation is a biofilm disruptor.

[0020] An aspect of the present disclosure relates to a formulation for use in the treatment of infections comprising: methyl ethyl ketone and an organic co-solvent or an essential oil,

wherein the essential oil is select from a list consisting of: orange oil, lemon oil, oregano oil, thyme oil or mixture thereof;

wherein the organic co-solvent is selected from a list consisting of: tetrachloroethylene, dichloroethane, hydrocarbon solvent, oxygenated solvents, glycol ether; or mixtures thereof.

[0021] Surprisingly the composition described in the present disclosure may be used for killing, inhibiting or preventing the growth of a microbial biofilm; namely endodontic microbiological films.

[0022] In an embodiment, the anti-biofilm formulation is an endodontic formulation.

[0023] In an embodiment, the infection is a dental infection.

[0024] In an embodiment, the biofilm is a *Candida albicans* biofilm or a *E. faecalis* biofilm, as refractory biofilm examples.

[0025] In an embodiment, the anti-biofilm formulation comprises methyl ethyl ketone (MEK) and a co-solvent.

[0026] In an embodiment, the co-solvent is an organic solvent (for example tetrachloroethylene), or an essential oil (for example orange oil).

[0027] In an embodiment, the amount of methyl ethyl ketone (MEK) and co-solvent ranges from 20% –75% (v/v) of each component, preferably 40% – 60% (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

[0028] In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of co-solvent is about 50% (v/v) of each component.

[0029] In an embodiment, the organic co-solvent is selected from a list consisting of tetrachloroethylene, dichloroethane, hydrocarbon solvent, oxygenated solvents, glycol ether, or mixtures thereof.

[0030] In an embodiment the essential oil is select from a list consisting of: orange oil, lemon oil, oregano oil, thyme, or mixtures thereof, preferably orange oil, preferably the limonene is in the form of orange oil. In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of tetrachloroethylene in the mixture (herein after "MEK/tetrachloroethylene mixture") ranges from 20% – 75% (v/v) of each component, preferably 40% – 60% (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

[0031] In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of essential oil in the mixture (herein after "MEK/ orange oil mixture") ranges from 20% –75% (v/v) of each component, preferably 40% – 60% (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

[0032] In an embodiment, the organic solvent is selected from a list consisting of tetrachloroethylene, dichloroethene, hydrocarbon solvent, oxygenated solvents, glycol ether, or mixtures thereof.

[0033] In an embodiment, the essential oil is select from a list consisting of: orange oil, lemon oil, oregano oil, thyme, or mixtures thereof, preferably orange oil, preferably the limonene is in the form of orange oil.

[0034] In an embodiment, the formulation of the present disclosure may be used in medicine, namely in dental medicine and oral medicine.

[0035] In an embodiment, the formulation of the present disclosure may be used in the treatment of dental infection associated with biofilm.

[0036] In an embodiment, the formulation of the present disclosure may be used as a biofilm disruptor to disrupt biofilm, prevent biofilm formation, or for dissolution of endodontic filling materials.

[0037] In an embodiment, the formulation of the present disclosure may be used in the treatment of the infections associated with biofilm such as an endodontic infection.

[0038] In an embodiment, the formulation of the present disclosure may be used in the treatment of pulpal and perirradicular infections, including acute forms, such as, acute apical abscesses, or chronic phases of intrarradicular root canal infection, as granuloma or cyst of endodontic origin.

[0039] In an embodiment, the biofilm comprises *Candida albicans* or *E. faecalis* biofilms, amongst others.

[0040] In an embodiment, the biofilm is a biofilm that is resistant to conventional biofilm disruptors.

[0041] Another aspect of the present disclosure relates to an endodontic filling material comprising the formulation disclosed in the present disclosure.

[0042] Another aspect of the present disclosure relates to the use of an anti-biofilm endodontic formulation comprising methyl ethyl ketone and an organic co-solvent or an essential oil, as an anti-biofilm agent.

[0043] Another aspect of the present disclosure relates to method of disrupting biofilm or preventing biofilm formation comprising applying the anti-biofilm formulation of the present disclosure onto a surface or a surface with a biofilm.

[0044] In an embodiment, the formulation of the present disclosure treats and prevents the development of refractory endodontic biofilms in a surface, namely biofilms previously treated with traditional endodontic irrigants (NaOCl + EDTA).

[0045] In an embodiment, MEK was observed to not be able to dissolve gutta-percha on its own.

[0046] In an embodiment, MEK was observed to be able to dissolve, with a single step-procedure, both filling materials gutta-percha and sealer remnants, when used in combination with a co-solvent, namely in a complementary procedure.

[0047] In an embodiment, tetrachloroethylene and orange oil were selected to be mixed with MEK in the experimental assay. Both anti-biofilm endodontic composition (MEK/tetrachloroethylene, MEK/orange oil) presented significantly higher efficacy as compared to their individual solvents, thus suggesting a synergistic effect.

[0048] In an embodiment, the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil), when tested in human osteoblastic cells, showed high cytocompatibility, while chloroform was shown to be very toxic.

[0049] In an embodiment, the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) have an antibiofilm activity against a 24-hour growth biofilm of *Candida albicans and E. faecalis*, considered as examples of the most difficult microorganisms to eradicate in persistent endodontic infections, resistant to endodontic treatment.

[0050] In an embodiment, the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) has the advantage of being able to promote the disruption of the biofilm matrix beyond having ability of endodontic filling materials dissolution. Traditionally, the main purpose of solvents was limited to softening of filling materials, in particular gutta-percha's, to enable the initial penetration of treatment instruments.

[0051] In an embodiment, the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) exhibits antibiofilm

property, namely against *Candida albicans* and *E. faecalis* biofilm, while simultaneously exhibiting high dissolution of both main filling materials efficacy that is similar to chloroform, and at the same time have a low cytotoxicity profile.

[0052] In an embodiment, micro-CT studies show that the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) is able to produce a cleanliness effect similar to the enlargement of the next apical instrument size and hence more dentin structure could be preserved. This might decrease the risk of fracture.

[0053] In an embodiment, the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) exhibits a synergistic effect between MEK and the co-solvents to produce a higher dissolution value in both filling materials (epoxy resinous sealer and gutta-percha) similar or even higher than the chloroform. In fact, the dissolution efficacy of the formulation was superior to the effect of individual solvents, independent of any agitation.

[0054] In an embodiment, it was observed that the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) exhibits improved antibiofilm activity than MEK on its own.

[0055] In an embodiment, it was observed that the anti-biofilm endodontic formulations of the present disclosure (such as MEK/tetrachloroethylene, MEK/orange oil) exhibits lower cytotoxicity than the individual solvents.

Brief Description of the Drawings

[0056] The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of invention.

[0100] **Figure 1 -** shows the SEM analysis of biofilm development, with and without treatment with the different irrigation protocols.

[0101] **Figure 2 -** shows the result of cell count performed after 24 hours of aerobic incubation at 37 °C. Values of cultivable sessile cells were expressed as Log CFU per area

(cm^2). [MEK - methyl ethyl ketone; TCE - tetrachloroethylene; OOil - orange oil / mixtures: MEK/TCE and MEK/OOil]

[0102] **Figure 3A-** shows the result of the weight loss of gutta-percha and AH Plus samples after immersion in the tested solutions for 2 and 5 minutes. **Figure 3B** shows representative SEM images of gutta-percha (secondary electrons mode) and AH Plus (backscattered electrons mode) samples before (control) and after 5 minutes of immersion in the tested solutions [$CHCl_3$ - chloroform; MEK - methyl ethyl ketone; TCE - tetrachloroethylene; OOil - orange oil and mixtures: MEK/TCE and MEK/OOil].

[0103] **Figure 4 -** shows fluorescent-based live-dead cell staining images of cell viability/survival of MG63 osteoblastic cells after exposure to the tested solutions. (A), Fluorescence images of live (green) and dead (red) cells (Scale bar: 100 μm); (B) - Percentage of live and dead cells. [MEK - methyl ethyl ketone; TCE - tetrachloroethylene; OOil - orange oil and mixtures: MEK/TCE and MEK/OOil]

[0104] **Figure 5 -** shows FTIR spectroscopic plots of the isolated solvents and binary mixtures [MEK - methyl ethyl ketone; TCE - tetrachloroethylene; OOil - orange oil and mixtures: MEK/TCE and MEK/OOil].

Detailed Description

[0057] The present disclosure relates to an anti-biofilm formulation, preferably an endodontic formulation that disrupts endodontic biofilm. In particular, for the treatment of dental infections, for example, persistent endodontic *Candida albicans* or *E. faecalis* biofilms.

[0058] In an embodiment, the anti-biofilm endodontic disrupts persistent endodontic biofilms, for example *Candida albicans* or *E. faecalis* biofilms, usually resistant to conventional endodontic treatment procedures.

[0059] In an embodiment, the anti-biofilm endodontic formulation comprises methyl ethyl ketone (MEK) and a co-solvent.

[0060] In an embodiment, the co-solvent is an organic solvent (for example tetrachloroethylene), or an essential oil (for example orange oil).

[0061] In an embodiment, the amount of methyl ethyl ketone (MEK) ranges from 20% – 75% (v/v), preferably 40% – 60% (v/v), more preferably 50% - 55% (v/v).

[0062] In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of co-solvent is about 50% (v/v) each.

[0063] In an embodiment, the anti-biofilm endodontic formulation comprises methyl ethyl ketone and tetrachloroethylene (herein after "MEK/tetrachloroethylene mixture") in a volume ratio of 1:1 (v:v).

[0064] In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of tetrachloroethylene in the mixture (herein after "MEK/ tetrachloroethylene mixture") ranges from 20% – 75% (v/v), preferably 40% – 60% (v/v), more preferably 50% - 55% (v/v) each.

[0065] In an embodiment, the anti-biofilm endodontic formulation comprises methyl ethyl ketone and essential oil (herein after "MEK/orange oil mixture") in a volume ratio of 1:1 (v:v).

[0066] In an embodiment, the amount of methyl ethyl ketone (MEK) and the amount of essential oil in the mixture (herein after "MEK/ orange oil mixture") ranges from 20% –75% (v/v), preferably 40% – 60% (v/v), more preferably 50% - 55% (v/v) each.

[0067] In an embodiment, the antibiofilm activity of MEK/tetrachloroethylene mixture and MEK/orange oil mixture against a *Candida albicans* or *E. faecalis* biofilm, cultivated for 24 hours was evaluated.

[0068] In an embodiment, a notable reduction in biomass was observed after the biofilm came into contact with either of the anti-biofilm endodontic formulation. This shows excellent potential for eliminating heterogeneous and resistant biofilm structures.

[0069] In an embodiment, the colony forming unit (CFU) counts were almost null after exposure to either of the two antibiofilm endodontic formulations (**Figures 1 and 2**).

[0070] In an embodiment, a Kirby-Baur disk diffusion test was conducted. It was observed that both anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) exhibit a very significant inhibition of bacteria growth (4.50±0.50 for methyl ethyl ketone/orange oil; 1.38±0.18 for methyl ethyl ketone/tetrachlorethylene).

[0071] In an embodiment, both anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) and the individual co-solvents (tetrachloroethylene and orange oil) were tested for dissolution of gutta-percha and epoxy resinous "AH Plus" sealer (immersion time, 2 and 5 minutes; ultrasonic agitation), (weight loss, surface topography and mechanical properties).

[0072] Both anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) showed significantly higher dissolution efficacy as compared to their individual co-solvents (tetrachloroethylene and orange oil). This suggests a synergistic effect between MEK and the co-solvents (tetrachloroethylene and orange oil). Dissolution efficacy was either similar (MEK/orange oil) or higher (MEK/tetrachloroethylene) as compared to chloroform. Epoxy resinous sealer (AH Plus) presented significantly higher weight loss and time-dependent dissolution as compared to gutta-percha (**Figure 3**).

[0073] In an embodiment, the anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) and the individual co-solvents (tetrachloroethylene and orange oil) were tested for cytotoxicity (MG63 osteoblastic cells, dead/live assay) and chemical composition (Fourier transform infrared spectroscopy).

[0074] In an embodiment, for the cytotoxicity evaluation, human osteoblastic cells were cultivated for 48 hours and incubated with different dilutions (1:1, 1:10 or 1:20) of anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture), MEK or the co-solvents, before fluorescent-based live-dead cell assessment is being performed. At 1:10 dilution, MEK and the anti-biofilm endodontic formulations showed a higher proportion of live cells (around 80%), quantitatively there is no significant differences as compared to the control. At 1:20 dilution, high cell viability (within the range of 90%) was observed, revealing no significant differences as compared to the control. As such, the anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) showed high cytocompatibility while chloroform was shown to be very toxic (**Figure 4**).

[0075] In an embodiment, the anti-biofilm endodontic formulations (MEK/tetrachloroethylene mixture and MEK/orange oil mixture) did not show the formation of new compounds upon mixing (FTIR analysis – **Figure 5**).

[0076] In an embodiment, micro-CT studies were performed to evaluate the persistence of filling material remnants. In other words, the ability of the mixture of solvents to clean or dissolve filling materials from the root canal during retreatment.

[0077] In an embodiment, supplementary cleaning procedure with application of the presently disclosed anti-biofilm endodontic formulations showed a statistically significant reduction in filling material remnants, in extracted teeth as compared to control (without anti-biofilm endodontic formulations).

[0078] As an example, the anti-biofilm endodontic formulations can be applied as follows:

a) Root canals prepared to a larger size (40) were as clean, refering filling residues, as smaller preparations (30), when solvent mixture was sonically agitated.

b) After removing the bulk of filling materials with rotary or manual instrumentation, re-preparation and final irrigation protocol with NaOCl and EDTA, we propose a supplementary binary solvents' mixture (MEK/tetrachloroethylene mixture or MEK/orange oil mixture) irrigating procedure, enhanced by agitation, in view of improving the removal of filling material remnants, potentially infected, and biofilm disruption, achieving a better cleanliness of root canal system before obturation.

c) The mixture is applied with a syringe-and-needle in the re-prepared root canal and agitated through various possible endodontic devices (sonic or ultrasonic tips, rotary instruments), with renewal of the solvent solution. The filling remnants dissolution effect is time dependent, especially for the sealer. Then the root canal is prepared to be filled again.

[0079] The term "comprising" whenever used in this document is intended to indicate the presence of stated features, integers, steps, components, but not to preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

[0080] The disclosure should not be seen in any way restricted to the embodiments described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof.

[0081] The embodiments described above are combinable.

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C L A I M S

1. An anti-biofilm formulation for use in the treatment of infections comprising: methyl ethyl ketone and an organic co-solvent or an essential oil, wherein the anti-biofilm formulation is a biofilm disruptor.

2. An anti-biofilm formulation for use in the treatment of infections comprising: methyl ethyl ketone and an organic co-solvent or an essential oil,
   wherein the essential oil is select from a list consisting of: orange oil, lemon oil, oregano oil, thyme oil or mixture thereof;
   wherein the organic co-solvent is selected from a list consisting of: tetrachloroethylene, dichloroethane, hydrocarbon solvent, oxygenated solvents, glycol ether; or mixtures thereof.

3. The formulation for use according to any of the previous claims wherein the anti-biofilm formulation is an endodontic formulation.

4. The formulation for use according to any of the previous claims wherein the infection is a dental infection.

5. The formulation for use according to any of the previous claims wherein the biofilm is a *Candida albicans* biofilm or a *E. faecalis* biofilm.

6. The formulation for use according to any of the previous claims wherein the organic co-solvent is selected from a list consisting of tetrachloroethylene, dichloroethane, hydrocarbon solvent, oxygenated solvents, glycol ether; or mixtures thereof.

7. The formulation for use according to any of the previous claims wherein the essential oil is select from a list consisting of: orange oil, lemon oil, oregano oil, thyme or mixture thereof; preferably orange oil.

8. The formulation for use according to any of the previous claims wherein the methyl ethyl ketone and organic co-solvent is in an amount ranging from 20% –75% (v/v) of each component, preferably 40% – 60 % (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

9. The formulation for use according to any of the previous claims comprising methyl ethyl ketone and tetrachloroethylene.

10. The formulation for use according to any of the previous claims comprising methyl ethyl ketone and orange oil.

11. The formulation for use according to any of the previous claims comprising methyl ethyl ketone and tetrachloroethylene in an amount ranging from 20% – 75% (v/v) of each component, preferably 40% – 60 % (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

12. The formulation for use according to any of the previous claims comprising methyl ethyl ketone and orange oil in an amount ranging from 20% –75 % (v/v) of each component, preferably 40% – 60 % (v/v) of each component, more preferably 50% - 55% (v/v) of each component.

13. The formulation for use according to any of the previous claims for use in medicine.

14. The formulation for use according to any of the previous claims for use in the treatment of dental infection associated with biofilm.

15. The formulation for use according to any of the previous claims for use as a biofilm disruptor to disrupt biofilm, prevent biofilm formation, for dissolution of endodontic filling materials, or enhanced by its agitation.

16. The formulation for use according to any of the previous claims for use in the treatment of the root canal infections associated with biofilms.

17. The formulation for use according to any of the previous claims for use in the treatment of pulpal and perirradicular infections, preferably including acute forms, such as, acute apical abscesses, or chronic phases of intrarradicular root canal infection, preferably granuloma or cyst of endodontic origin.

18. The formulation for use according to any of the previous claims, wherein the biofilm is a biofilm that is resistant to conventional biofilm disruptors.

19. An endodontic filling material comprising the formulation for use according to any of the previous claims.

20. Use of an anti-biofilm endodontic formulation comprising methyl ethyl ketone and organic co-solvent or an essential oil, as an anti-biofilm agent.

21. A method for disrupting biofilm or preventing biofilm formation comprising applying the anti-biofilm formulation according to any of the previous claims 1 – 19 onto a surface or a surface with a biofilm.

22. The method for disrupting biofilm or preventing biofilm formation according to the previous claim, wherein the surface with a biofilm is a surface with a biofilm, preferably a refractory endodontic biofilm comprising *Candida albicans,* or *E. faecalis*.

D R A W I N G S

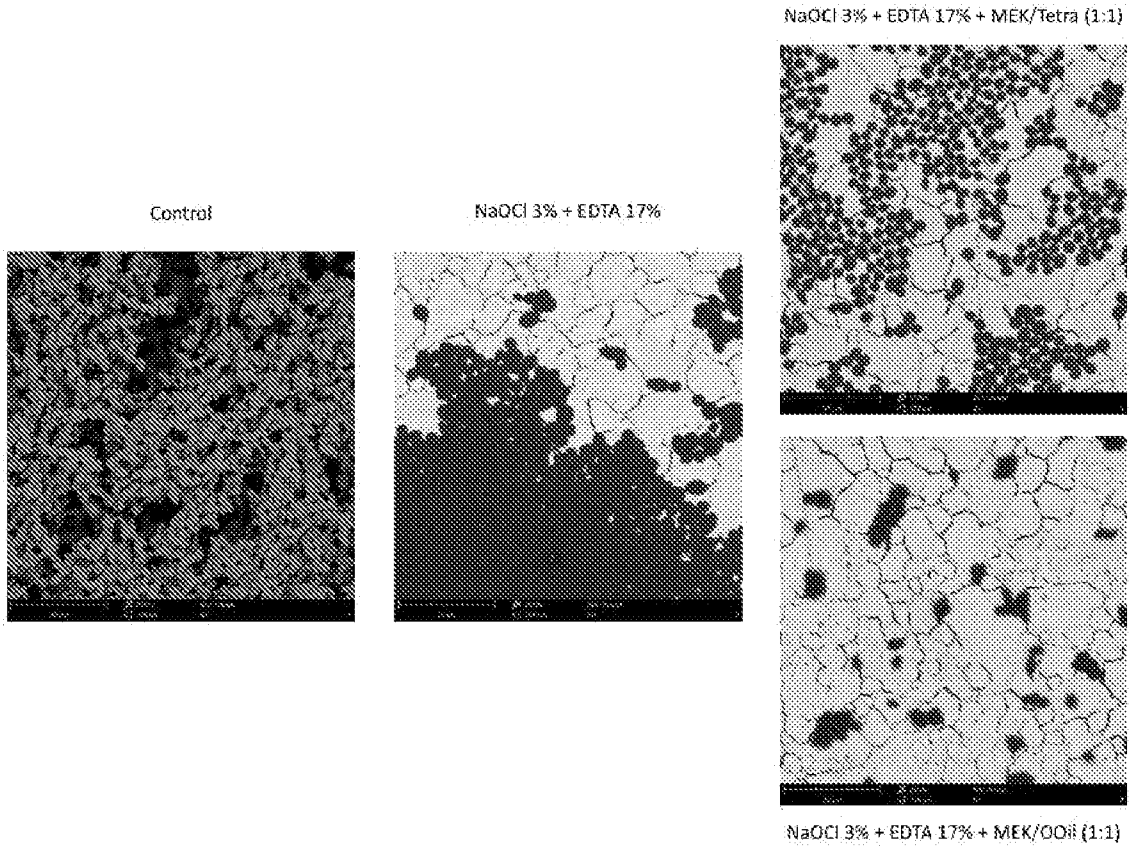


Fig. 1

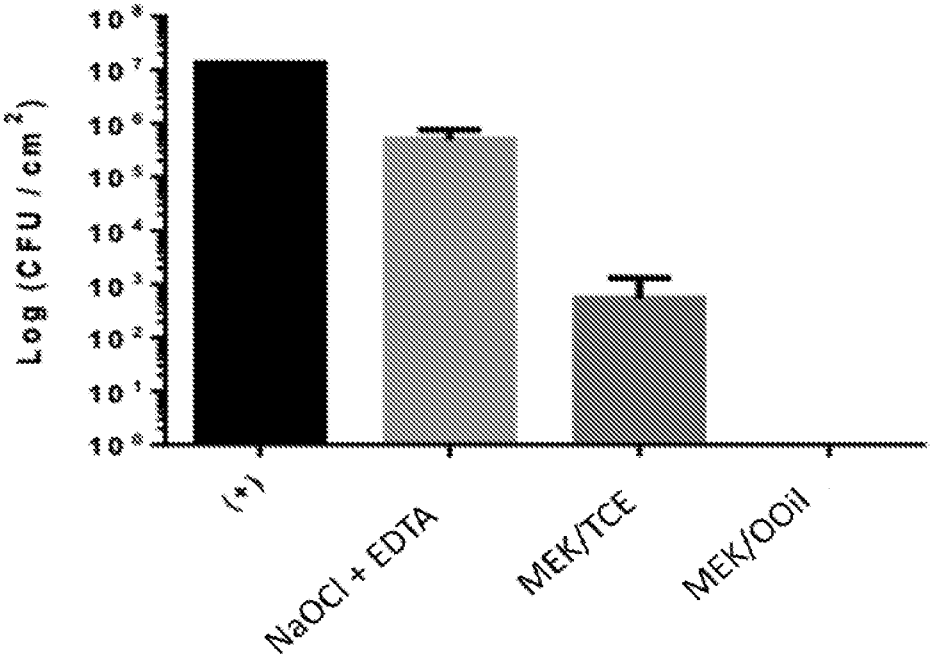
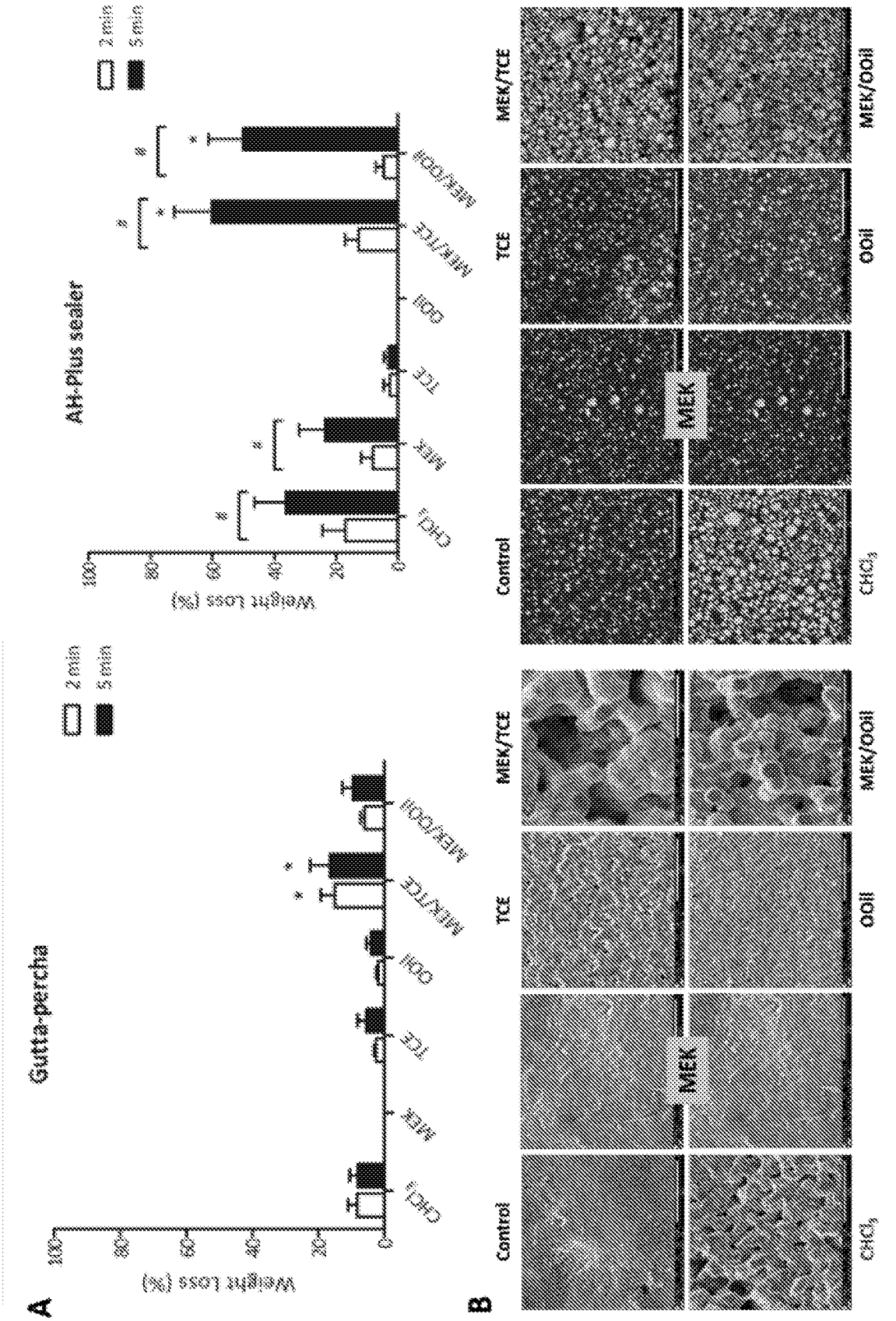


Fig. 2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Weight loss (%)
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5 min
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MEK
TCE
OOil
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B
Control
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TCE
OOil
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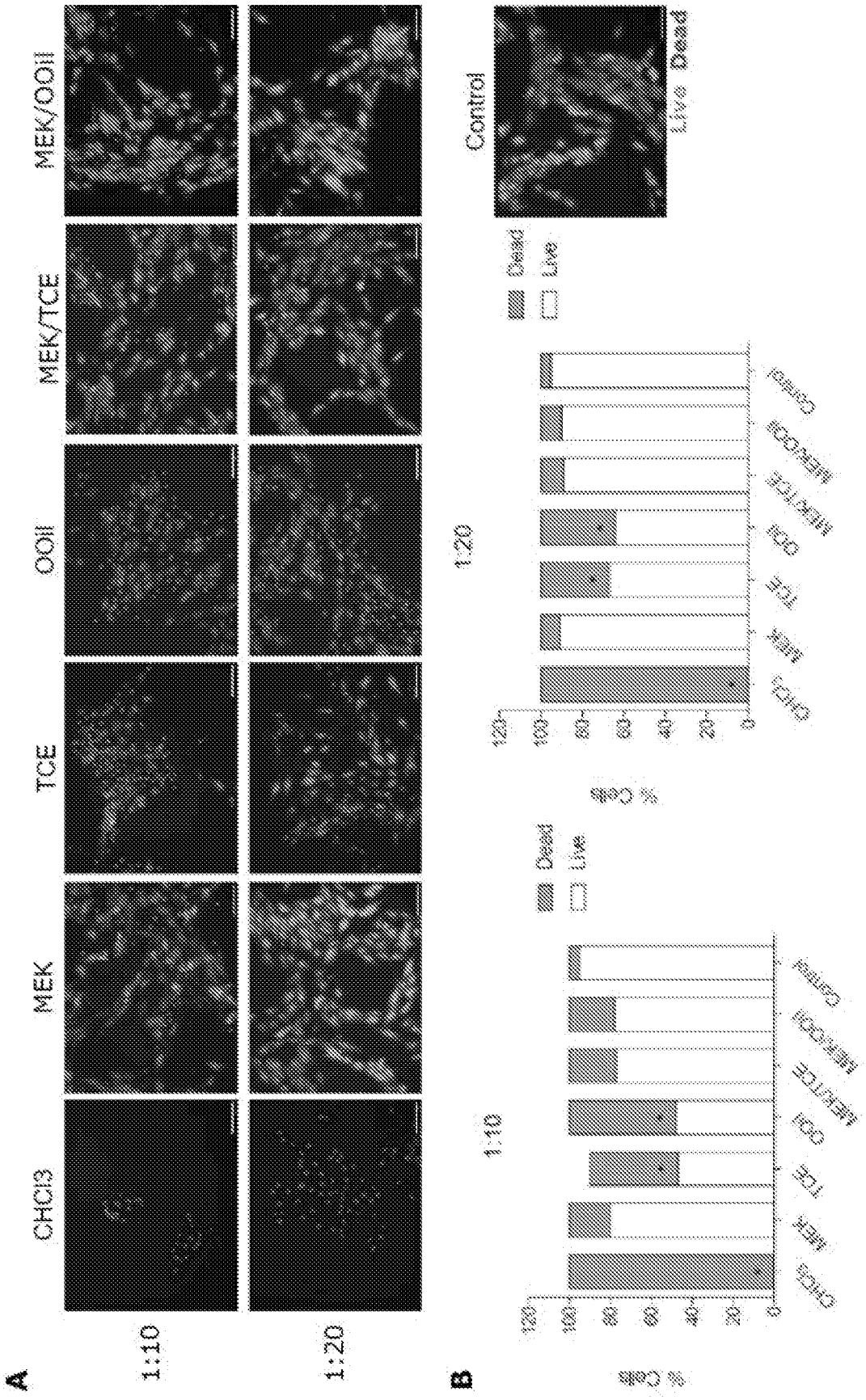
MEK/OOil
CHCl3

Fig. 3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TCE
OOil
MEK/TCE
MEK/OOil
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B
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MEK
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OOil
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MEK/OOil
Control

Fig. 4

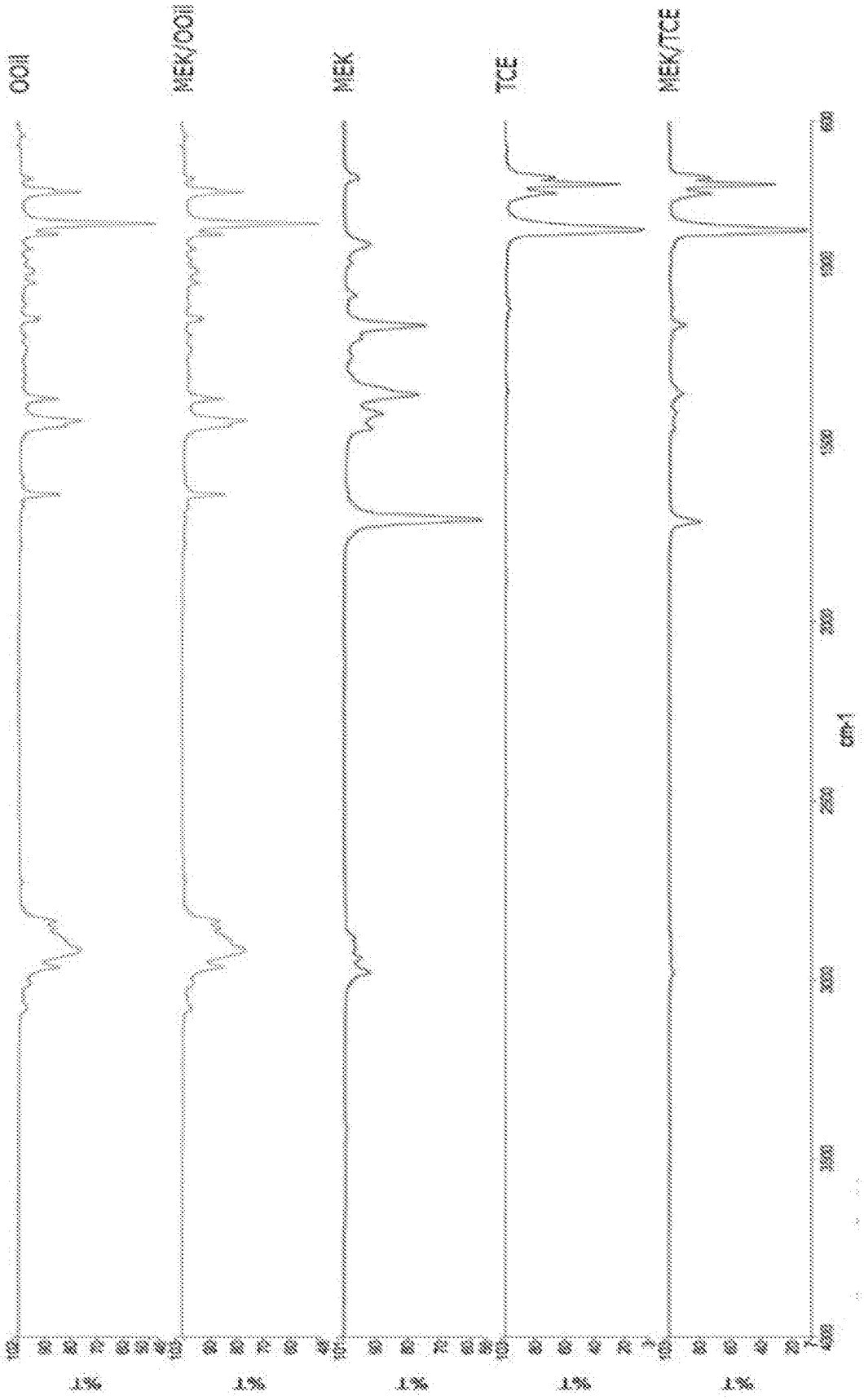

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/080378

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K8/49 A61K31/121 A61Q11/00 A61K8/35 A61K36/752
A61P31/04
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61Q A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERREIRA INÊS ET AL: "Effect of Sonic Agitation of a Binary Mixture of Solvents on Filling Remnants Removal as an Alternative to Apical Enlargement-A Micro-CT Study", JOURNAL OF CLINICAL MEDICINE, vol. 9, no. 8, 1 August 2020 (2020-08-01), page 2465, XP055890114, DOI: 10.3390/jcm9082465 page 5, paragraph 5 abstract page 6, paragraphs 4,6 ----- -/--	13,19

[x] Further documents are listed in the continuation of Box C.
[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2022	01/03/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Escolar Blasco, P

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERREIRA INÊS ET AL: "Efficacy and Cytotoxicity of Binary Mixtures as Root Canal Filling Solvents", MATERIALS, vol. 13, no. 14, 21 July 2020 (2020-07-21), page 3237, XP055890211, DOI: 10.3390/ma13143237	13,19
Y	abstract page 1, paragraph 1 - page 2, paragraph 5 page 8	1-12, 14-18, 20-22
X,P	FERREIRA INÊS ET AL: "Adjunctive procedure with solvent mixtures in non-surgical endodontic retreatment: does it affect root dentin hardness?", ODONTOLOGY, SPRINGER JAPAN, TOKYO, vol. 109, no. 4, 2 April 2021 (2021-04-02), pages 812-818, XP037547047, ISSN: 1618-1247, DOI: 10.1007/S10266-021-00603-6 [retrieved on 2021-04-02] abstract page 812, right-hand column - page 813, left-hand column	1-22
Y	MARTOS JOSUÉ ET AL: "Antimicrobial activity of essential oils and chloroform alone and combinated with cetrimide against Enterococcus faecalis biofilm", EUROPEAN JOURNAL OF MICROBIOLOGY AND IMMUNOLOGY, vol. 3, no. 1, 1 March 2013 (2013-03-01), pages 44-48, XP055890292, HU ISSN: 2062-509X, DOI: 10.1556/EuJMI.3.2013.1.6 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3832082/pdf/EuJMI-03-044.pdf> page 47, left-hand column, paragraph 3 - page 48, left-hand column, paragraph 3; table 1 abstract page 44	1-12, 14-18, 20-22

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MANKAR CHETANA ET AL: "Evaluation of antimicrobial activity of orange peel extract against oral biofilm forming organisms: an in vitro microbial study and scanning electron microscopic assessment", INTERNATIONAL JOURNAL OF BASIC AND CLINICAL PHARMACOLOGY, 1 January 2016 (2016-01-01), pages 1917-1923, XP055890497, ISSN: 2319-2003, DOI: 10.18203/2319-2003.ijbcp20163212 page 1918, left-hand column, paragraph 1-3 page 1920, right-hand column - page 1921, right-hand column	1-22
A	MANCONI MARIA ET AL: "Thymusessential oil extraction, characterization and incorporation in phospholipid vesicles for the antioxidant/antibacterial treatment of oral cavity diseases", COLLOIDS AND SURFACES B: BIOINTERFACES, ELSEVIER AMSTERDAM, NL, vol. 171, 11 July 2018 (2018-07-11), pages 115-122, XP085500237, ISSN: 0927-7765, DOI: 10.1016/J.COLSURFB.2018.07.021 abstract page 121, last paragraph	1-22
A	LIU TING ET AL: "Thymol as a critical component of Thymus vulgaris L. essential oil combats Pseudomonas aeruginosa by intercalating DNA and inactivating biofilm", LWT- FOOD SCIENCE AND TECHNOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 136, 8 October 2020 (2020-10-08), XP086395299, ISSN: 0023-6438, DOI: 10.1016/J.LWT.2020.110354 [retrieved on 2020-10-08] page 7	1-22

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