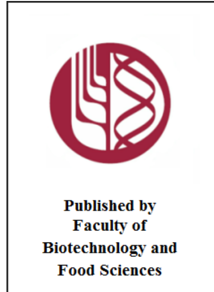
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ALUMINA NANOPARTICLE INCORPORATED *Melaleuca alternifolia* OIL FORMULATION FOR CONTROL OF *Streptococcus mutans* ISOLATED FROM DENTAL CARIES

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ABSTRACT

Occurrence of dental caries is an issue of prime concern in civic health. It is a multifactorial infection with pathological characteristics like salivary dysfunction, swelling of gums and degradation of periodontal tissues. Caries are caused primarily by *Streptococcus mutans* that forms oral biofilms and exhibits certain phenotypic characteristics like acidogenesis that is favorable for their growth. Clinical use of antibiotics for its control may cause hypersensitivity reactions, supra-infections and teeth staining with the organisms eventually also developing resistance to the same. Alternatively, development of green formulations that could effectively inhibit the development of caries could be an active area of research. In this study, synthesis and characterization of *Melaleuca alternifolia* oil in water formulation with a sorbitan ester and its ethoxylate have been attempted. Its mean particle diameter and zeta potential have been measured along with FTIR and AFM analysis. Its antibacterial activity and that of alumina nanoparticles at different concentrations has been assessed separately by broth microdilution method against an MTCC culture and clinical isolates of *Streptococcus mutans*. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration for a range of concentrations have been recorded. Thereafter, a novel nanoparticle-incorporated emulsion has been formulated. Significant enhancement in bactericidal efficiency and decrease in biofilm formation has been observed with it. Additionally, the killing kinetics has been studied and the results clearly demonstrated a time-dependent killing pattern. It is inferred that this alumina nanoparticle incorporated tea tree oil formulation can be further explored as a pharmaceutical product for the treatment of dental caries.

Keywords: Dental caries, *Streptococcus mutans*, *Melaleuca alternifolia*, sorbitan esters, Al_2O_3 nanoparticles

INTRODUCTION

Essential oils are organic liquids that are extracted from various plant sources and typically aromatic in nature. They are composed of different combinations of a wide range of complex organic compounds that contribute to their antimicrobial and aromatic properties. Till date, although 3000 of such oils are known to man, only about 10% are commercially used on a regular basis **(Van de Braak *et al.*, 1999)**.Their antibacterial properties have long since been known to mankind apart from their highly exploited aromatic and food-preservative traits. There are also reports of the utilization of some oils for treatment of cancer **(Da Silva *et al.*, 2008)**.

Tea tree oil (TTO) is one of the most commonly used essential oils and obtained primarily from the plant *Melaleuca alternifolia* that is native to Australia. In Australia, Europe, North America and most parts of Asia, the oil is traditionally considered to be a remedy for various ailments **(Carson *et al.*, 2006)**. It is typically a complex mixture of about 100 different organic components comprising chiefly of terpenes that are volatile aromatic hydrocarbons considered as polymers of isoprene with the formula C_5H_8. Among these, monoterpenes and sesquiterpenes along with the associated alcohols are mostly present in considerable amounts **(Botha and Lall, 2013)**.The potent biological activity of TTO is attributed largely to the presence of terpinen-4-ol that is the chief component apart from other important components like 1,8-cineole, α-terpinene, γ-terpinene and terpinolene **(Swamy *et al.*, 2016)**.The antimicrobial activity demonstrated by almost all of the components, especially by the small terpenoids and their oxygenated derivatives, along with certain phenolic compounds, correlates with the high solubility of the components in biological membranes owing to the presence of alcoholic functional groups **(Carson *et al.*, 2006)**. Contemporary data demonstrates substantially strong fungicidal, antiviral as well as anti-protozoal properties apart from a broad spectrum antibacterial activity. A vast majority of bacterial species have been found to be vulnerable to the effect of TTO at low concentrations of 1.0% or below. The nature of activity is principally bactericidal although bacteriostatic effect has been observed at lower concentrations **(Subhashini and Reetha, 2015)**.

In recent times, multi-drug resistant strains of several deadly pathogens have collectively emerged as a grave threat to the general wellbeing of mankind. World Health Organization reports that a large portion of the world population is still dependent primarily on traditional medicines for basic protection against such pathogens. TTO is specifically of importance in this regard since its complex, multi-component nature reduces the propensity to the development of natural resistance as multiple and simultaneous mutations are required which is highly improbable **(Carson *et al.*, 2006)**. The preferential partitioning of hydrocarbons into biological membranes followed by disruption of the latter's vital functions **(Sikkema *et al.*, 1995)**, is presumed to be the general mode of bactericidal action of TTO, not unlike other hydrocarbons. This proposition is further reinforced by experimental data showing the permeabilization of model liposomal systems by TTO **(Thosar *et al.*, 2013)**.The effect also includes respiratory inhibition as a result of increased membrane permeability **(Carson *et al.*, 2006)**. Thus, this oil is responsible for causing the loss of membrane integrity as well as function, manifested by the uncontrolled ionic outflow, ultimately leading to cell lysis **(Thosar *et al.*, 2013)**.

'Dental Caries' can medically be defined as localized destruction of the dental tissues by the corrosive action of acids produced during bacterial fermentation of carbohydrates present in diet. This is manifested by demineralization of the enamel layer of teeth eventually followed by the dentine **(Botha and Lall, 2013)**. The cavities may be of a number of different colors from yellow to black. Associated symptoms may comprise of pain resulting in difficulty during eating.

Apart from this, several other complications like inflammation of surrounding tissues, loss of teeth and the development of infection and /or abscess formation may also be manifested (**Thosar *et al.*, 2013**). Dental cavities occur when the rate of demineralization far exceeds the rate of natural re-mineralization process. The acidogenic and aciduric (acid-tolerant) properties of *Streptococci* such as *S. mutans* cause reduction in plaque-pH levels thus facilitating the attachment of other biofilm-forming bacteria. Fall of the pH below a magnitude of 5.5 facilitates demineralization of the dentine layer. Hence, one of the most important properties of any antimicrobial substance used for the prevention of dental caries is its need to inhibit the bacterial adhesion that is crucial to bacterial survival and pathogenesis. This is because adherence confers higher resistance against the bacteriolytic action of enzymes and antibiotics (**Botha and Lall, 2013**).

S. mutans is considered to be the primary and one the most cariogenic bacteria and its mode of action involves the formation of biofilm on tooth surface by the synthesis of considerable amounts of extra- as well as intra-cellular polysaccharides. Bacterial interaction with alpha-galactosides present in the biofilm layer, through the mediation of certain proteins, leads to *S. mutans* accumulation that is followed closely by "co-adhesion or co-aggregation" of fresh bacteria (**Freires *et al.*, 2015**). TTO shows considerable microbicidal activity against *S.mutans* that includes active destruction of mature biofilms and inhibition of further biofilm formation (**Lorene *et al.*, 2017**). The value of minimum inhibitory concentration (MIC) against *S.mutans* has been reported to be around 0.0125% (**Groppo *et al.,* 2002**). Nanomaterials, especially metal oxide nanoparticles, in recent times have been recognized as one of the most promising antimicrobial agents in general owing to an exponential increase in surface area with decrease in particle size that in turn leads to a considerably higher surface reactivity as compared to their bulk counterparts. This effectively implies the specific reaction between 40-50% of molecules or ions present on the surface and their target moieties on the microbial cell surface (**Manyasree *et al.*, 2018**). A wide range of different target sites ensures that the development of resistance against the action of nanoparticles is highly unlikely since it would require microorganisms to undergo multiple spontaneous mutations simultaneously (**Mukherjee *et al.*, 2011**). Although the amount of experimental data available currently is fairly limited, alumina nanoparticles have been shown to possess a broad range antibacterial activity. They possess thermodynamic stability across a broad temperature range. The corundum-like structure is characterized by close hexagonal arrangement of oxygen atoms, with alumina ions occupying two thirds of the octahedral lattice-sites. The current theory on the mode of action suggests that the inhibition of the apoptotic pathway may in turn inhibit reactive oxygen species (ROS) production thus causing inhibition of an ROS defense system before completion of the glutamate-induced cell death program. It also describes the attachment of nanoparticles to bacterial cell surface due to electrostatic interaction (**Sadiq *et al.*, 2009**).

Emulsions, both oil in water and water in oil ones, stabilized by suitable surfactants improves solubility of actives, that in turn enhances the latter compounds' bioavailability. Emulsion formulations thus form a more effective way of administration as compared to the plain oil. Surfactants are used as stabilizing agents to create a dispersion system involving two immiscible liquids. The choice of the former is critical for the prevention of spontaneous phase separation that would inevitably take place immediately upon cessation of agitation. The hydrophilic-lipophilic balance (HLB) system of Griffin is an important tool utilized for the selection of the most appropriate surfactant combination wherein the goal is to achieve a final HLB value equal to that of the material being emulsified. Previous studies have proven that a surfactant combination, comprising of a low HLB type, along with one of a high HLB one, provides greater stability as compared to a single surfactant with the same HLB value (**Takamura *et al.*, 1979**). Tweens and Spans are classes of non-ionic surfactants that provide significant advantages over ionic similars, like enhanced stability, non-reactivity with ionic ingredients and flexibility in terms of formulation along with wider compatibility (**Wang *et al.*, 2009**). Spans are chemically sorbitan esters produced by esterification of fatty acids with the product formed by the dehydration of sorbitol while Tweens are ethoxylated forms of Spans. Apart from the HLB value, the primary consideration while choosing a particular combination of surfactants is the underlying chemistry involved. Spans in combination with the corresponding Tweens act as efficient stabilizers for oil-in-water systems (**Takamura *et al.*, 1979**).

In the present study, a tea tree oil emulsion (TTE) with a combination of Tween 80 and Span 80 to achieve an HLB of 8 was formulated, loaded with alumina nanoparticles (NP) and characterized for its particle size, zeta potential, electrophoretic mobility and poly-dispersity index. Its FTIR spectrum has been recorded and its AFM image captured. It was then evaluated for its activity against clinical samples of *Streptococcus mutans,* isolated from dental caries, by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values. The ability of the emulsion to inhibit biofilm formation was also assessed. To the best of our knowledge, such a TTE loaded with NP for control of *Streptococcus mutans* with promising results has not been reported so far.

MATERIAL AND METHODS

Chemicals and media

TTO was purchased from Falcon, India. Tween80, Span80 and NP were procured from SRL, India. All media were procured from Hi Media Pvt. Ltd. Milli-Q water was utilized for formulation of the emulsion. Chlorhexidine gluconate mouthwash (0.2%) (CHX; used as positive control) was procured from the local medical store.

Bacterial strains and growth conditions

Dental plaque samples were collected from patients visiting the Department of Conservative Dentistry and Endodontics, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, Bengaluru, after due ethical clearance (No: MSRDC/EC/2012-14/CONS/F/019 dated 29.08.2013) and their consent. The plaque samples from molar teeth were collected with gloved hands, using sterile swabs. They were carefully transferred into sterile Todd Hewitt broth, transported to the laboratory at 4˚C and kept in the biosafety cabinet of class II type A2. Then from these plaques samples, five dental clinical isolates of *Streptococcus mutans* were isolated by using Mitis Salivarius bacitracin agar plates. The organisms were confirmed by routine biochemical tests (**Syed *et al.*, 1975**; **Jain *et al.*, 2015**). A standard MTCC 890 was also obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh. All the organism stocks were preserved at -4°C on Brain-Heart Infusion (BHI) agar slants. Aliquots of the different stock-cultures were inoculated separately in fresh BHI broth and incubated for 48 hours at 37°C whenever required for further studies.

GC-MS analysis of TTO

GC7890A/MS5975C instrument model (Agilent Technologies, USA) was used for GC-MS analysis of the oil. Agilent DB5MS ultra-inert chromatography column, with a length of 30m, internal dia of 0.25mm and film thickness of 0.25µm was employed. Pure helium gas was chosen as the carrier gas and a standard type front-injection system was used with an injection volume of 1µl. The inlet temperature was set at 325°C while the temperature of the oven was maintained at 40°C for a minute and then gradually ramped up to 300°C at a steady rate of 10°C/min. The temperature of both the GC interface as well as the ion chamber was set to 250°C. Total run time was 30 minutes for the entire GC analysis procedure. EMV mode was employed with a gain factor value of 1.00. Additionally, quadruple-double focusing mass analyzer and photo multiplier tube were utilized. Finally, identification of the compounds was done by referring to the NIST library.

Formulation of TTE

The final surfactant-mixture used for emulsion formulation [43.92% (v/v) of Tween80 and 56.07% (v/v) of Span80] was prepared so as to achieve an HLB value of 8.0. Here, the oil and surfactant were both warmed to 40ºC before addition of the latter in increments of 0.5ml gradually with stirring in different volumetric flasks. The total volume was made up to 50ml by gradual and regulated addition of water. The visually stable (without phase separation) formulations were chosen and sonicated (Vibra Cell, Sonics and Materials Inc., USA) for 5 minutes at a pulse rate of 2 per minute and duty cycle of 16.66%. They were then left undisturbed for 24 hours at 37ºC. Thus, a range of emulsions were prepared with different oil-to-surfactant ratios (**Banerjee *et al*., 2018**). The particle sizes for these emulsions were taken in triplicate using an SZ-100 particle size analyzer [Horiba Scientific accompanied by Windows (Z type) version 2.00 software]. One way ANOVA and Tukey tests were performed using Origin software (version 9.0) to find significant differences (if any) between the particle sizes of the samples for selection of a suitable emulsion with the least particle size.

Characterization studies of TTE

Determination of particle size, zeta potential and electrophoretic mobility of the selected TTE

Horiba Scientific SZ-100 instrument were used for the analysis of zeta-potential of TTE along with its electrophoretic mobility. An emulsion dilution of 1:100 was used. Thereafter, mean particle diameter and polydispersity index were also recorded with the same instrument at a scattering angle of 90º at 25°C. The software was used for generating a plot of percentage-frequency of particles against their diameters. Following optimal dilution, the electrophoretic mobility was recorded using the same instrument.

Fourier Transform Infrared (FTIR) Spectroscopic and Atomic Force Microscopic (AFM) analyses of TTE

FTIR spectroscopic analyses of the TTO and TTE were performed using FTIR Spectrophotometer (Bruker Optics, Germany). Attenuated total reflection (ATR) technique was employed and the transmittance spectra were recorded over a frequency range of 4000 to 500cm^{-1}. Topographical characterization of TTE was done using AFM analysis (Easyscan 2 model, Nanosurf AG, Switzerland). A thin smear was prepared, dried for 24 hours and then placed on the sample stage for analysis.

Tests for shelf-life stability and centrifugation test for TTE

The TTE was taken in a beaker and maintained at 40°C and -18°C for 48 hours. It was then brought to 37°C and observed for any visual destabilization signs like creaming, cracking, oil-droplet formation and (/or) phase separation. The same test procedure was performed for three consecutive cycles. Shelf-life stability was assessed by maintaining the emulsions at 37°C for a period of 90 days followed by observation for any visual sign(s) of destabilization. The emulsion was also centrifuged (REMI Microprocessor Research Centrifuge PR-24) for 30 minutes at 10,000 rpm and examined visually for signs of destabilization **(Banerjee *et al.,* 2018).**

Determination of MIC and MBC of TTE and NP with clinical isolates and MTCC culture of *S. mutans*

MIC studies for TTE, NP and a commercially available mouthwash containing 0.2% (w/v) of CHX (positive control, were carried out for all the clinical isolates and MTCC culture of *S. mutans* following CLSI guidelines using broth microdilution method with BHI (**Li *et al.,* 2014**). All experiments were carried out in a biosafety cabinet of class II type A2 (**Jain *et al.*, 2015**). The concentrations used were 200, 400, 600, 800 and 1000ppm. 180µl of the different dilutions were placed in respective wells of a 96-well microtiter plate. 20µl of bacterial suspension with a final concentration of 1x10^{5}CFU/ml was inoculated in each well and the microplate was incubated at 37°C for 24hrs. The optical density (OD) readings were recorded using enzyme-linked immunosorbent reader (BioRad 6.0) at 595nm and plotted against the respective concentrations in each case. The ppm concentrations, wherein a drop of 50% and 90% in the bacterial growth were observed, (as calculated from the OD values) were recorded as the MIC and MBC of TTE and NP.

Formulation of NP incorporated TTE emulsion (NIE) and determination of its MIC and MBC with clinical isolates and MTCC culture of *S. mutans*

For this, the MIC of nanoparticles was sonicated in the aqueous phase. It was then used as for the formulation of NIE by the same procedure as was described for the formulation of TTE. Its MIC and MBC were also determined for all the clinical isolates and MTCC culture of *S. mutans* as per the CLSI guidelines.

Studies on inhibition of biofilm formation of clinical isolates and MTCC culture of *S. mutans* by TTE, NP and NIE

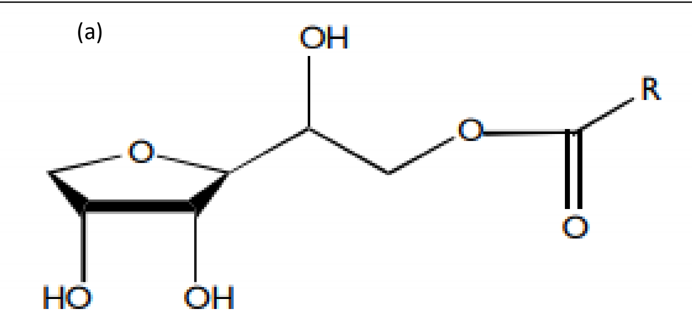
This test was performed as formerly described by **Krzysciak *et al.* (2014),** with a few modifications. 0.2ml volume of 24 hour-old broth cultures of *S. mutans* strains (10^6 CFU/ml) were seeded in respective wells of a 96-well microtiter plate with each containing 1.8ml of TTE, NP or NIE. After 48 hours of incubation, the supernatants were drained and the wells were rinsed twice with previously-sterilized distilled water. 1mL of methanol per well (incubated for 15 minutes) was used for fixation of the adhered bacteria. After disposal of the methanol followed by air-drying, staining was done for 15 minutes using 0.2ml of 0.1%(w/w) crystal violet and excess stain was later washed off by running tap water. Subsequently, removal of the previously-bound dye was achieved by dispensing 0.2mL of 30%(v/v) glacial acetic acid in each well. The solutions thus obtained, were evaluated by recording the OD at 595nm using a microplate reader (**Li *et al.*, 2015**). The OD in each case was plotted against the corresponding concentrations of the respective samples. The percentage inhibition of biofilm formation was calculated thereafter using the equation: Biofilm inhibition percentage = (1- OD value for test sample / OD value for negative control) x 100 **(Nikolić *et al.,* 2014).**

Time kill assay of NIE with clinical isolates and MTCC culture of *S. mutans*

This was performed as per the procedure described by **Carson *et al.* (2006)** with certain modifications. Each of the different clinical isolates as well as the MTCC strain was suitably diluted with fresh BHI broth to give a final concentration of 1.5×10^5 CFU/ml. They were thereafter allowed to grow in fresh BHI broth containing the MIC concentration NIE for 0, 5, 10, 15, 30, 60, 120, 240, 480, and 720 minutes at 37°C. 20µl aliquots of the different strains were plated onto BHI agar at each time point as mentioned previously and incubated at 37°C for 24hrs in aerobic condition. Post-incubation, the CFU was calculated using the following formula: CFU = (Number of colonies x Dilution factor) / Volume of sample on the plate Thereafter, the time-kill effect of NIE was determined by plotting the $\log_{10}$ value of previously calculated CFU versus the specific time points as mentioned above.

RESULTS AND DISCUSSION

The versatile curative properties of *Melaleuca alternifolia* or TTO that include antifungal, anti-inflammatory as well as antiseptic ones for cuts and burns are well documented. However, certain concentration-based symptoms of toxicity have been demonstrated in recent studies **(Payzar *et al.,* 2013)**. A wide variation in the degree of these effects is noted across a range of parameters such as test subjects, organs, cells and conditions studied. TTO based emulsion formulations have been reported earlier, although in such systems several supplementary synthetic chemicals incorporated for stability, make a noxious contribution **(Reichling *et al.*, 2006).** In this context, the TTE formulated in this study involved the non-ionic compounds, Tween 80 and Span 80 (figure 1) without the addition of any synthetic chemicals.



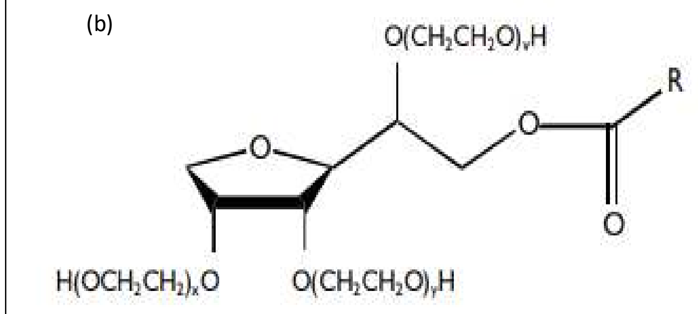


Figure 1 (a) Polysorbate monooleate (Tween 80) (b) Sorbate monooleate (Span 80)

GC-MS Analysis of TTO

The abundance versus retention time spectra generated after GC-MS analysis of TTO is shown in figure 2.

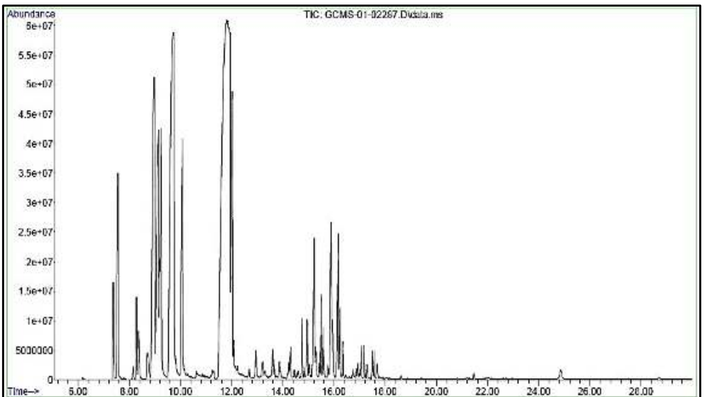


Figure 2 GC spectrum of TTO

Different constituent organic compounds in TTO (mainly terpene hydrocarbons) as revealed by GC-MS analysis along with their corresponding relative abundance (represented as % peak area) and their major biological activities are shown in table 1.

Table 1 Compounds identified by GC/MS in TTO with retention times, relative peak area (%) and their biological activities

Retention time (mins)	Identified compound name (Common name)	2D Molecular Structure	CAS No.	Peak area (%)	Major biological activity
7.366	4-methyl-1-propan-2-ylbicyclo[3.1.0]hex-3-ene (α-Thujene)		2867-05-2	1.31	Antioxidant, Anti-viral **(Kelen *et al.*, 2008)**
7.546	2,6,6-Trimethyl bicyclo [3.1.1] hept-2-ene (α-Pinene)		7785-70-8	3.28	Anti-inflammatory, Chondroprotective **(Rufino *et al.*, 2014)**
8.963	3,7,7-trimethylbicyclo[4.1.0]hept-4-ene (4-Carene)		29050-33-7	10.15	Antioxidant, Cytoprotective **(Gagnon *et al.*, 2017)**
9.132	1,2,4,5 tetramethyl benzene (Durene)		95-93-2	7.10	Morphology control, Electrocatalytic activity **(Tang *et al.*, 2011)**
9.184	(+)-(R)-4-isopropenyl-1-methylcyclohexene (Limonene)		138-86-3	1.27	Antifungal, Bacteriostatic **(Smeriglio *et al.*, 2017)**
9.248	2,2,4-trimethyl-3-oxabicyclo[2.2.2]octane (Eucalyptol)		470-82-6	4.04	Anti-inflammatory, Analgesic, Anti-cancer, Antidermatophytic **(Korsak *et al.*, 1998)**
9.726	2,6,6-Trimethyl bicyclo-hept-2-ene (α-Pinene)	Already given above	7785-70-8	18.58	Anti-inflammatory, Chondroprotective **(Espina, L *et al.*, 2013)**
10.070	3,7,7-trimethylbicyclo[4.1.0]hept-4-ene (4-Carene)	Already given above	29050-33-7	4.22	Antioxidant, Cytoprotective **(Gagnon, M. C *et al.*, 2017)**
11.801	4-methyl-1-(1-methyl ethyl)- 3-Cyclohexen-1-ol (Terpinen-4-ol)		20126-76-5	27.40	Antifungal **(Kumar Sahoo *et al.*, 2011)**

12.011	2-(4-methylcyclohex-3-en-1-yl)propan-2-ol {α-Terpineol		98-55-5	4.35	Anticancer, Gastroprotective **(Mondello *et al.*, 2006)**
15.223/ 15.485	(1aR,4aR,7S,7aS,7bS)-1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene (Alloaromadendrene)		25246-27-9	2.97	Antiproliferative, Antioxidant **(Chavan *et al.*, 2010)**
15.876	(3S,3aS,5R)-3,8-dimethyl-5-prop-1-en-2-yl-1,2,3,3a,4,5,6,7-octahydroazulene (α-Bulnesene)		3691-11-0	2.80	PAF Inhibitor **(Tsai *et al.*, 2007)**
16.155	(1S,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,3,5,6,8a-hexahydronaphthalene (Cadinene)		483-76-1	2.79	Antioxidants **(Damasceno *et al.*, 2017)**
16.225	1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydronaphthalene (Calamenene)		483-77-2	2.79	Antioxidants **(Azevedo *et al.*, 2013)**

Terpinen-4-ol is the principal active ingredient and also the most abundant (typically a concentration of 35.0-48.0%) constituent present in the oil. It has strong antibacterial as well as anti-fungal properties **(Kumar Sahoo *et al.*, 2011).** α-Terpineole (2.0-5.0%), that is an isomer of terpinen-4-ol has been reported to have a wide variety of biological actions including anti-cancer, anti-ulcer, anti-hypertensive and anti-oxidant effects **(Mondello *et al.*, 2006).** α-Terpinene is another important constituent present in the oil (typically 6.0-12.0%) that contributes to the characteristic odor of TTO **(Rudbäck *et al.*, 2012).** Apart from these, Eucalyptol is a notable component constituting upto 10.0%(w/v) of the oil and is known to possess substantial antibacterial as well as antifungal activities **(Gilles *et al.*, 2010)**. It also exhibits analgesic and anti-inflammatory effects and is routinely used in the preparation of mouthwashes and cough-suppressants **(Korsac *et al.*, 1998)**. TTO also contains limonene (0.5-1.5%) that is found widely in citrus fruits and has anti-cancer properties. It is currently, also being used for preparation of bronchitis medications **(Smeriglio *et al.*, 2017).** Apart from these, certain other compounds are also present in trace amounts (peak area<1.0%) in the oil *viz.,* α-Terpinene, beta-Myrcene, α -Phellandrene, α-Gurjunene, Caryophyllene, Epi-bicyclosesquiphellandrene and Bicyclogermacrene.

Selection of TTE

The selection of a specific oil-to-surfactant ratio was based on the findings of the one-way ANOVA and Tukey tests that did not reveal any significant differences between the mean particle sizes at $p<0.05$. This implied that changes in the oil:surfactant ratio within the stable range, did not influence the particle size. Thus, the TTE with oil-to-surfactant ratio of 1:0.6 was used in the present study as it involved lower amount of the surfactant which would be more economical.

Determination of particle size, zeta potential and electrophoretic mobility of the selected TTE

The method of dynamic light scattering was utilized for the determination of the size-distribution profile of TTE (figure 3a) and the mean particle size obtained was 108.3±0.53nm. The level of charge distribution in the diffuse layer surrounding the constituent droplets as expressed by the mean zeta potential (figure 3b), was found to be -52.1±1.84mV. The zeta potential values corresponding to a stable emulsion system are known to lie outside the range of -30 to +30mV. The TTE constitutes a polydisperse system with both surfactant coated oil droplets and plain surfactant droplets. The polydispersity index (PI) and electrophoretic mobility was found to be 0.284±0.033 and -4.05±1.49 respectively. These values indicate the stable nature of the emulsion.

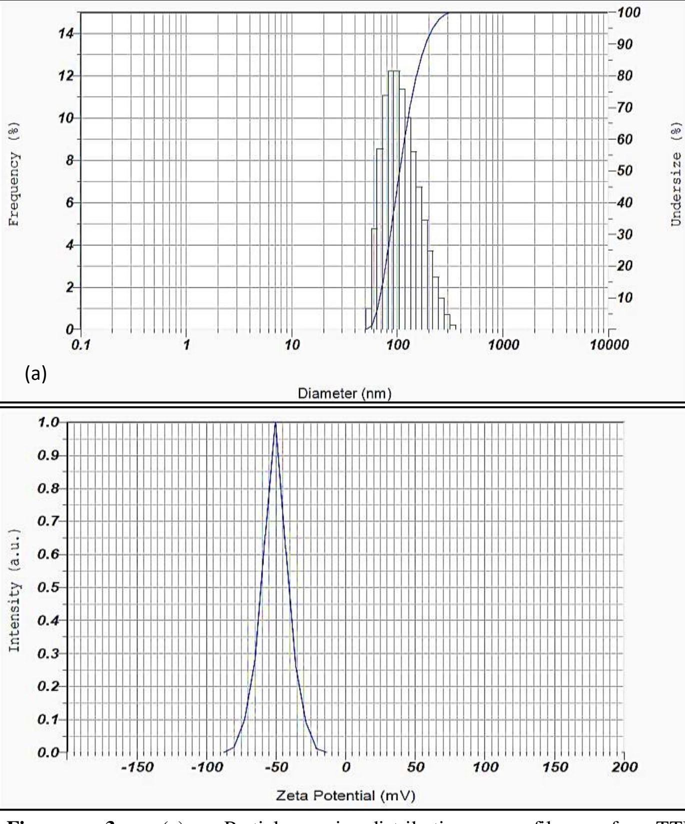


Figure 3: (a) Particle size-distribution profile of TTE (b) Zeta potential measurements of TTE

Fourier Transform Infrared (FTIR) Spectroscopic analyses of TTO and TTE & Atomic Force Microscopic (AFM) analysis of TTE

FTIR spectroscopic analysis revealed the different functional groups present in the constituents of TTO and TTE.

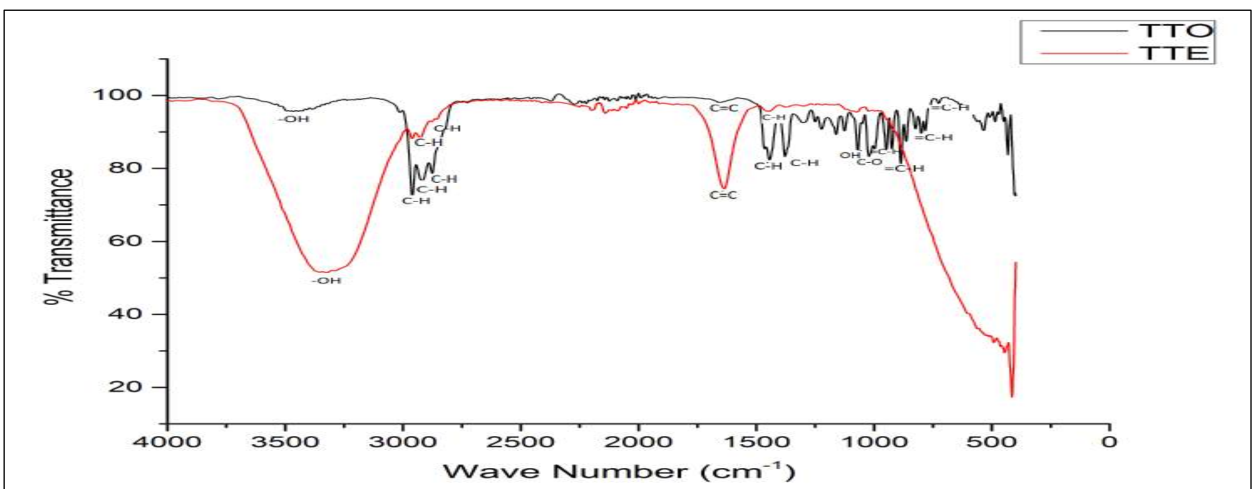


Figure 4 FTIR spectra of TTO and TTE

The FTIR spectrum showed the presence of specific functional groups in the constituent compounds of TTO as identified initially by GC/MS analysis (table 1). The -C-H (2960.73 cm^{-1} to 2875.86 cm^{-1}), C=C (1647.21cm^{-1}) and =C-H bonds (725.23cm^{-1} to 948.98cm^{-1}) present in the compounds show bands at their characteristic wave numbers. The broad peak (strong intensity) produced at 3464.15cm^{-1} is majorly contributed by the -OH of Eucalyptol, Terpinen-4-ol and α-Terpineol. Additionally, the alcoholic C-O bonds in these compounds also contributed to the formation of a sharp peak at 1068.56cm^{-1}. The three sharp peaks of strong intensity produced at 2960.73, 2920.23 and 2875.86cm^{-1} correspond to the stretching vibrations caused by the presence of –C-H bonds in almost all the components. Similarly, bending vibrations of the =C-H bonds (present in almost all components) result in the formation of sharp peaks (strong) at 725.23, 783.10, 887.26 and 948.98cm^{-1}. –C-H bending vibrations in compounds like α-Pinene, 4-Carene, Terpinen-4-ol, Alloaromadendrene, α-Bulnesene and naphthalene derivatives like Cadinene and Calamenene etc. contribute to the formation of sharp peaks at 1973.10cm^{-1} and 1444.68cm^{-1}. The ether bond (C-O) present in Eucalyptol contributes to the sharp peak of strong intensity at 1022.27cm^{-1}.

AFM was done for studying the surface topology of TTE. The graphs and surface scans generated are shown in figure 5. It reveals the fairly smooth surface of the TTE. Deflection scans give information about the line-width, side-wall and line-edge roughness of the TTE surface.

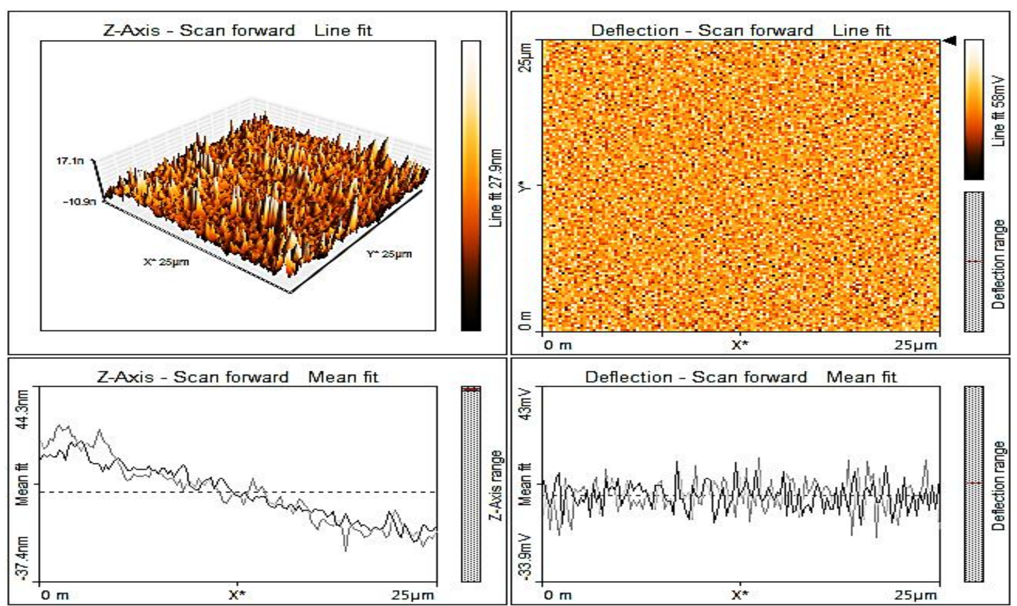


Figure 5 AFM image, deflected three dimensional image and surface topology of TTE

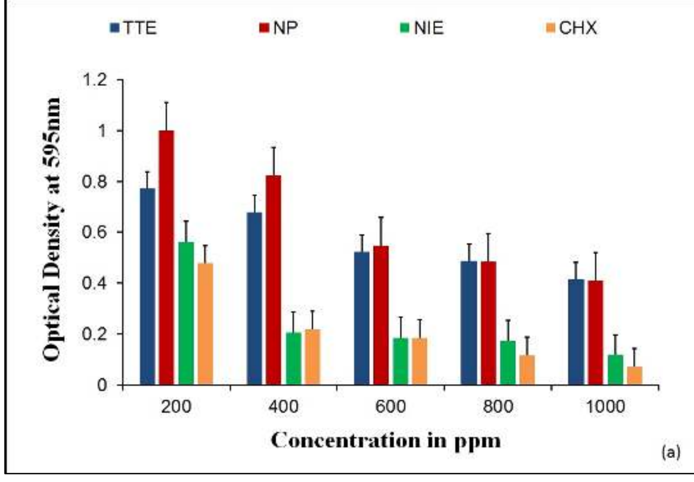
Tests for shelf-life stability and centrifugation test for TTE

Different decay mechanisms of emulsions like creaming, flocculation, coalescence, and Ostwald ripening have previously been described **(Sjoblom *et al.*, 2005).** No visual indication of destabilization *i.e.,* cracking, creaming, phase separation, oil droplet formation was observed in the selected TTE after being stored for a period of 90 days at 37ºC. Its texture and color also appeared to be unaffected. No changes were observed after heating, cooling and freeze-thawing test cycles. Additionally, no visible sign of destabilization was noted after the centrifugation test. The TTE was thus stable under all the different test conditions **(Sjoblom *et al.*, 2005).**

Determination of MIC and MBC of TTE, NP and NIE with clinical isolates and MTCC culture of ***S. mutans***

The MIC for both TTE and NP was found to be 600 ppm (figure 6a and 6b). TTO and NP separately have antibacterial activities that can reduce cell concentrations and inhibit biofilm formation **(Groppo *et al.,* 2002 & Manyasree *et al.,* 2018)**. After determination of the MIC values, the MIC concentrations of the two were combined to produce the NIE. According to CLSI-M26-A guidelines, the value obtained should theoretically be higher than the MIC. Also a particular substance is considered to have antibacterial activity as long as the MBC is upto 4 times the MIC. The MIC of the NIE was found to be around 400ppm while the MBC was

approximately 800ppm. Nearly identical values for both MIC and MBC were obtained with the positive control, *viz.*,0.2%w/v solution of CHX. It was chosen as a positive control in the current study since its strong antimicrobial activity against dental pathogens have been reported **(Autio-Gold, 2008)**. MIC value obtained for the NIE was hence found to be lower than that of both TTE and the NP when tested separately. The synergistic effect of TTE and nanoparticles is thus observed here.



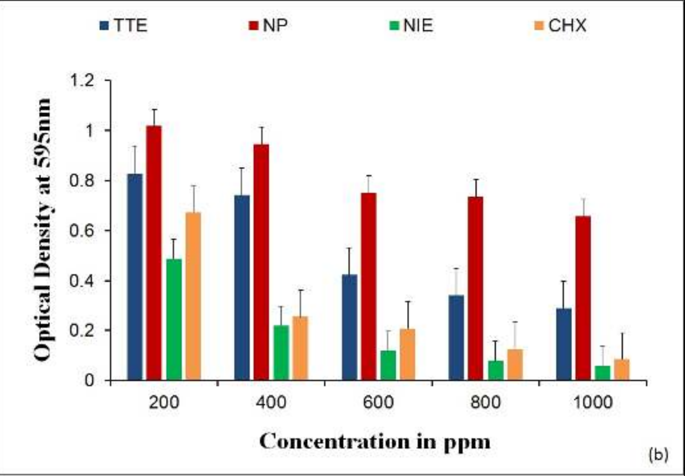


Figure 6 Optical density values for determining (a) MIC of clinical isolates (b) MIC of MTCC 890 (CHX is taken as the positive control)

Studies on inhibition of biofilm formation of clinical isolates and MTCC 890 culture of ***S. mutans*** **by NIE**

Assay for inhibition of biofilm formation was performed separately for TTE, nanoparticle suspension and NIE. Percentage inhibition of biofilm formation in presence of MIC was calculated for each test strain. The results indicated considerable impediment in biofilm formation by NIE (figure 7). This % was higher than obtained with TTE and nanoparticle suspensions. Such inhibitory action displayed by NIE is perhaps a synergistic function of the antimicrobial actions of both the TTE as well as the nanoparticles. The overall mechanism of action probably involves the disruption of bacterial cell membrane by either the induction of oxidative stress, release of metal ions or penetration of cell membrane followed by DNA interactions **(Carson *et al.*, 2006).**

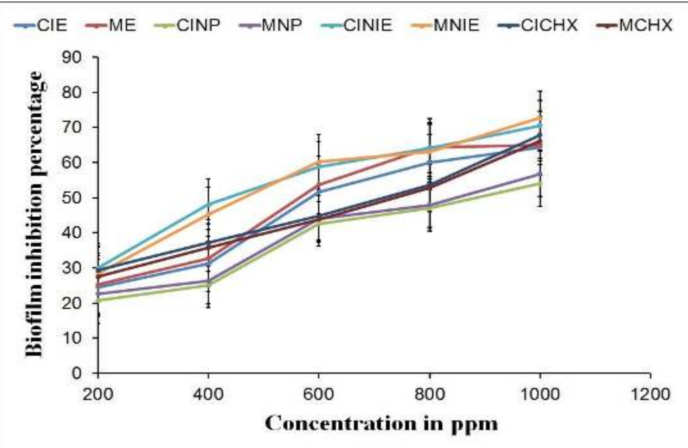


Figure 7: Biofilm inhibition percentage of five clinical isolates of *S. mutans* (average values) and of MTCC 890 by TTE (CIE, ME), NP (CINP, MNP), NIE (CINIE,MNIE) and positive control CHX (CICHX,MCHX) respectively.

Time kill assay of NIE with clinical isolates and MTCC 890 culture of ***S. mutans***

This was carried out with 400ppm concentration (MIC) of NIE. The time and concentration dependent killing pattern was evident from the sharp drop in the $\log_{10}$ value of CFU/ml within (60-120) minutes post-incubation signifying the inhibitory effect on *S. mutans* caused by the NIE (figure 8). The control plate, without NIE, showed gradual but steady growth over time which eventually stabilizes.

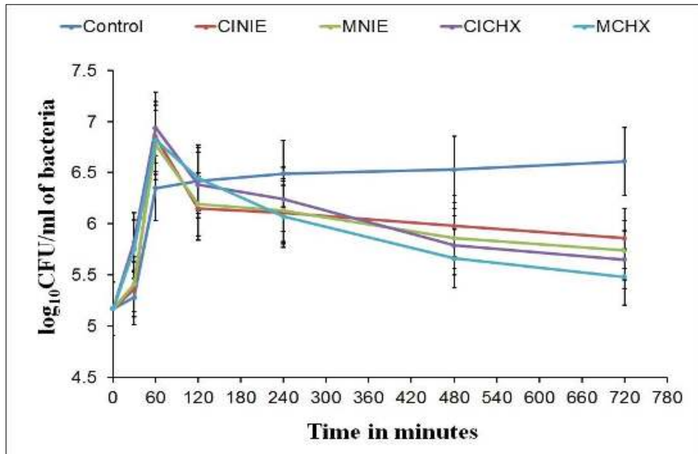


Figure 8 Time kill assay with NIE Time kill assay of five clinical isolates of *S. mutans* (average values) and of MTCC 890 by NIE (CINIE and MNIE respectively) and by the positive control CHX (CICHX and MCHX respectively).

CONCLUSION

The volatile TTO extracted from the leaves and terminal branchlets of the Australian-native species *Melaleuca alternifolia* by steam distillation has been known to possess manifold biological activities including inhibitory activities against bacteria, viruses and fungi in addition to anti-inflammatory, analgesic as well as insecticidal effects **(De Groot & Schmidt, 2016).** Although certain genotypic variations cause a significant difference in relative proportions of the constituents (which determines the particular chemotype), the same chemical spectrum is majorly observed. Commercially available TTOs almost always belong to the terpinen-4-ol chemotypic category. The major chemicals along with the corresponding concentration limits in case of commercial TTOs include terpinen-4-ol (35.0-48.0%), γ-terpinene (14.0-28.0%), 1,8-cineole (up to 10.0%), α-terpinene (6.0-12.0%), α-terpineol (8.0%), α-pinene (1.0-4.0%) and limonene (0.5-1.5%). **(De Groot & Schmidt, 2016).**

MIC of TTE when tested against clinical isolates of *S. mutans* was determined to be around 600ppm whereas MBC was approximately twice that value. A very similar set of results were obtained in case of NP. Preliminary tests revealed a significant enhancement of antibacterial activity in case of NIE and the MIC had a value of 400ppm with the MBC being twice of that. Moreover, the NIE clearly

showed higher biofilm inhibition as compared to both nano-alumina as well as TTE when taken separately. The antibacterial potency displayed *in-vitro* by the NIE against cariogenic strains of *S. mutans* was a significant improvement over the activities exhibited individually by both TTE as well as nano-alumina. Moreover, the activity was comparable to a commercially available chlorhexidine gluconate (CHX) preparation that was used as a positive control in this study. Assessment of the level of inhibition of biofilm formation in simulated mouth-system using artificial saliva along with the amount of cell-membrane damage caused by the formulation by *in-vitro* techniques requires further investigation. In this regard, identification of the gene(s) in *S. mutans* responsible for biofilm formation and investigation of any potential inhibitory effect of the NIE on gene-function can be explored in future. The NIE may find future application as mouthwash formulations intended for caries prevention. It could thus, potentially act as a safer alternative to several commercially popular synthetic preparations that may lead to deleterious side effects upon prolonged usage.

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CONFLICT OF INTEREST: Nil

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