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Fluid properties of solvents and oils used in *in situ* forming microparticles

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ABSTRACT

In situ forming microparticle (ISM) has been recently used as the injectable drug delivery system prepared from polymeric non-aqueous phase in oil emulsion. Solvents and oils used in ISM including N-methyl pyrrolidone (NMP), 2-pyrrolidone (PYR), dimethyl sulfoxide (DMSO), glycofurol, triacetin, olive oil, camellia oil and isopropyl myristate (IPM) were evaluated for pH, density, viscosity, flow time, surface tension, contact angle, color distribution, injectability, antimicrobial activities and cytotoxicity. The most viscous camellia oil and olive oil showed the highest flow time with lowest color diffusion and lower injectability; thus, they were suitable for performing as external phase of ISM to prolong drug release. Lower viscosity of NMP with low contact angle promoted its rapid wetting and faster color diffusion rate. All the solvents inhibited *C. albicans* more effective than bacteria. DMSO showed the lowest cytotoxicity against HCT116 human colorectal carcinoma cell among tested solvents which was followed by PYR, NMP, triacetin and glycofurol, respectively. These obtained fluid properties of solvents provide the useful information for preparation of ISM for drug delivery. Camellia oil and olive oil demonstrated their potency for using as external phase while NMP with its good injectability/antimicrobial activities exhibited the most suitable solvent of internal phase of ISM.

Keywords: *In situ* forming microparticles, Physicochemical, Antimicrobial activities, Injectability, Cytotoxicity

INTRODUCTION

Pharmaceutical manufacturers use solvents as the vehicle, wetting agent, and granulating fluid. The physicochemical characteristics of solvents such as density, viscosity, pH, injectability, and solubility directly influence the properties of pharmaceutical dosage forms.[1,2] The solvent affinity to the polymer could affect the injectability of the *in situ* forming systems. For a good solvent, the polymer-solvent interaction should be dominant over the polymer-polymer interaction which leads to lowering the viscosity.[1] Therefore, the study of the viscosity of the solvents used for *in situ* drug delivery system is necessary. Typically, the preparation with the applied force lower than 50 N was acceptable as injectable dosage forms.[3] The ease of injection is a critical factor together with an optimal rheological behavior of the injectable dosage forms that should be a Newtonian or pseudoplastic flow. Theoretically, the density is one of the crucial factors in stability of emulsion. The density of the internal phase and the external phase of the emulsion system should not be that different to inhibit the phase separation process.[4] In addition, the contact angle, surface tension, and interfacial tension are important parameters for an emulsion system such as an aqueous in oil emulsion of *in situ* forming microparticles (ISM). Furthermore, the wettability data are indicative in compatibility among a formula vehicle, and it is described by the value of contact angle of a liquid on a solid surface.[5] ISM is an injectable

dosage form using emulsion template with the internal phase consisting drug dissolved in polymer solution whereas the external phase consists of injectable oil with proper emulsifier, and these two phases are mixed using two syringe connectors before administration.[6] ISM exhibited beneficial manner over the *in situ* forming gel, such as minimized cytotoxicity, better reproducibility, lowered burst release and proper injectability, because the component especially drug and solvent did not directly expose the cell, and the lubricating property of the external phase.[7] Developed doxycycline hyclate (DH)-loaded bleached shellac ISM exhibited a sustainable drug release for 47 days with Fickian drug diffusion and effectively inhibited *Porphyromonas gingivalis, Streptococcus mutans,* and *Staphylococcus aureus*.[8]

Practically, the solvent-exchange induced-ISM is in liquid form before administration but when in contact with body fluid, the influx of water from environment and diffusion out of solvent leads to the phase separation of the polymer emulsion droplet into microparticles. When ISM exposed to the surrounding aqueous environment, the water miscible organic solvent exchanged with water leading to the formation of a solid/semisolid depot at the site of injection followed by sustained release of the drug. Between the time of injection and complete depot formation, the initial drug burst release occurred which can be controlled by modulating the hydrophobicity of the solvent.[9] The diffusion pattern of solute into the solvents could be assessed using the water soluble dye solution and oil soluble color. By comparison with *in situ* forming gel, the advantage of ISM includes: The decreased myotoxicity,[10] the minimized burst-free drug release patterns[11] with better injectability[12] from a lower viscosity of the emulsion due to the presence of external oil phase.

The ideal solvent for *in situ* forming systems needs to gain appropriate properties in terms of proper water affinity, low viscosity with good injectability, high ability to dissolve the active compound/polymer, and its safety with biocompatibility.[1,12] In the present study, the various solvents such as *N*-methyl pyrrolidone (NMP), 2-pyrrolidone (PYR), dimethyl sulfoxide (DMSO), triacetin, and glycofurol showed interesting qualities as the injectable solvents as the internal phase and the oils such as olive oil, camellia oil and isopropyl myristate (IPM) were selected as the oil phase of ISM. Being the amphipathic molecule, DMSO is miscible with water and organic solvents which is a very efficient solvent for water-insoluble compounds through hydrogen-bound disruption.[13] DMSO has been used in the formulation of an injection and subcutaneously implant.[14,15] LD_{50} for intravenous and subcutaneous injections of DMSO in rat is 5.3 and 12 g/kg, respectively.[16] In addition, it can be used as a polymer vehicle in *in situ* forming implant and ISM.[17] A polar disubstituted cyclic amide group present in NMP molecules is responsible for interactions with water molecules for their complete miscibility. In addition, it has the ability to act as a co-solvent due to presence of the non-polar carbons which can weaken the hydrogen-bonded structure of water.[18] It is thermally stable, biocompatible and has a high solubilizing power which can be used in the parenteral and oral medications as an acceptable solvent.[15,19] Its solubility is similar to that of ethanol and DMSO.[20] NMP shows low toxicity both orally and parenterally.[21] The LD_{50} of NMP is 3.9 g/kg in rat when given by oral route.[16] The PYR is miscible with a wide variety of other solvents with greater solubilizing power than a conventional solvent such as ethanol, glycerin, or propylene glycol due to a complexation and/or co-solvency mechanism.[22] PYR is often used as a solvent in the parenteral formulation including *in situ* forming gel system[1,22] and ISM[10,23] and was used as a plasticizer to improve mechanical properties and reduce the drug burst release of the spider silk films.[24] LD_{50} value, when tested in rat by oral route, of PYR is 6.5 g/kg.[16] The other biocompatible solvents such as triacetin and glycofurol were also investigated in this study. Triacetin, the triester of glycerol and acetylating agents, such as acetic acid and acetic anhydride,[25] have been used as a plasticizer in *in situ* forming implant systems.[17] Using triacetin as a solvent can lower a burst effect and extend the drug release of *in situ* forming biodegradable PLGA microspheres.[23] The initial burst release was reduced when triacetin was used as the co-solvent in hydrophilic polymer depot containing either NMP or DMSO as the solvents.[26] Triacetin was recognized as safe (FDA's GRAS list)[17] which is not toxic in short-term inhalation or parenteral routes.[27] Moreover, it was used as a potential parenteral nutrient.[28,29] The LD_{50} of triacetin is higher than 2 mL/kg which is acceptable for using in the injectable implant systems.[11] Glycofurol was known as a parenteral solvent[30,31] which has the ability to dissolve a wide range of water insoluble drugs.[32] It was used as a co-solvent for Eudragit and Eudragit to obtain the high viscous systems of controlled release properties,[32] as the injectable solvent of the *in situ* forming implant system[33] and also used as the vehicle for the naproxen loaded topical gel[34] and for the intranasal preparation of insulin[35] and butemide[36] to enhance drug absorption. Glycofurol is a nontoxic and nonirritant material when it is diluted with water.[37] The LD_{50} of glycofurol by intravenous injection in mice is 3.78 g/kg.[37] Olive oil has been used as an oily vehicle in the injectable solution.[38] The main composition of olive oil are the mixed triglyceride esters of oleic acid and palmitic acid.[39] IPM has been used as a vehicle for injectable solution as an alternative of a vegetable oil.[38,40] In addition, IPM showed very low irritability and no sensitizing properties in animals after topical and parenteral administration.[41] Camellia oil, obtained from *Camellia oleifera*, has several therapeutic effects and it has the comparable health benefits to olive oil and better than sunflower oil.[42] Moreover, it possesses a remarkable antioxidant activity.[43] Because of the fatty acid constituents such as palmitic, linoleic, oleic and stearic acids, this oil is important in cosmetics used for skin and hair. Chemical structure of NMP, PYR, DMSO, triacetin, glycofurol, and IPM are shown in Figure 1. The investigation on antimicrobial activities and cytotoxicity of above solvents should be considered for using them as components in ISM. Therefore, the objective of the present study is to investigate physicochemical properties of various solvents and oils used in ISM preparation.

MATERIALS AND METHODS

Materials

NMP (lot no. A0251390, Fluka, New Jersey, USA), DMSO (lot no. 453035, Fluka, Switzerland) and PYR (lot no. BCBF5715V, Fluka, Germany) were used as received. Glycofurol (T3396) was supplied by SIGMA-ALDRICH Co., St. Louis, USA. Olive

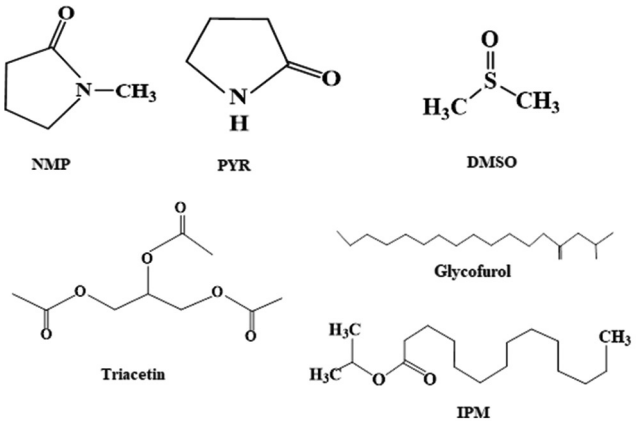

Figure 1: Chemical structure of solvents and isopropyl myristate

oil (Lot no. L5506T H1700, ConAgra Foods Inc.), IPM (batch no. 1993KNLE, PC drug centre Co.Ltd.), camellia oil (Lot no. 11-1-20123-0185, Naturel, P.K.Trading, Thailand) and triacetin (Lot no. A0375797, SIGMA-ALDRICH) were used as received. Amaranth (lot No. A0293188, ACROS organic, New Jersey, USA) was used for measurement of diffusion pattern of solvents. Agarose (Lot H7014714, Vivantis, Malaysia) was used to study the solvent diffusion. Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, 10 Australia) and sodium hydroxide (lot no. AF 310204, Ajax Finechem, Australia) were used for preparation of phosphate buffer (PBS) pH 6.8.

For antimicrobial activity of solvents, the bacterial strains such as *S. aureus* ATCC 25923, *S. aureus* DMST 6935, *S. aureus* ATCC 43300, *Klebsiella pneumoniae* ATCC 700603, *Enterococcus faecium* UCLA192, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DMST 37166, and *Stenotrophomonas maltophilia* DMST 19079 were purchased from the Department of Medical Science, Ministry of Public Health, Thailand (DMST). *P. gingivalis* was used as anaerobic bacteria in this study. For determination of cytotoxic activity of solvents, HCT116 colorectal carcinoma cell line was purchased from American Type Culture Collection (ATCC) USA. The culture medium components including DMEM, L-glutamine, penicillin streptomycin and fetal bovine serum were purchased from Invitrogen, Paisley, UK.

Measurement of Density and pH

The density of solvents was determined by pycnometer (Densito 30PX, 21 Mettler Toledo [Thailand] Ltd, PortableLab TM) (n = 3) at room temperature. The pH values of the preparations were measured with the pH meter (Seven compact, Mettler-Toledo, Thailand Ltd.) by setting the automatic read reader to room temperature and the pH values were recorded (n = 3).

Viscosity Measurement

Viscosity measurement was carried out using a Brookfield viscometer (Brookfield DV-lll ULTRA programmable rheometer, BROOKFIELD ENGINEERING LABORATORIES, INC., Middleboro, United States). Shear rate was changed slowly and then the correlation between shear rate and shear stress was recorded.

Measurement of Surface Tension and Contact Angle

The surface tension and contact angle of solvents were measured using a goniometer (FTA 1000, First Ten Angstroms, USA) at room temperature (n = 3).

Measurement of Flow Time

The determination of flow time for the various solvents were carried out using a Ubbelohde viscometer (Calibrated viscometer, model 9721-R53, Cannon) (n = 3).

Injectability Test

The injectability test was used to indicate the difficulty of injection using a texture analyzer in a compression mode (TA.XT plus, Stable Micro Systems, UK). Briefly, each solvent was inserted into a 1 mL syringe with a 27-gauge needle which was clamped with a stand. The upper probe of the texture analyzer moved downwards at a constant speed (1.0 mm/s) and a constant force of 0.1 N was applied to the base. Force displacement profiles were recorded, with the forces at a distance of 10-mm being selected for analysis (n = 3).

Measurement of Diffusion Pattern of Color and Miscibility Test

In order to observe the behavior of diffusion pattern in various solvents, the 0.3% w/w of water soluble dye (amaranth solution) and the water insoluble dye (nile red) were added into the 10 ml solvent filled glass bottle. Then, the changes of diffusion pattern in 0, 1, 2, 3, 5, 10, 15, 20, 25, 30 min were recorded. The oil soluble colors used to investigate their diffusion patterns into the various solvents and oils were green, blue and red. For miscibility study, 3 ml of each solvent dyed with 0.01% amaranth (distilled water, NMP, PYR, DMSO and glycofurol) was individually mixed with 3 ml of triacetin, IPM, olive oil and camellia oil in 10 ml test tubes. Then, the two phases were mixed by vortex mixer for 1 min and the miscibility of the two phases was examined visually. Then, each of mixture was filled into 10 ml cylinders and allowed to set for 5 min. The volume miscibilities after setting for 5 min were recorded.

Antimicrobial Study

The antimicrobial activities of five solvents were tested using a cylinder plate method. This technique is based on diffusion of the solvent from a stainless-steel cylinder (6 mm. in diameter) through agar gel inoculated with tested microorganisms. Briefly, the 0.5 McFarland turbidity of studied bacterial strain was spread on Muller Hinton agar (MHA) (Oxoid, UK). The 100 µL of tested preparation was diffused from a cylinder placed on the surface of an inoculated MHA plate and incubated at 35°C for 18–24 h based on the type of organism. The test for anaerobic bacteria was conducted using an anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA). The inhibition zone (mm.) against the tested organism around the cylinder was measured as the diameter (mm) (n = 3).

Determination of Cytotoxic Activity

HCT116 cell line was cultured, according to standard protocol, in DMEM 4.5 g/l glucose supplemented with 10% heat–inactivated fetal bovine serum, 1% glutamine and 150 U/ml penicillin, 5 μG/ml streptomycin. The HCT116 culture flask was incubated in the humidified incubator at 37°C, 5% CO_2. For MTT assay using colorectal HCT 116 cell line, three independent passages of HCT116 were seeded at a density of 10,000 cells/well in 100 μl medium in a 96-well plate. 24 h after seeding, the culture medium was changed to the 100 μl solvent containing medium at different concentrations from 0 to 200 μg/mL. The medium was used as a control group. After 72 h incubation, the viability of each well was evaluated by adding 10 μl MTT and further incubated at 37°C for 3 h. After the incubation, the medium was gently removed and 100 μL of DMSO was added into each well. After mixing, the absorbance was measured by a microplate reader at 550 nm. The toxic concentrations of solvents were determined and compared.

Statistical Analysis

The statistical significance of the obtained data was examined using one-way analysis of variance (ANOVA) followed by a least significant difference post hoc test. The significance level was set at $P < 0.05$. The analysis was performed using SPSS for Windows (version 11.5).

Statement of Human and Animal Rights

The present article does not contain any studies in related with human or animal subjects performed by any of the authors.

RESULTS AND DISCUSSION

Density

Density is one of the crucial factors related with the stability of an emulsion. The difference in density between two phases of an emulsion creates the rising up of oil. Then, the droplet of oil appears at the top and coalesces to form a separate layer of oil on the top. To prevent this phase separation, the density gradient between the external and the internal phase should not be much different.[4] The olive oil had a lower density while bleached shellac in different solvents showed a higher unique density depending on the type of solvents.[2,8] According to the experiment, IPM showed the lowest value of density because this synthetic hydrophobic compound is lipid-like material and its molecular weight is only 270.5 g/mol; thus, its density is rather low. Camellia oil and olive oil also showed the lower density values which was followed by distilled water, NMP, glycofurol, DMSO, PYR and triacetin, respectively as shown in Table 1. Thus, the emulsion containing NMP, glycofurol and DMSO as injectable solvents could be more stable than other solvents.

pH

The pH value of each solvent is shown in Table 1. Typically, the amides compound possessing coplanar structures are not basic but for NMP and PYR, their carbonyl groups and lone pair electrons of nitrogen atoms are not coplanar and the resonance effect did not occur. Therefore, the water molecules can protonate the hydrogen atoms with those electrons that lead to an increase in acidity.[44] Therefore, solvents such as NMP, PYR showed the high pH or alkalinity. DMSO also showed high pH whereas the others exhibited pH of lower than 7. Some polymers with acidic structure such as bleached shellac is practically soluble in these high alkaline solutions for fabrication into the *in situ* forming gel and microparticles.[2,8] Olive oil is mainly composed with the triglycerides and the amount of free fatty acids; therefore, it showed the lowest pH value whereas the pH of the IPM was slightly lower but higher than that of olive oil. The trend for pH of various solvents was olive oil< IPM< triacetin < camellia oil < glycofurol < distilled water< DMSO< PYR< NMP, respectively.

Viscosity

The viscous nature of the solvent could directly affect not only the viscosity of the polymer solution but also the formulation,[2,45] but also the diffusion rate and the character of the solvent exchange and the injectability of *in situ* drug delivery system. Moreover, in non-aqueous ISM emulsion, the oils are used as the external phase and the results revealed that the most viscous one was the camellia oil which was followed by olive oil as shown in Table 1. The choice of oil for the external phase is also important for the drug release property of the system because the oil could retard the immediate drug release from the system thereby the burst effect in the *in situ* forming system can be reduced. It had been reported that the oil with medium viscosity was selected for the preparation of non-aqueous/oil emulsion.[12] Glycofurol showed the highest viscosity which was followed by the triacetin while DMSO gave the lowest value. The trend of viscosity of solvents was glycofurol > triacetin > PYR > IPM > NMP > DMSO. PYR gave the highest viscosity among three solvents such as PYR, NMP, and DMSO. It may be due to the stronger interaction of PYR via H-bonding of H atom in C-H. Typically, a good solvent should have the strongest interaction with the polymer. Moreover, the replacement of some solvents by the drug could lead to the higher viscosity of the system in the drug-loaded formula.[44] The previous study reported that the lower amount of solvent in the DH-loaded *in situ* forming gel increased the viscosity of the system than the drug free formula.[8,45] DMSO and NMP have a more positive charge than N-H in PYR which is liable to interact with oxygen atom. The distilled water with the smaller molecules gave the lowest viscosity. The longer chains of large hydrocarbon molecules are easily entangled than smaller molecules with shorter chains. Typically, the tighter the molecules are linked, the more the substance will resist deformation.[46]

Flow Time

Among the various solvents, the most viscous camellia oil gave the highest flow time which was followed by the olive oil while the distilled water showed the lowest value due to its low viscosity [Table 1]. This proved that the increased viscosity retarded the flow time of the solvents. In addition, the high flow time supported the phase separation rate of the *in situ* forming emulsion composition. The most viscous solvent retarded the flow time which could also resist the separation flow from the oil in the system. The trend for the flow times were camellia oil > olive oil> IPM> glycofurol > triacetin > PYR > DMSO > NMO > distilled water.

Table 1: The physicochemical properties of various solvents and oils (Mean±SD) (n=3)

Solvent	Density (g/ml)	pH	Viscosity (cPs)	Flow time (min)	Injectability (N.m)	Contact angle (degree) at 5 s	Surface Tension (mM/m)
Glycofurol	1.0800±0.0003	6.59±0.20	17.20±0.20	91.42±3.70	17.74±0.50	16.65±0.50	41.74±3.02
NMP	1.0300±0.0004	11.87±0.10	4.88±0.20	9.17±0.03	6.11±0.30	16.84±0.90	49.68±0.30
PYR	1.1100±0.0002	11.58±0.10	13.76±0.20	65.39±1.50	14.28±0.70	21.66±1.80	49.06±1.80
Triacetin	1.1500±0.0005	4.58±0.03	15.75±0.20	72.21±0.80	13.92±0.03	19.92±1.90	37.48±0.60
DMSO	1.0900±0.0003	9.75±0.10	4.52±0.01	10.32±0.10	5.42±0.30	29.96±6.80	51.63±1.10
Distilled water	0.9900±0.0001	6.70±0.04	1.57±0.03	5.23±0.03	3.07±0.20	44.79±1.60	72.62±0.70
Olive oil	0.9100±0.0001	2.36±0.10	69.98±0.20	375.64±5.13	84.03±3.02	21.74±0.80	32.81±0.20
IPM	0.8500±0.0001	3.58±0.02	8.47±0.01	244.9±6.13	76.26±5.40	8.97±1.30	30.73±0.90
Camellia oil	0.9110±0.0002	5.822±0.03	70.403±5.90	387.33±2.52	64.944±7.80	35.68±0.49	21.67±1.60

Injectability

For an effective and pharmaceutically acceptable injectable formulation, the administration of the *in situ* forming system with a proper needle and syringe should be easily and rapidly carried out without pain.[47,48] The determination of injectability indicates the force required to expel the product.[8] All the solvents, except the olive oil, IPM and camellia oil, were acceptable as injectable products because the forces were lower than 50 N.[12] Due to the most viscous nature of the oil, the work force required for expulsions was apparently high indicating lower injectability while the distilled water with the lowest viscosity showed the lowest work force which was followed by DMSO, NMP, triacetin, PYR and glycofurol, respectively [Table 1]. The amount of polymer used in the *in situ* forming system could affect the injectability of the solvent and eventually on the final formula as the increased polymer content increased the work force to expel indicating a lower injectability.[49] However, the injectability is facilitated by the solvent affinity to the polymer. For a good solvent, the polymer-solvent interaction should be dominant over the polymer-polymer thus lowering the viscosity.[1,2]

Contact Angle and Surface Tension

Typically, the lower the contact angle and the surface tension lead to better wettability.[4] However, the contact angle between liquids decreased with time. The distilled water exhibited the highest contact angle due to the high surface tension whereas the lowest surface energy at air/liquid of IPM gave the lowest contact angle [Figure 2]. In addition, the solvents with higher viscosity such as olive oil and PYR increased the contact angle indicating the decreased wettability which in turn reduced the spreading rate. Although the surface tension of NMP was high, the lower viscosity behavior of NMP possessed the lower contact angle. The higher contact angle of DMSO with its high surface tension contributed to the difficulty in wettability of this solvent. DMSO exhibits dipole-dipole molecular interaction,[13,16] this unique characteristic might disturb the force between them and surface of glass slide; thus, there was high variation value of contact angle as shown in Figure 2.

The surface tension of water was high due to the strong hydrogen bond formation of water molecules,[4,5] whereas the surface tension of three solvents such as NMP, PYR and DMSO showed to be comparatively low which ensured the weaker molecular interaction between molecules than that of water. The DMSO showed the lowest surface tension. Due to the low surface tension of DMSO, it would minimize the free energy thereafter the stable ISM system prepared with this solvent could be obtained.

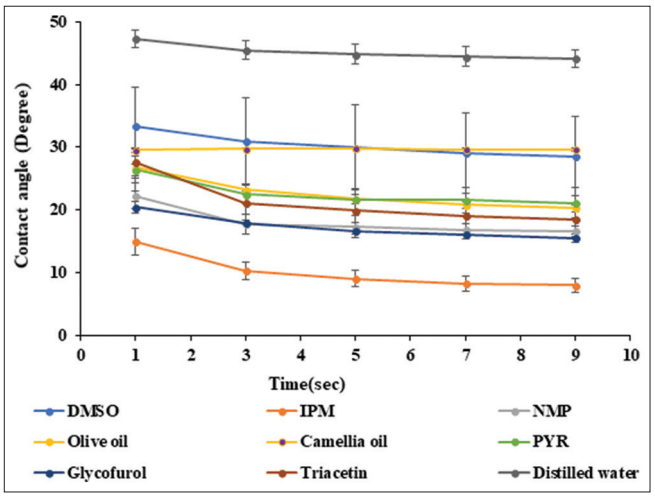

Figure 2: Contact angle for various solvents and oils

Measurement of Color Diffusion Pattern and Solvent/Oil Miscibility

Typically, the movement of molecules for the diffusion process occurred from the higher concentration to the lower concentration area. The water soluble dye, amaranth, freely diffused into the distilled water with Brownian motion [Figure 3a]. The diffusion of the coloring agent happened till it spread out evenly throughout the distilled water until uniform colored water was obtained. The amaranth also diffused in DMSO in the same manner. Another factor of diffusion of dye was the density and viscosity of the diffusion medium. Thus, having the lowest viscosity of distilled water and DMSO among different solvents, amaranth diffused and dispersed easily into these solvents. The high viscosity dissipation of PYR and triacetin slowed the diffusion of the amaranth. Because

glycofurol exhibited the higher viscosity, the differences in the density gradient caused the amaranth solution to be precipitated while the amaranth solution was immiscible with the oils.

The nile red, a lipophilic stain, gives a fluorescent manner depending on solvent polarity. Nile red is almost non-fluorescent in water and also in most polar solvents. However, in a lipid-rich environment, it becomes fluorescent with varying shades from deep red to a strong yellow-gold emission. Therefore, the color of nile red in distilled water disappeared [Figure 3b]. In addition, owing to the lipophilic nature of the nile red it did not diffuse in the polar solvents such as DMSO, NMP, PYR and glycofurol. The nile red solution in IPM, olive oil and camellia oil gave a yellow color because of the lipid nature but it could not diffuse into the olive oil and camellia oil while the slight diffusion occurred in IPM because the viscosity of the olive oil and camellia oil was higher than that of the IPM. The oil soluble colors did not diffuse but floated on the surface of the distilled water [Figure 4]. All the oil soluble colors apparently diffused into DMSO greater than that in NMP. However, the little diffusion of dyes in PYR was due to the higher viscous nature of PYR. Although the oil soluble colors were used in this study, the high viscosity of the oil retarded the diffusion of the oil soluble colors. Nevertheless, the three oil soluble colors diffused freely into all solvents and oil except distilled water after mixing with a vortex mixer [Figure 5]. Due to the apparently high viscosity nature among tested solvents and oils, the camellia and olive oils took about 20 min to obtain a complete diffusion.

The type of solvent used in the emulsion should have an appreciable polarity to make it immiscible with oil. Thus, the miscibility and phase separation behavior of solvents such as DMSO, NMP, PYR, glycofurol and water with the oil phases such as the olive oil, camellia oil, IPM and triacetin were investigated. In general, a polar solvent dissolves or mixes in other polar components to comply with the "like dissolves like" rule. Polar solvents can form a single phase mixture with other polar substances whereas no interaction will be formed between polar and non-polar compounds; thus, there is a clearly defined surface that appears between these two immiscible phases. The density gradient, the polar/non-polar interaction, solubility, miscibility are the critical factors for the phase separation and miscibility. The miscibility and phase separation behavior of the solvents with the oil phase were studied. The experiment showed that all the solvents except distilled water mixed well with the triacetin and no separation occurred. The more similar the densities between the two phases were, the longer the time it took for phase separation. When the density gradient between triacetin and solvents was not much different, the volume of separation showed zero to longer than 12 h. It has been reported that the water affinity of solvents was reduced by water-immiscible or partially water

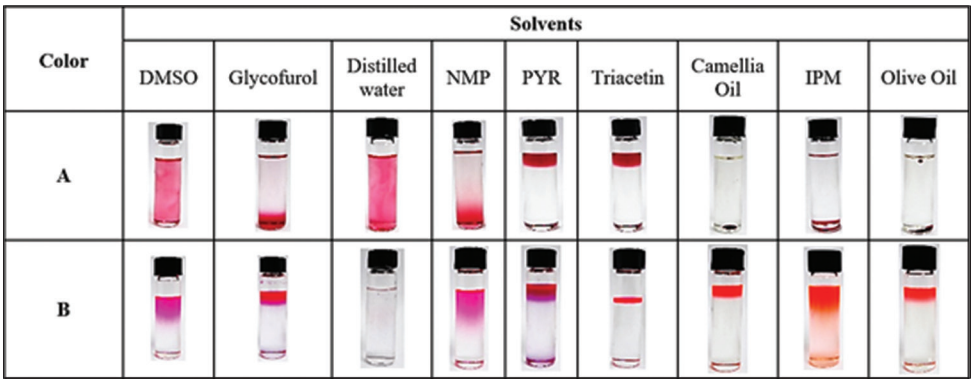

Figure 3: Diffusion pattern of 0.3%w/w amaranth solution (a) and 0.3%w/w nile red solution (b)

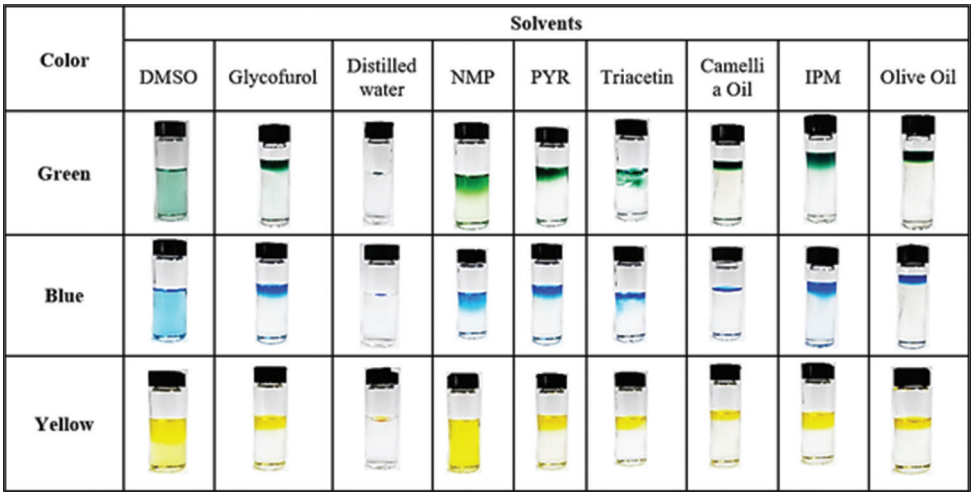

Figure 4: Diffusion pattern of oil soluble dyes in different solvents and oil after 24 h

miscible components.[50] Thus, the phase separation occurred between triacetin and distilled water. Because the density of distilled water was lower than the triacetin, the distilled water layer appeared at the upper part. When polar and non-polar compounds are mixed, they tend to separate with minimal surface area between them. The solvents and oils (camellia oil, olive oil, IPM) exhibited the phase separation which oil appeared at the upper layer. Significantly, when the oils were mixed with glycofurol, the olive oil and camellia oil layer were slightly cloudy at first, whereas the clear separation of the olive oil phase was obtained after 12 h.

Antimicrobial Study

The inhibition zone of tested solvents against different microbes is shown in Table 2. *S. aureous* could be isolated form periodontitis,[51] and it has the ability to form a biofilm which can lead to antibacterial drug resistance.[52,53] In addition, *E. coli* is the microorganism which was found in patients with periodontitis.[54] It has been reported that *S. aureus* and *P aeruginosa* occurred in high numbers within the buccal/gingival crevice cells from periodontitis patients.[55] Although *E. faecium* is rarely found in a healthy human oral cavity, they have emerged as an increasingly important cause of nosocomial infections.[56] Moreover, it has been reported that refractory periodontitis could be provoked with an infection with *Candida albicans*.[57] Thus, these standard microbes were used in this study. NMP and PYR exhibited outstanding antibacterial activities against all bacterial strains [Table 2]. Because NMP and PYR are the dipole aprotic, solvents could dissolve many substances including polymers and drugs as well as they can solubilize the lipids in microbial cell membranes and consequently enhance the drug penetration.[18,45,58] Thus, NMP as well as PYR showed a concordant potency based on the similar clear zone on tested microorganisms. The aqueous

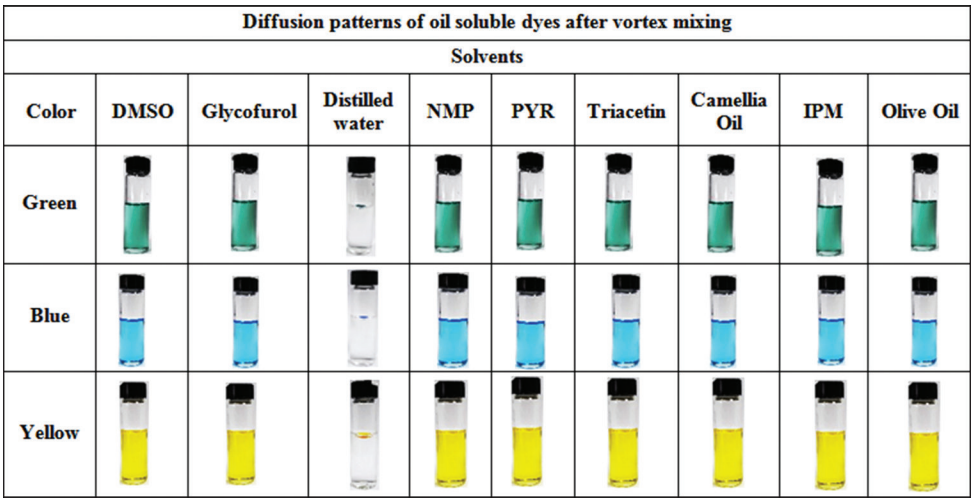

Figure 5: Diffusion pattern of oil soluble colors in different solvents and oil after vortex mixing

Table 2: Inhibition zone diameter (mm.) of tested formulations against different microbes (n=3)

Microbes	Clear zone diameter (mean±S.D.) (mm)				
	Glycofurol	NMP	PYR	Triacetin	DMSO
Gram positive bacteria					
Staphylococcus aureus ATCC 25923	13.33±0.94	20.67±1.15	21.33±1.15	7.00±1.00	-
Staphylococcus aureus DMST 6935	12.67±0.47	14.33±0.58	12.33±0.58	7.00±0.00	-
Staphylococcus aureus ATCC 43300	11.50±0.50	17.20±0.30	21.00±1.00	-	-
Enterococci faecium 192	15.33±0.47	15.33±0.58	18.33±0.58	-	-
Gram negative bacteria					
Escherichia coli ATCC 25922	15.00±0.00	18.33±0.58	18.67±1.15	7.00±0.00	-
Escherichia coli	13.00±1.00	20.00±0.50	20.00±0.00	-	13.20±1.26
Klebsiella pneumoniae ATCC 700063	14.33±0.94	17.33±1.15	18.00±0.00	-	-
Pseudomonas aeruginosa DMST 37166	8.67±0.47	20.33±0.58	20.33±0.58	12.00±1.00	-
Stenotrophomonas malttophilia ATCC 19079	18.67±0.94	23.33±1.15	25.33±1.15	19.33±1.15	-
Porphyromonas gingivalis ATCC 33277	12.00±1.00	20.33±0.58	22.00±1.00	-	-
Yeast					
Candida albicans	19.3±0.60	30.70±1.80	32.20±2.80	11.70±0.60	17.80±1.80

-=No clear zone

NMP-loaded lutrol® theremosensitive gel inhibited *E. coli* and *C. albicans* with the dose-dependent manner.[58-60] Glycofural, NMP and PYR inhibited a methicillin-resistant *S. aureus* (MRSA) such as *S. aureus* ATCC 43300 in this study. PYR inhibited this MRSA strain more effectively than NMP and glycofural, respectively. Thus, these solvents were unique for using as the solvent for the ISM for delivery of antimicrobial active compound for treatment of infectious diseases because they could promote the efficacy of antimicrobial activities. DMSO did not show antibacterial activities except against *E. coli* and *C. albicans*. DMSO exhibited a high permeability through the cell membrane of *E. coli* and *C. albicans*.[61] All the solvents inhibited the larger inhibition zones against *C. albicans* than bacteria. Glycofurol showed the smaller zone diameter against the test microorganisms than NMP and PYR whereas triacetin showed the smallest zone diameter. However, triacetin showed more inhibitory effect on *P. aeruginosa* DMST 37166 and *Stenotrophomonas malttophilia* ATCC 19079 than glycofurol. The smaller zone diameter of triacetin and glycofurol was owing to the viscous nature of these solvents that retarded their diffusion into the medium.

Determination of Cytotoxic Activity of Solvents

Typically, the cell culture systems are used to assess the bioactivity of compounds whereas the organic solvent is used to dissolve these compounds.[62,63] The cytotoxic effect of solvents was investigated against colorectal HCT116 cells [Figure 6]. The different concentrations (0.8–200 µg/ml) of the individual solvents exhibited the inhibitory effect on cell growth in HCT 116 cells with concentration-dependent fashion whereas the effect of cytotoxicity increased with dose dependently. It has been reported that the cytotoxic activity of the solvents are dose dependent.[61] The trend for viability of cells was glycofurol < triacetin < NMP < PYR < DMSO. The previous study stated that DMSO, at concentrations <0.5% (v/v) was a compatible solvent vehicle toward the examined cells.[64] The 80% viability were observed at 0.8 µg/ml for glycofurol and triacetin, 1.6 µg/ml for NMP, 3.1 µg/ml for PYR and 25 µg/ml for DMSO, respectively. Therefore, glycofurol showed the highest cytotoxic activity among solvents.

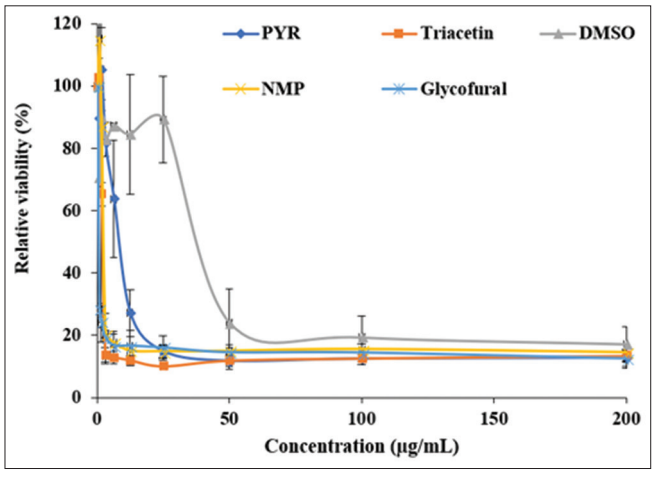

Figure 6: Viability percentage of colorectal HCT 116 cells exposed to different solvents

DMSO displayed the least cytotoxicity against HCT 116 cell line which was consistent with Q3C solvent classification in which DMSO was claimed to be in the safest category, class 3 solvents.[62] DMSO showed the lowest cytotoxicity among the tested solvents which was followed by PYR, NMP, triacetin and glycofurol, respectively.

CONCLUSION

The physicochemical properties such as pH, density, viscosities, surface tension and contact angle measurements provide useful information of various solvents and oils used in ISM. Moreover, the water-soluble dye could dissolve in DMSO which this diffusion behavior proved that DMSO can be used as the solvent of choice. Furthermore, the low viscosity of the DMSO provided the ease of injectability. Moreover, DMSO showed its lowest cytotoxic activity against colorectal HCT 116 cell line. In addition, the appropriate density, viscosity, and surface tension data proved that the olive oil and camellia oil exhibited the potential use as the external phase in emulsification while the low viscous NMP with its good injectability/antimicrobial activities exhibited the most suitable solvent of the internal phase of ISM.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this research.

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