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Poloxamer and Chitosan-based *In Situ* Gels Loaded with Orthosiphon Stamineus Extracts Containing Rosmarinic Acid for the Treatment of Ocular Infections

Short title: *In Situ* Gels for Ocular Infections

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ABSTRACT
INTRODUCTION: Orthosiphon stamineus (OS) is a commonly used medicinal plant to curb bacterial infections globally. The objective of this work was to fabricate poloxamer and chitosan-based in situ gels loaded with standardized aqueous-ethanolic OS leaf extracts and investigate their antimicrobial efficacy as a potential remedy against ocular infections.
METHODS: In situ gels containing 0.5% w/v OS extract prepared using cold dispersion method were subjected to physicochemical characterization including in vitro release studies. Antimicrobial efficacy was tested against S. aureus, M. luteus, E. coli and P. aeruginosa using agar diffusion method.
RESULTS: TLC and HPLC chromatograms confirmed the presence of rosmarinic acid (RA) and sinensitin in OS extract with same Rf (0.26 and 0.49) and retention times (12.2 and 20.7 min) against reference standards. A homogenous brown coloured in situ gels exhibited low viscosity as a solution and increased viscosity in gel form at ocular temperature. The optimized formulations, P7 (21% P407/4% P188), P8 (21% P407/5% P188) and F5 (1.5% chitosan and 45% β-glycerophosphate) exhibited ideal ocular pH (7.27-7.46), phase transition

at ocular temperature (33-37ºC) and prolonged RA release up to 12 h. Formulation F5 demonstrated an inhibition zone of 4.3 mm against M. luteus.
DISCUSSION AND CONCLUSION: Among all, formulation F5 alone exhibited modest antimicrobial activity against M. luteus. OS extracts at 5 and 10% were most active against tested bacteria however, loading them into in situ gels resulted in sedimentation. Hence, isolation of RA from OS extract is suggested prior to loading into formulations for a better antimicrobial activity.
Keywords: Chitosan, in situ gels, ocular infections, orthosiphon stamineus, poloxamer

1. INTRODUCTION

Ocular infections such as conjunctivitis, keratitis, endophthalmitis, blepharitis and orbital cellulitis if left untreated damage the structures of the eye which may possibly lead to visual impairments and blindness.[1,2] Conventional ocular dosage forms such as eye drops therapy requires frequent administration, has limited duration of action and encounters rapid drainage from the eyes leading to poor ocular drug bioavailability (<5%).[3] To overcome these limitations, *in situ* gels can be a potential alternative to eye drops as they exist as free-flowing liquid before instillation into the eyes and transform into semi-solid gel upon exposure to ocular conditions such as pH, temperature or ionic concentration.[4] Although numerous *in situ* gelling systems have been developed, thermosensitive *in situ* gels are attracting popularity because of their swift response to a change in the temperature of surrounding environment. Thermosensitive *in situ* gels exist as a solution at room temperature (25ºC) but transform at ocular temperature (33-37 ºC) into gel.[5,6]

Several polymers have been employed in the development of *in situ* gelling system intended for ocular delivery. However, being biocompatible, biodegradable, non-toxic and bio-adhesive, chitosan is an ideal polymer.[3,7] Nevertheless, because of pH-dependent the activity of chitosan it would not render a thermosensitive *in situ* gel.[8] However, complexation of chitosan with β-glycerophosphate (β-GP) which is a polyol salt could yield a thermosensitive *in situ* gel system.[9,10] Apart from chitosan, poloxamers (Pluronics®) are also employed in the preparation of thermosensitive *in situ* gels. Poloxamers are tri-block copolymer exhibiting amphiphilic nature attributed to hydrophilic polyethylene oxide (PEO) attached to central hydrophobic polypropylene oxide (PPO).[11] Poloxamers are available in various grades owing to different weight ratio of PEO and PPO.[3] Among them, poloxamer 188 (P188) and poloxamer 407 (P407) grades are equally effective due to safety in the ophthalmic region and clarity of their aqueous solution.[11] Hence, these grades were selected in the present research to prepare thermosensitive *in situ* gels.

Despite the significant role of antibiotics and other antimicrobial agents in the treatment of infectious diseases, side effects as well as emerging resistance have compromised their efficacy.[12,13] Hence, this situation has called for urgent action to turn the search lights on the development of naturally derived novel bioactive antimicrobial compounds from plants to fight against new and re-emerging infectious diseases with greater efficacy, lower toxicity, and lower resistance. *Orthosiphon stamineus* (OS), also known as 'Misai Kucing', is a popular plant (Family: Lamiaceae) in the Southeast Asian and tropical countries including Malaysia for curbing various ailments such as diabetes, inflammations, abdominal pain, oedema, and gout.[14] OS has been reported to be rich in phenolic bioactive compounds namely rosmarinic acid (RA), sinensetin (SIN) and eupatorine, which present profoundly in the leaves of OS.[15] OS extract loaded into ethosomal formulation using sophorolipid as a surfactant has exhibited anti-angiogenic and anti-melanoma effects in albino mice after topical application.[16] Hitherto, no published reports are available on the loading of OS extract into *in situ* gel

formulation to evaluate the antimicrobial profile. Hence, this hypothesis-driven preliminary study was undertaken with an objective to investigate the antimicrobial efficacy of OS extract loaded thermosensitive *in situ* gelling systems against the selected pathogenic microorganisms responsible for various ocular infections.

2. MATERIALS AND METHODS

Experimental part

2.1. Materials

Dried OS leaves were obtained from Ethno Resources, Malaysia. The standards of RA and SIN were purchased from Chemfaces, China. Chitosan, β-GP disodium salt, poloxamers and solvents including ethanol, methanol, acetonitrile, lactic acid, toluene, ethyl acetate, formic acid, benzalkonium chloride were purchased from Sigma-Aldrich, USA. Sodium chloride was purchased from Friendemann Schmidt, Australia. Mueller-Hinton agar was procured from Oxoid, Hampshire, UK. All reagents and chemicals were utilised as received.

2.2. Preparation of aqueous-ethanolic OS leaf extract

An aliquot (50 g) of dried OS leaves was blended (Waring 800S, USA) followed by maceration in 500 mL of ethanol-water (50:50) solvent (EWS) followed by filtration performed by a vacuum pump (Rocker 300, Malaysia). The evaporation of the filtrate was conducted using a rotary evaporator (Heidoph-36001270, Heidolph^{TM}, Germany) followed by lyophilization using a freeze dryer (Scanvac CoolSafe 9L, Denmark) for 48 h. The crude-dried extract was stored in desiccator at an ambient room temperature until further use.

2.3. Thin-layer chromatography

Extract of OS was dissolved in EWS to prepare standard solutions of 1, 5 and 10 mg/mL whilst the reference standards were RA and SIN standards which were dissolved in methanol (HPLC grade) to prepare a solution of 1 mg/mL. The stationary phase was thin-layer chromatography (TLC) plates (20 cm x 10 cm) pre-coated with silica gel 60 F254 (E. Merck, Germany) whereas the mobile phase was comprised of ethyl acetate: toluene: formic acid at a ratio of 7:3:0.1, respectively.[17] Following the air-drying of the TLC plate, images were taken under UV light at 254 nm and 366 nm. The band separation was observed followed by the measurement of the Rf (retention factor) values of the compounds using the formula as given below.[17]

$$Rf = \frac{\textbf{Distance travelled by compound}}{\textbf{Distance travelled by solvent}} \quad \text{(Eq. 1)}$$

2.4. Fingerprinting and quantification of aqueous-ethanolic OS extract using HPLC

The test samples were prepared by dissolving 5 mg freeze-dried OS extract in 1 mL of EWS followed by dilution to 1 mg/mL. Standard solutions of RA and SIN at a strength of 0.5 mg/mL were prepared using methanol (HPLC grade) followed by serial dilutions to prepare a concentration range between 12.5 and 500 µg/mL. An injection volume of 20 µL was introduced into C18 column (5 µm, 4.6 mm x 250 mm) in triplicate. The gradient mobile phase of 0.1% formic acid solution and acetonitrile (Table 1) was used at flow rate of 1.5 mL/min.[18] The analysis was performed using HPLC (Agilent Technologies 1200, USA) at a

detection wavelength of 320 nm. The identification of RA and SIN in OS extracts was performed by comparing retention times with reference standards. The quantification was done using the peak area of chromatograms obtained.

2.5. Preparation of OS extract loaded poloxamer and chitosan-based in situ gels

Cold dispersion method was employed to prepare poloxamer-based *in situ* gels. In this method, accurately weighed poloxamer grades P188 and P407 were dissolved in phosphate buffer solution (PBS, pH 7.4) using magnetic stirrer at 300 rpm for 1 h followed by overnight refrigeration to allow complete hydration at 4°C (Table 2). OS extract along with sodium chloride and benzalkonium chloride was incorporated into hydrated formulation with constant stirring until a homogenous solution was seen. Chitosan-based *in situ* gels were fabricated by adding chitosan polymer (1.5% w/v) in lactic acid (1.5% v/v) with constant stirring at 300 rpm to obtain a homogenous solution (Table 2). β-GP solutions (10-50% w/w) were formed using (PBS, pH 6.8) followed by an overnight storage at 4° C overnight to get a clear solution. The OS extract along with sodium chloride and benzalkonium chloride was incorporated into β-GP solution followed by constant stirring until a homogenous solution is obtained. This solution was incorporated dropwise into chitosan solution with constant stirring until a homogenous solution was obtained. All prepared formulations were subjected to storage at 4°C till required for use.

2.6. Physicochemical characterization

Physicochemical characterization of the prepared *in situ* gels was performed in terms of appearance, pH, sol-to-gel transition temperature and viscosity.[3] The appearance of the *in situ* gels was observed against background (black and white) while the pH was determined using a pH meter (EUTECH Instrument, USA). To determine the temperature of sol-to-gel transition, solution form of *in situ* gel was transferred into an empty vial followed by immersion into a water bath adjusted initially at a temperature of 25ºC followed by gradual increase up to 50ºC. The vial was overturned frequently to observe the gel formation and the temperature of sol-to-gel transition was recorded once the solution converted into a complete gel with no flow upon wobbling of the vial. The viscosity of the *in situ* gels formulations before and after gelation was determined using a rheometer (Anton Paar, Stokholm, Sweden) with spindle type CC17 at a speed between10 and 100 rpm with a shear rate of 0-100 mm/s. To determine the suitable sterilization technique for the sterility of prepared *in situ* gels (P7 and F5 formulations), two sterilization methods namely sterilization using an autoclave at 121ºC for 15 min (Tomy SX-500, Japan) and sterilization using 0.20 µm sterile regenerated cellulose syringe filters (Sartorius, Germany) under aseptic conditions were employed.

2.7. In vitro release of RA

The *In vitro* release of RA was performed using dialysis membrane technique reported in our earlier publications with suitable modifications.[3,19] The first ever drawn schematic representation of dialysis membrane method which illustrates the *in vitro* RA release measurement procedure via a step-by-step manner is shown in Figure 1. Dialysis membrane with a molecular weight cut-off (MWCO) of 3500 Da (Fisher Scientific, New Hampshire, USA) was soaked in the dissolution medium of simulated tear fluid (STF, pH 6.8) overnight. About 1 mL formulation was transferred into the dialysis bag which was subsequently placed into a beaker filled with a 50 mL STF, pH 6.8 and placed in shaking water bath maintained at 37ºC and 50 rpm. A fixed volume of 1 mL sample was taken at predetermined time intervals between 0.5 and 12 h followed by replacement of with equal amount of fresh STF to preserve the sink condition. RA released from *in situ* gels was analysed at a wavelength of 320 nm

using UV-Visible spectrophotometer (GENESYS™ 10, Thermo Scientific, USA). Percentage cumulative RA released versus time was plotted the drug release profile was characterized by estimating the time taken to release 50% of RA ($t_{50\%}$).[20]

2.8. Antimicrobial efficacy of OS extract loaded in situ gels

The antimicrobial efficacy of standardized aqueous-ethanolic OS leaf extracts loaded *in situ* gels against Gram-negative bacteria (*E. coli*, ATCC 10798 and *P. aeruginosa*, ATCC 9721) and Gram-positive bacteria (*M. luteus*, ATCC 49732 and *S. aureus*, ATCC 6538) was determined using the agar well diffusion method. Autoclaved Mueller-Hinton agar (Oxoid, Hampshire, UK) was transferred into sterile petri dishes followed by inoculation using 100 µL bacterial broth culture (0.5 McFarland standard). Agar wells (6 mm in diameter) were prepared using the tip of sterile Pasteur pipette followed by filling with 70 µL tested OS extracts (0.5% to 10%) and OS extracts loaded *in situ* gel formulations, respectively. RA (1 mg/mL). Xepanicol®, 0.5% (chloramphenicol) and Ciplox®, 0.3% (ciprofloxacin) eye drops were employed as a positive control. The incubation of petri dishes was performed for 24 h at 37°C followed by the measurement of the resultant zones of inhibition (ZOI).

2.9. Statistical analysis

The results were evaluated statistically using One-way Multivariate Analysis of Variance (ANOVA) followed by post hoc Tukey's test (GraphPad Prism version 6.01, GraphPad Software, La Jolla, California). The value $p<0.05$ was considered as statistically significant difference.

3. RESULTS

3.1. TLC and HPLC analysis of OS EWS extract

OS EWS extract exhibited a percentage yield of 14.5% (*w/w*) on dry weight basis. TLC chromatograms demonstrated that both OS extract and reference standards (RA and SIN) exhibited same Rf values at 0.26 and 0.49, respectively when viewed under UV light at wavelengths of 254 nm (Figure 2a) and 366 nm (Figure 2b) confirming the presence of bioactive compounds RA and SIN. The HPLC chromatograms of reference standards (RA and SIN) and aqueous-ethanolic OS leaf extracts were shown in Figure 3. Both RA and SIN were eluted at the same retention times as of reference standards, RA (12.2 min) and SIN (20.7 min). A relatively higher content of RA (11.50 ± 0.23% w/w) was found in aqueous-ethanolic OS leaves of 5 mg/mL compared to SIN (0.88 ± 0.02% w/w). However, SIN was not quantifiable in aqueous-ethanolic OS extract of 1mg/mL whereas the content of RA was measured as 11.37 ± 0.76% w/w.

3.2. Physicochemical characterization of in situ gel formulations

Except for formulation P3 prepared using 21% poloxamer 407, all *in situ* gel formulations were found to be clear, homogenous and free flowing solution with brown colour suggesting easy instillation into the eyes at an ambient room temperature (Table 3). The pH of poloxamer based *in situ* gels was in a range between 7.21 and 7.32. The sol-to-gel phase transition temperature of *in situ* gel formulations is depicted in Figure 4. *In situ* gels comprising merely of P407 at 21% w/v content exhibited highest viscosity of 2440 mPas at room temperature. Nevertheless, the introduction of P188 (1-5%) at 21% P407 caused a gradual reduction in the viscosity from 166 to 116 mPas before gelation and increased viscosities from 337 to 5750

mPas after the gelation (Table 3). Chitosan based formulations demonstrated a substantial reduction in the viscosity from 25.9 to 11.5 mPas before gelation and an increased viscosity after gelation from 211 to 111 mPas with an increased β-GP concentration from 30 to 50% (F3-F6). The two sterilization methods employed did not affect the appearance, flow property, sol-to-gel temperature and viscosity results, however compared with autoclave sterilization, syringe filter sterilization demonstrated a small reduction of 0.1 units in pH value. In addition, the sterilized P7 and F5 *in situ* gel formulations exhibited an increased RA content (47.67% and 45.13%) and reduced RA content (6.23% and 5.39%) following the sterilization using autoclave and filtration techniques, respectively in comparison to the freshly prepared *in situ* gels.

3.3. In vitro release study of RA

Based on the physicochemical characterization especially sol-to-gel temperature findings, F5, P7 and P8 formulations were preferred for *in vitro* RA release and antimicrobial efficacy investigations. The *in vitro* release of RA from chitosan-β-GP and poloxamer-based *in situ* gel formulations are depicted in Figure 5. A substantial burst release of 35%, 32.81% and 28.73% within the first 60 min and thereafter a gradual and complete RA release in a sustained manner for a period of 12 h was observed from F5, P7 and P8 formulations, respectively.

3.4. Antimicrobial efficacy of standardized aqueous-ethanolic OS leaf extracts loaded in situ gel formulations

Table 4 and Figure 6 show the ZOI of the tested samples against the Gram-positive and negative bacteria. The formulation F5 loaded with 0.5% w/v OS extract exhibited modest antimicrobial efficacy against Gram-positive *M. luteus* (ZOI = 4.3 mm). It was, however, inactive against *S. aureus* and *P. aeruginosa*. To further test the antimicrobial efficacy of OS extract, the loading concentration of OS extract in P7 and P8 formulations was increased from 0.5% to 1% w/v. However, both formulations were also found to be inactive against all tested bacteria (ZOI = 0.3 mm against *S. aureus*). Subsequent tests which involved the use of OS leaf extracts at 0.5%-10% (i.e., E0.5%-10%) yielded concentration-dependent antimicrobial activity against all tested bacteria except *P. aeruginosa*. The extracts, especially E5% and E10%, were most active against *M. luteus* (highest ZOI = 16.0 mm), followed by *S. aureus* (highest ZOI = 9.3 mm) and *E. coli* (highest ZOI = 3.3 mm). As expected, reference products (i.e., positive controls), chloramphenicol (Xepanicol®, 0.5%) and ciprofloxacin (Ciplox®, 0.3%) elicited very active antimicrobial efficacy against all the tested bacteria (ZOI $\geq$ 20.3 mm). The standard RA, on the other hand, produced modest antimicrobial activity when tested against both Gram-positive and negative bacteria at 1 mg/mL (ZOI $\leq$ 4.7 mm).

4. DISCUSSION

The extraction of OS leaves was successfully with a percentage yield of approximately 14.5% w/w on dry weight basis which was consistent with an earlier reported study.[21] The TLC analysis of OS extract exhibited robust band separation of the intended bioactive compounds namely RA and SIN and these results are in agreement with previous study. [17] Also, considerable amount of RA (11.5% w/w) was quantified by HPLC analysis of OS extract at 5 mg/mL which is primarily accountable for the antimicrobial activity of OS extract.[14] The use of water and ethanol binary mixture at 50:50 ratio as a solvent has been reported to extract high content of phenolic compounds including RA.[22]

Apart from formulation F1 (0% β-GP), chitosan and β-GP based *in situ* gel formulations homogenous, clear and with pH values within a range (6.8-7.4) ideal for the ophthalmic tissues depicting that *in situ* gels were non-irritating to eye.[23] All chitosan-based *in situ* gel formulations exhibited low viscosity at 25ºC pivotal for their existence in the solution form to ease the expulsion from the eye drop container.[3] Increasing the β-GP concentration from 10% to 50% w/v led to a further reduction in the viscosity ($p<0.05$) which could be ascribed to the attachment of amine group of chitosan with phosphate group of the β-GP.[24] The sol-to-gel transition temperature of these formulations dropped significantly to 32.4°C from 38.7°C upon increasing the β-GP concentrations from 30 to 50% w/w. In the absence of β-GP, chitosan did not exhibit sol-to-gel phase transition (Formulation F0) indicating the thermal insensitivity of chitosan to the surrounding temperature. Interestingly, only F5 (1.5% chitosan and 45% β-GP w/v) transformed into gel at the temperature range between 33 and 37°C which might be attributable to the enhancement in hydrogen bonding among chitosan chains upon a rise in temperature rise leading to gelation.[25] This gelation caused an enhancement in viscosity to 120 mPas from 12.3 mPas before gelation which aided in increasing the reside time of formulation at pre-corneal surface [19]. Based on the ideal characteristics of *in situ* gels, formulation F5 was selected for further evaluation in terms of *in vitro* release and the antimicrobial activity.

Except for P3 (P407 at 21% w/v), poloxamer-based *in situ* gels were clear, homogenous as well as free-slowing at 25ºC, whilst P3 formed viscous gel even at room temperature (Table 3). This was not surprising as it was reported that P407 would form a gel at an ambient room temperature at a content above 20% w/v sol-to-gel and gelation phenomenon is reversible.[26,27] The pH of all poloxamer-based formulations was within a limit pivotal for ocular surface and suitable for ocular application without any irritation.[28] The viscosity of poloxamer-based formulations enhanced significantly by increasing the P407 concentration to 21% w/v. Apart from P5, a significant increase in the viscosity of *in situ* gels from 337 to 5750 mPas after gelation was observed when compared to before gelation at room temperature ($p<0.05$). These findings were in agreement with a previous report stating that the size and number of micelles within gel increase with a rise in the polymer concentration which thereafter leads to enhanced cross-link between micelles by shortening the inter-micellar distance, resulting in the formation of three-dimensional gel structure with higher viscosity.[29] Among all, P7 and P8 formulations demonstrated the highest viscosities upon transition into a gel. An optimum *in situ* gel must exhibit sol-gel transition at a temperature above 25°C but below the temperature of eye surface (33-37°C). Nonetheless, increasing the concentration of P407 from 18% w/v to 21% w/v, led to the incapability to undergo gelation until 50°C and formulation P3 was formed gel-like structure at room temperature. This is attributed to the formation of additional micelles at P407 concentration above its critical micelle concentration which caused a reduction in energy required for endothermic micellar crystallisation and consequently sol-gel temperature was decreased.[30] Nonetheless, an addition of P188 at a content between 1-5% w/w enhanced the sol-to-gel transition temperature for the subsequent formulations (P4- P9) to 34.9°C from 27.9°C. This is ascribable to the disruption in the formation of P407 micelles upon incorporation of hydrophilic P188 leading to accumulation of excessive water in the proximity of hydrophobic PPO units of P407. This could have increased the required energy to initiate hydrophobic interactions among P407 micelles and consequently increased the temperature of sol-gel transition.[26] Only P7 and P8 formulations demonstrated optimum pH and phase transition at ocular temperature and hence were chosen for further studies.

Autoclave-sterilization dramatically increased the content of phenolic compound, RA to almost double with respect to the freshly prepared and unsterilized *in situ* gel formulations. This could be ascribed to the heat employed during the autoclave process (121°C) which promoted the release of phenolic compounds due to the breakdown of cell walls and cellular components. The dissociation of conjugated polyphenols, such as tannins to simpler phenolic compounds due to the thermal process could have also contributed to the increased amount of RA.[31] The attained results are in similar agreement with the previously reported literature on the sterilization effect on the bioactive compounds present in the natural plant extracts.[32,33] The sterilization of *in situ* gel formulations by filtration technique demonstrated a small decrease in the RA content which might be due to the sorption where the substance is adsorbed onto the filter at the solid-liquid interface and removes the active constituent from the formulation.[34] Besides, the filtration of *in situ* gel formulations through 0.2 µm was found to be cumbersome due to the slightly viscous nature of the formulations because of the presence of poloxamer and chitosan polymeric content. Hence, this study confirms that autoclave sterilization is a suitable technique for the sterility of *in situ* gels as it did not exert any negative effect on the formulation characteristics and rather increased the content of RA which could aid in further enhancing the antimicrobial efficacy of the prepared formulations.

The *in vitro* release studies of RA from the optimized *in situ gel* formulations demonstrated an initial burst release at first hour followed by a gradual and sustained release for 12 h which is pivotal for the effective treatment of ocular infections. The $t_{50\%}$ of the formulations F5, P7 and P8 were found to be 2.26 h, 2.55 h and 3.06 h, respectively. No statistically significant differences ($p>0.05$) between the release profiles of RA from F5, P7 and P8 in terms of $t_{50\%}$ values were found, suggesting a closely similar *in vitro* release profiles.[20] The sustained release of RA from formulation F5 was accounted to chitosan polymer in the *in situ* gelling system capable of not only enhancing the viscosity of the formulation but also strong adhesion to the mucous layer of the precorneal area.[8] The sustained delivery of RA from poloxamer based formulations might be attributed to enhanced rheological properties of formulations after gelation which led the formation of tightly packed micellar structure while initiating increased intermolecular interactions in the *in situ* gelling system.[35]

The chitosan-based *in situ* gel formulation (F5) loaded with 0.5% w/v OS extract exhibited modest antimicrobial activity against the Gram-positive *M. luteus*. On the other hand, poloxamer-based *in situ* gels formulations, P7 and P8, loaded with either 0.5% w/v or 1% w/v OS extracts, however, were inactive against all tested bacteria. Subsequently, the resultant antimicrobial activity of OS extract at concentrations ranging between 0.5%-10% w/v showed concentration-dependent antimicrobial activity against all tested bacteria except *P. aeruginosa*. The antimicrobial activity of OS is attributed primarily to the pro-oxidative properties of the RA.[36] In addition, RA could also induce bacterial cell death by altering the charge and hydrophobicity of the bacterial membrane surface.[37] The present findings indicated OS extract, especially E5% and E10%, to be most active against *M. luteus*. This is in line with previous findings documenting the partial to full effectiveness of OS extracts against Gram-positive bacteria especially *M. luteus* at a concentration above 3%.[38] The ineffectiveness of OS extract against Gram-negative bacteria, was also consistent with previously reported findings[39]. This could be ascribed to an additional fortification for the microorganisms provided by extra outer membrane.[40] An attempt to incorporate 5-10% w/v OS extract into *in situ* gels resulted in dark-colored formulations with insoluble sediments. This could be due to the presence of elevated content of lipophilic compounds in the OS

extract.[41] Dark coloured formulations could impair vision and mitigate the treatment adherence because clarity is a pivotal characteristic of any ocular formulation.[42] Nevertheless, the solubility of OS extract at relatively higher concentrations in the *in situ* gel formulation could be enhanced using various methods such as particle size reduction, inclusion of surfactants and complexation.[43,44] Moreover, purified RA isolated from OS extract might also demonstrate promising antimicrobial activity.

5. CONCLUSION

Standardized aqueous-ethanolic leaf extract of OS was obtained successfully using water: ethanol binary mixture (50:50) as solvent via maceration method. The presence of bioactive phenolic compounds namely RA and SIN in the OS extract was confirmed through TLC and HPLC method. The optimized thermosensitive poloxamer and chitosan-based *in situ* gels loaded with OS extract, P7, P8 and F5 demonstrated a pH and sol-to-gel temperature suitable for ocular region. In addition, release of RA was sustained up to a period of 12 h. Sterilization by autoclave was found to be a better technique than sterilization by filtration for achieving the sterility of *in situ* gel formulations. Formulation F5 loaded with 0.5% OS extract exhibited modest antimicrobial activity against *M. luteus* which appeared to be concentration dependent and indicated that increasing OS extract to 10% w/v may improve the antimicrobial activity especially against Gram positive bacteria. However, high concentration of OS extract in the *in situ* gelling system induced dark-coloured sediments. In conclusion, preliminary findings of this study could serve as a new source in a race to find novel plant-derived antimicrobial compounds to fight against the new and re-emerging infections caused by antibiotic-resistant pathogens which is posing an alarming concern across the globe.

Study limitations

The OS extract exhibited moderate antimicrobial activity indicating concentration dependent improvement against the antimicrobial activity especially against Gram positive bacteria. Nonetheless, elevating the content OS extract in the *in situ* gelling system resulted in dark-coloured sediments. This raises the need for further research to improve the clarity of *in situ* gel formulations loaded with OS extract at high concentrations. Further research is also recommended for isolation of RA from OS extract prior to incorporation into *in situ* gels for the achievement of a potential antimicrobial activity.

Conflict of interest

The authors declared no conflicts of interest.

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Table 1. Gradient elution system in separation of bioactive compounds present in OS extract.

Time	0.1% Formic acid in water (%)	Acetonitrile (%)
0.01	95	5

25.00	50	50
26.00	20	80
35.01	95	5
45.00	95	5

Poloxamer-based *in situ* gels containing OS extract								
Ingredients (% w/v)	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
OS extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poloxamer 407	15	18	21	21	21	21	21	21
Poloxamer 188	-	-	-	1	2	3	4	5
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium chloride	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
PBS pH 7.4 qs ad	100	100	100	100	100	100	100	100

Chitosan-based *in situ* gels containing OS extract							
Ingredients (% w/v)	**F0**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
OS extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chitosan in 1.5% lactic acid	1.5	1.5	1.5	1.5	1.5	1.5	1.5
β-glycerophosphate	0	10	20	30	40	45	50
Sodium chloride	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01
PBS pH 6.8 qs ad	100	100	100	100	100	100	100

Table 2. Composition of poloxamer and chitosan-based *in situ* gels loaded with OS extract.

Table 3. Physicochemical characterisation of poloxamer and chitosan based *in situ* gels loaded with OS extract (Mean ± SD, n=3).

Formulation code	Appearance and clarity	pH	Sol-to-gel temperature (°C)	Viscosity (mPas)	
				Before gelation	After gelation
Poloxamer-based *in situ* gel formulations					
P1	Clear & Free flowing	7.21 ± 0.01	No gelation until 50 °C	27.30 ± 0.38	-
P2	Clear & Free flowing	7.23 ± 0.01	No gelation until 50 °C	58.01 ± 0.46	-
P3	Clear & Viscous (Gel)	7.29 ± 0.01	24.9 ± 0.06	2440.00 ± 17.52	-
P4	Clear & Free flowing	7.32 ± 0.01	27.9 ± 0.10	166.18 ± 3.15	337.52 ± 4.16
P5	Clear & Free flowing	7.30 ± 0.01	31.5 ± 0.21	150.37 ± 2.66	319.81 ± 2.43
P6	Clear & Free flowing	7.22 ± 0.01	32.9 ± 0.36	138.25 ± 2.01	1280.16 ± 8.66
P7	Clear & Free flowing	7.27 ± 0.01	33.6 ± 0.10	125.09 ± 2.45	3510.34 ± 11.02
P8	Clear & Free flowing	7.30 ± 0.01	34.9 ± 0.12	116.01 ± 3.38	5750.47 ± 18.49
Chitosan-based *in situ* gel formulations					
F0	Clear and free flowing	4.56 ± 0.01	No gelation until 50 °C	26.30 ± 0.77	-
F1	Clear and free flowing	7.09 ± 0.01	No gelation until 50 °C	58.90 ± 1.23	-
F2	Clear and free flowing	7.28 ± 0.01	No gelation until 50 °C	77.96 ± 1.84	-
F3	Clear and free flowing	7.41 ± 0.01	40.3 ± 1.27	25.90 ± 1.35	211.00 ± 4.81
F4	Clear and free flowing	7.37 ± 0.01	38.7 ± 0.85	14.75 ± 0.81	126.81 ± 3.42
F5	Clear and free flowing	7.46 ± 0.01	34.5 ± 0.07	12.37 ± 0.35	120.00 ± 6.19
F6	Clear and free flowing	7.47 ± 0.01	32.4 ± 0.71	11.50 ± 0.18	111.57 ± 5.05

Table 4. ZOI of the tested samples against Gram-positive and negative bacteria.

Treatments	ZOI (mm), Mean ± SD, n=3			
	Gram-positive bacteria		Gram-negative bacteria	
	M. luteus	*S. aureus*	*E. coli*	*P. aeruginosa*
Chloramphenicol (Xepanicol®; 0.5%)	42.3 ± 1.5[a]	26.0 ± 1.0[a]	29.7 ± 0.6[b]	24.7 ± 0.6[a]
Ciprofloxacin (Ciplox®; 0.3%)	32.7 ± 1.2[b]	26.3 ± 1.2[a]	33.3 ± 0.6[a]	20.3 ± 1.5[b]
Standard RA (1mg/mL)	3.0 ± 1.0[d]	1.3 ± 0.6[e]	3.0 ± 0.0[c]	4.7 ± 0.6[c]
F5 (0.5%)	4.3 ± 2.1[d]	0.0 ± 0.0[e]	NT	0.0 ± 0.0[d]
P7 (0.5%)	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P7 (1%)	0.0 ± 0.0[e]	0.3 ± 0.6[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P8 (0.5%)	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
P8 (1%)	0.0 ± 0.0[e]	0.3 ± 0.6[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E0.5%	3.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E1%	4.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E3%	15.3 ± 0.6[c]	4.3 ± 0.6[d]	0.0 ± 0.0[e]	0.0 ± 0.0[d]
E5 %	16.0 ± 1.0[c]	6.7 ± 0.6[c]	1.7 ± 0.6[d]	0.0 ± 0.0[d]
E10 %	16.0 ± 1.0[c]	9.3 ± 0.6[b]	3.3 ± 0.6[c]	0.0 ± 0.0[d]

[a-e] Different letters represent significant differences ($p < 0.05$) between treatments.
NT = Not tested

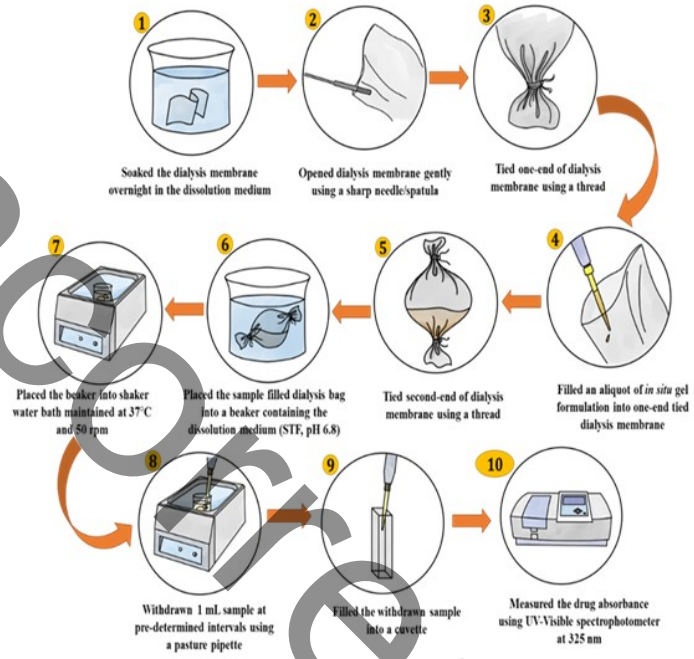

Fig. 1 Schematic representation of *in vitro* release testing of RA from *in situ* gel formulations using dialysis membrane method.

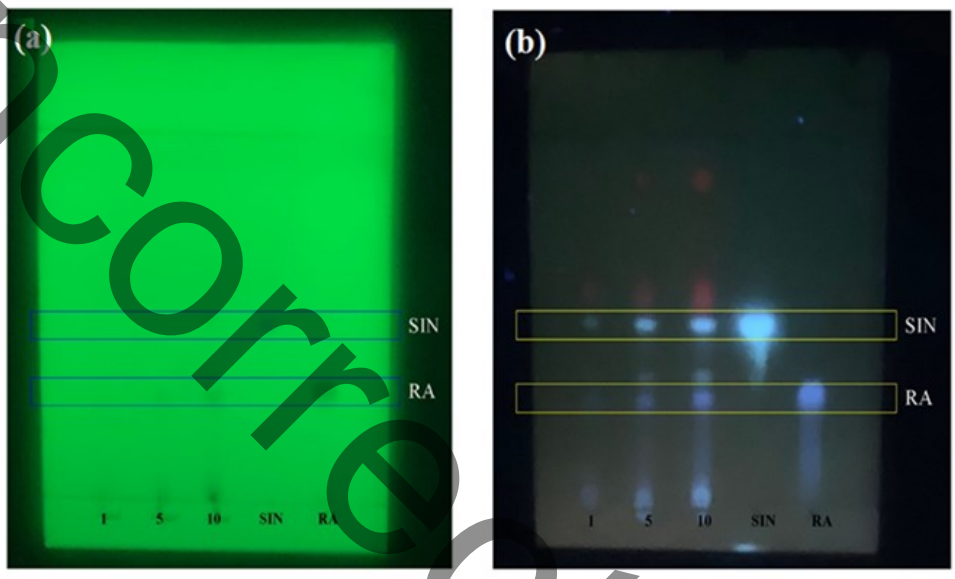

Fig. 2 TLC chromatograms of the aqueous-ethanolic leaf extract of OS (1mg/mL, 5mg/mL and 10mg/mL), and reference standards (RA and SIN) at 254 nm (a) and 366 nm (b).

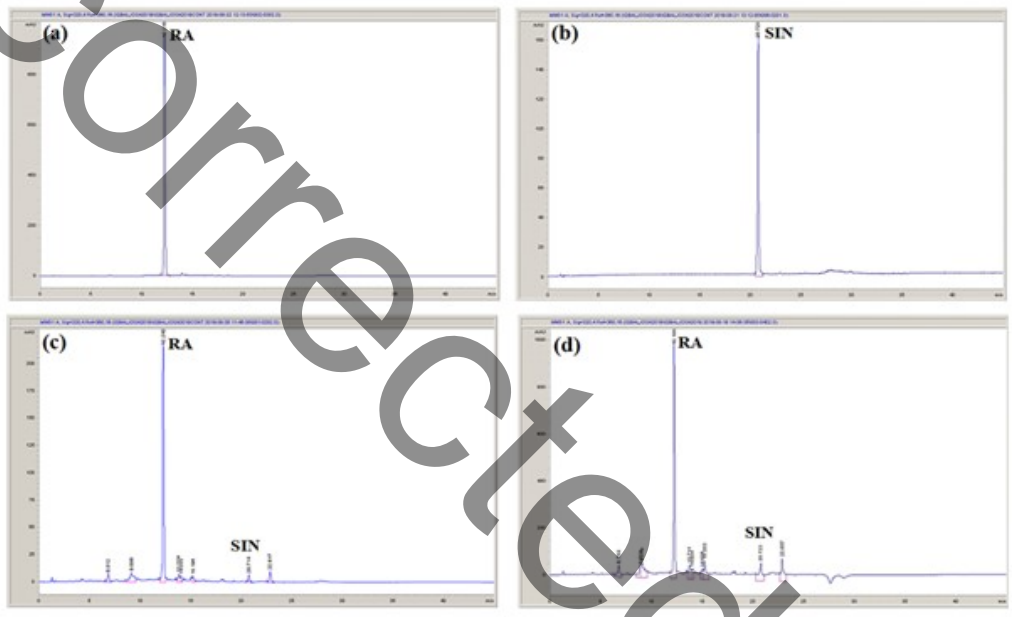

Fig. 3 HPLC chromatograms of (a) standard RA at t_r = 12.215 min; (b) standard SIN at t_r = 20.720 min; (c) aqueous-ethanolic leaf extract of *OS* (1 mg/mL) showing peaks corresponding to RA (t_r = 12.243 min) and SIN (t_r = 20.714 min); (d) aqueous-ethanolic leaf

extract of *OS* (5 mg/mL) showing peaks corresponding to RA (t_r = 12.188 min) and SIN (t_r = 20.723 min).

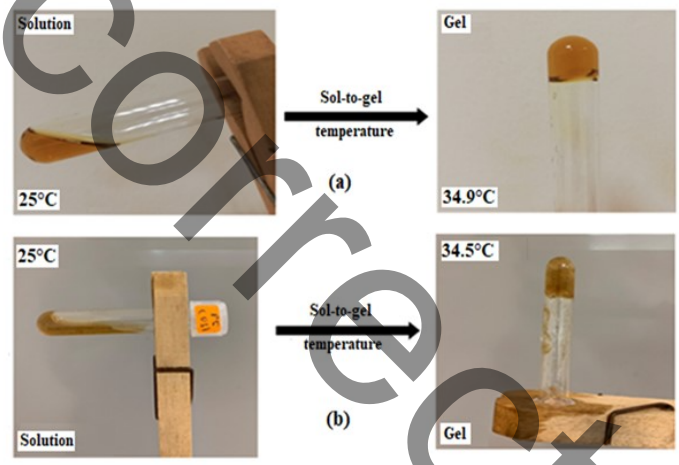

Fig. 4 Depiction of sol-to-gel phase transition temperature of poloxamer-(a) and chitosan-(b) based *in situ* gels loaded with OS extract.

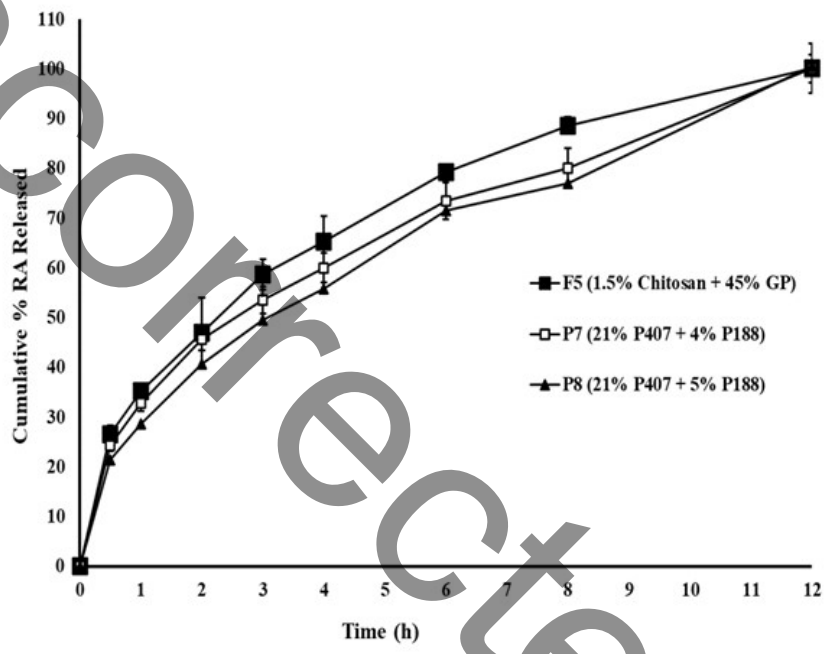

Fig. 5 *In vitro* release profile of RA from chitosan (F5) and poloxamer (P7 and P8)-based in *situ* gel formulations (Mean ± SD, n=3).

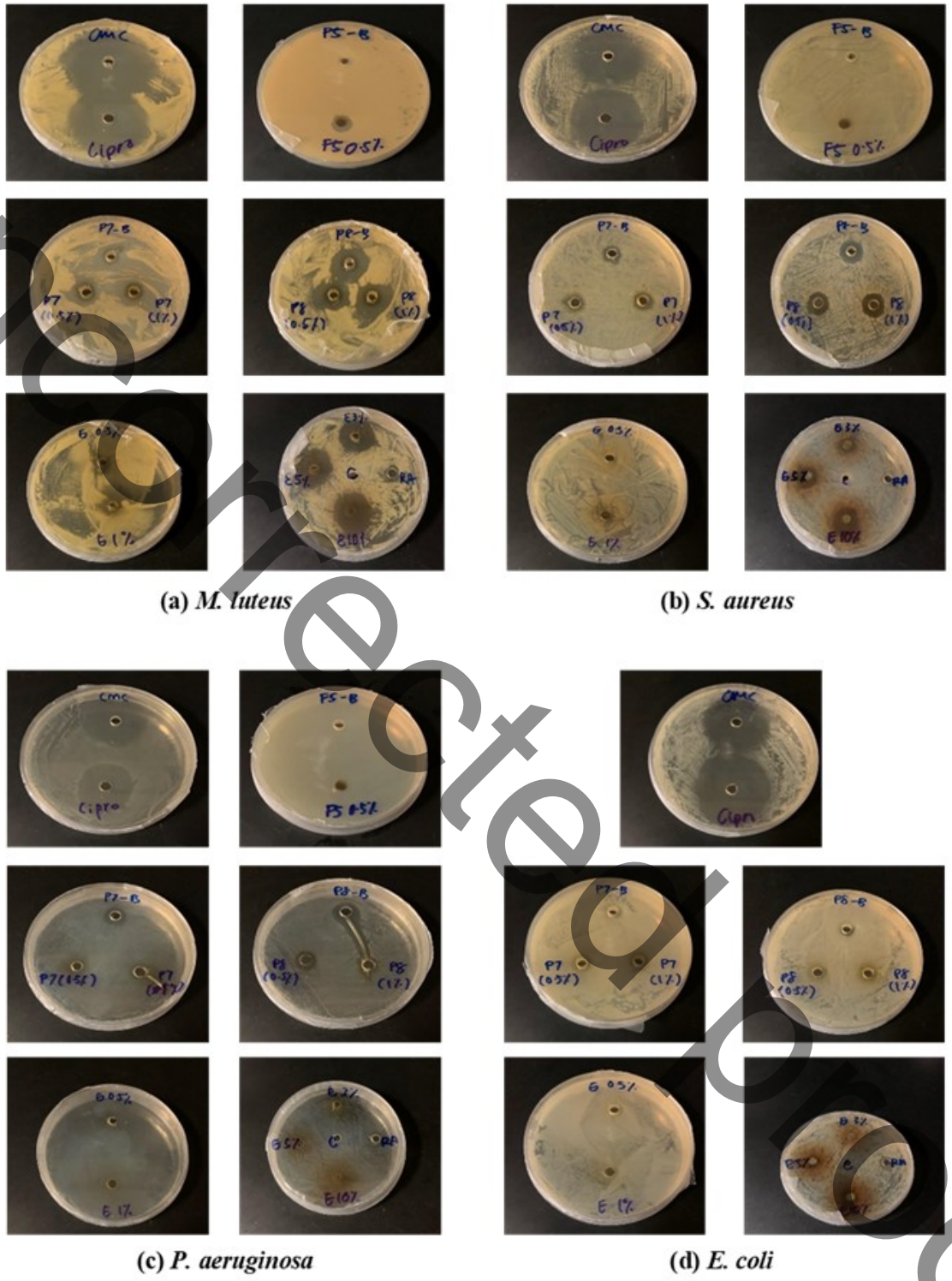

Fig. 6 Representative images indicating ZOI of the tested samples against Gram-positive bacteria (a) *M. luteus; (*b) *S. aureus* and Gram-negative bacteria (c) *P. aeruginosa; (d) E. Coli.*