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Formulation and Evaluation of *in situ* Gel Drug Delivery System of *Sesbania grandiflora* Flower Extract for the Treatment of Bacterial Conjunctivitis.

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Abstract
The purpose of the present investigation was to prepare and evaluate *in situ* gel for *Sesbania grandiflora* flower extract which acts as anti-bacterial and anti-fungal agent using thermo reversible polymer. For improved drug residence time with corneal epithelium, formulation was prepared in the liquid state at 4°C while turned into a gel at the temperature of the *Cul de sac*. Pluronic F127 and Chitosan were used as the polymers which exhibited the phase transition behavior. Natural polycationic polymer chitosan was used to improve residence time. The prepared formulations were characterized for pH, physical appearance, content uniformity, gelling temperature, viscosity, *in vitro* diffusion, anti-bacterial, anti-fungal activity, sterility testing, and eye safety test. The formulation exhibit drastic increase in the viscosity at the temperature of 37°C indicating there possible use in *in situ* gelling systems. Out of all the formulations studied the G4, i.e., Pluronic (17%) chitosan (0.3%) formulation shows the desired drug with prolong contact time with antimicrobial efficacy.

Keywords: *Sesbania Grandiflora*, flower extract, antimicrobial, *in situ* gel, conjunctivitis.

INTRODUCTION

In recent years, multiple drug resistance has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases. This problem demands that a renewed effort to seek the antibacterial agents effective against the pathogenic microorganisms resistant to current antibiotics (Soulsby, 2005). Besides, though synthetic antibiotics are strong medicines and save lives, they cause more harm than good when they are not used in right way (Neu, 1992). Therefore, there is also a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from other sources. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which *in vitro* show the antimicrobial properties and could serve as an alternative, effective, cheap and safe antibacterial for the treatment of microbial infections (Cowan, 1999). Now days the use of traditional medicinal plant material for the production of the new drug compounds is increasing very fastly.

Here we take our interest in the well known, commonly available plant *Sesbania grandiflora* (L.) Pers. It is commonly known as Hadaga, Agati, Agastya etc. belonging to family *Fabaceae*. By considering *Sesbania grandiflora* use as antimicrobial agent, we prepared the *in situ* gelling system for ocular use in treatment of conjunctivitis. Conjunctivitis is the inflammation of conjunctiva which is the mucous membrane covering the white of the eyes and the inner side of eyelid called conjunctiva. It is also known as Pink eyes because the white of the eye appear reddish. The common symptoms of conjunctivitis are redness in the eyes, burning sensation, sensitivity to light, dryness, grittiness sensation, itchy and scratchy feeling, watering of eyes, and swelling of eyelids. In severe cases there is sticky pus like discharge; it may cause the sticking of eyelashes & eyelids while sleeping. Conjunctivitis may cause due to the viruses that cause cold. The conjunctivitis caused due to bacteria known as bacterial conjunctivitis. Bacterias like *Pseudomonas, Chlamydia, Streptococci,* cause conjunctivitis (Mc Ewan, 2011; Therese and Madhvan, 2004)

Ophthalmic drug delivery system is extremely interesting and highly challenging endeavors (Ashim, 1993; Indu et al., 2004). Number of approaches have been tried to develop the sustained release in situ ophthalmic dosage form. The conventional liquid ophthalmic eye drops exhibit a short pre-corneal residence time and poor bioavailability due to rapid and extensive elimination of drugs from pre-corneal lachrymal fluid by solution drainage, lachrymation and non productive absorption by conjunctiva (Kamel, 2002). This problem can be overcome by using in situ gel forming ocular drug delivery system, prepared from polymer, exhibit sol-to-gel phase transition due to a change in a specific physico-chemical parameter like pH, temperature (Bain et al., 2010, Varshosaz et al, 2008). Polymers are very useful which undergo reversible sol to gel phase transition in response to physiological stimuli (Khurana et al., 2007).

MATERIALS AND METHODS

Pluronic F127 and Chitosan (High MW 60,000) obtained from Sigma Aldrich, Mumbai, Benzalkonium chloride, Petroleum ether, Ethyl acetate, Acetone from Merck chemicals, Triethanolamine from Rankem, Sodium chloride from Loba chemicals.

Collection of plant material

The flowers of *Sesbania grandiflora* were collected from the Shirpur and were authenticated by botanist Kalpana V.Wagh. Flowers were washed thoroughly with distilled

water and allowed to shade dry for 7-8 days in clean place. The dried flowers were grinded with the help of grinder.

Preparation of extract

The powdered flowers were subjected for extraction. Extraction was done in Soxhlet apparatus. Flowers were firstly extracted with the petroleum ether for defattion and then successively re-extracted with ethyl acetate and 70% acetone for 48 hrs. Obtained acetone extract was filtered and dried rota vapour.

Formulation of *in situ* gel

For preparation of gel dispersed the required amount of Pluronic F127 and chitosan were dispersed in cold distilled water and 0.2% acetic acid stirred it for 1hr. Pluronic solution was placed in refrigerator until it dissolve completely. The extract was weighed and dissolved it in small amount of water. Chitosan solution and solution of extract was added and stirred it vigorously for 1hr. After that the remaining ingredients were added while stirring. We prepared the gel containing various concentration of extract and the formulation codes are from G1 to G6.

Evaluation of *in situ* gelling system

Physical evaluation

The appearance of the formulation was observed which included clarity, transparency was determined visually and pH was measured using pH meter (Suh and Jun, 1996).

Measurement of pH

pH was measured with the help of pH meter (Systronic pH meter 802). The pH of ophthalmic formulation should be such that the formulation will be stable at that pH and at the same time there would be no irritation to the patient upon administration of the formulation.

Gelling capacity

The gelling capacity was measured by visual method. 100µl sample was placed in a vial containing 2 ml of artificial tear fluid freshly prepared and equilibrated at 35^0C and then visually assessing the gel formation and noting the time taken for gel formation (Qi et al., 2007).

Gelation temperature

10 ml of the sample solution and a magnetic bar were put into a transparent vial that was placed in a low-temperature water bath. A thermometer with accuracy of 0.1 °C was immersed in the sample solution. The solution was heated at the rate of 2°C/min with the continuous stirring. The temperature was determined as GT, at which the magnetic bar stopped moving due to gelation. Each sample was measured at least in triplicate (Qi et al., 2007).

Rheological study

Viscosity of formulation is an important factor in determining residence time of drug in the eye. The viscosity of the prepared formulations at 4ºC and 35±0.5ºC was measured with the help of Brookfield Viscometer (Brookfield DV-E) using spindle number 6 at 20 rpm.

Drug content

Content of flower extract was determined by dissolving an accurately weighed quantity of formulation (1g) in 50 ml of pH 6.8 STF. The solutions were then filtered through 0.45 m membrane filter and analyzed for extract content by UV sprectrophotometer at 272.5 nm.

***In vitro* Antibacterial activity of formulation**

The antimicrobial efficiency and prolonged effect of selected *in situ* gel were determined on *P. aeruginosa*, *S. aureus*, *E. coli*, and *C. albicans* strains as a function of time. The inhibitory effect of flower extract gel on the microorganisms was evaluated using agar well diffusion method. Wells were prepared into the nutrient agar previously seeded with test organisms and wells were filled with 100 µl of the samples. After allowing diffusion of solution for two hour the plates were incubated for 24 hr at 37°C and the diameters of inhibition zones were measured. The inhibitory effect of optimized gel formulation was compared with marketed Ciprofloxacin eye drops for bacteria and Ketoconazole for fungi.

***In vitro* diffusion study**

The *in vitro* diffusion of gel through a membrane was carried out by filling 1 gm of flower extract loaded poloxamer and chitosan *in situ* gel solution into the cellophane membrane (HIMEDIA), soaked overnight in artificial tear fluid pH 7.4 was stretched and fastened securely with rubber band. The gel was kept for gelation at 34 ^{0}C in an incubator and then weighed accurately. After gelation the above cellophane membrane was then placed in dissolution media as simulated tear fluid of same temperature. The temperature and stirring rate was maintained as 34^0C and 50 rpm respectively. Aliquots of 5 ml were withdrawn from release medium at specific time interval and replaced by an equal volume of fresh medium at each sampling time. Aliquots were diluted with simulated tear fluid and the amount of drug released was determined by using UV spectrophotometer at 272.5 nm.

Sterility testing

The sterility testing was performed with the help of two media namely, Alternate Thioglycolate Medium (ATGM) and Soya Bean-Casein Digest (SBCD) medium (IP) and investigated the presence or absence of aerobic, anaerobic bacteria and fungi in the formulated ophthalmic *in situ* gels.

RESULTS AND DISCUSSION

All parts of *Sesbania grandiflora* are utilized for medicine in Southeastern Asia and India including preparations derived from the roots, bark, gum, leaves, flowers, and fruit. In Folk Medicine it is resorted to be aperient, diuretic, emetic, emmenagogue, febrifuge, laxative, and tonic. Agati is a folk remedy for bruises, catarrh, dysentery, night blindness, eye sores, fevers, headaches, smallpox, sores, sore throat and stomatitis. Different parts of this plant are used in Siddha system of Indian traditional medicine for the treatment of a wide spectrum of ailments including anemia, bronchitis, fever, headache, ophthalmia, nasal catarrh, inflammation, leprosy, gout and rheumatism. It also possesses anxiolytic, antiulcer, antioxidant, analgesic, antipyretic, antimicrobial, anticancer, anticonvulsive and hepatoprotective properties (Wagh et al., 2009). It also used in formulation and development of sustained release tablet as a release retardant (Wagh et al., 2010). Even it has a dietary supplement in Night blindness and its treatment in ophthalmic diseases (Yogyata Pathare et al., 2012).

Table 1: Appearance, clarity, pH and drug content of *in situ* gel.

Sr. No.	Formulation Code / Evaluation	G1	G2	G3	G4	G5	G6
1	Clarity of solution	Clear	Clear	Clear	Clear	Clear	Clear
2	Clarity of gel	Clear	Clear	Clear	Clear	Clear	Clear
3	Appearance of solution	T	T	T	T	T	T
4	Appearance of gel	T	T	T	T	T	T
5	pH	7.52	7.29	7.35	7.40	7.320	7.18
6	Gelling capacity	-	+	++	+++	++++	++++
7	Drug content	89.34± 2.78	92.37±1.56	92.98±3.67	97.34± 2.16	91.81± 1.89	90.23± 3.32

- : No gelation, **+** : Gel forms after some time, **++** : Gel forms immediately, **+++:** Immediate gelation remains for 8 hrs, **++++** : Immediate gelation remains for more than 10hrs. **T** : Translucent

Table 2: Formulation batches of *in situ* gel

Ingredients	*G1*	*G2*	*G3*	*G4*	*G5*	*G6*
Pluronic F127	14	14	17	17	20	20
Chitosan	0.1	0.3	0.1	0.3	0.1	0.3
Extract	200	200	200	200	200	200
Sodium chloride	0.6	0.6	0.6	0.6	0.6	0.6
Benzalkonium chloride	0.002	0.002	0.002	0.002	0.002	0.002
Triethanolamine	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Water	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.

In present study the *in situ* gel was formed by using 14, 17 and 20% pluronic F127 and 0.1, 0.3% chitosan, and the flower extract of *Sesbania grandiflora*, the formulation batches were coded from G1 to G6. Among these batches G4 batch gives satisfactory results by forming gel at body temperature, appropriate viscosity in physiological and non physiological conditions, by sustaining the drug release up to 8 hrs. The gel also shows the good antimicrobial activity against the microbes used.

Appearance, clarity, pH, drug content and gelling capacity

These preliminary evaluations like appearance was done by observing the formulation against white background, clarity, gelling capacity were evaluated by visual observation. All batches of *in situ* gels were clear and translucent having drug content within the range of 89 – 97%, the pH of the formulation was adjusted 6.8 to 7.4 to avoid the ocular irritation, but there were slight variations in the pH of gels; G4 batch has the required pH of 7.4.

Formulation of in situ gel

Gelling triggered by a change in pH; the viscosities increase when the pH is raised from its native value to the eye environment (pH 7.4) like cellulose acetophthalate (CAP) latex (Guny, 1981; Guny et al., 1985), cross-linked polyacrylic acid derivatives such as carbomers and polycarbophil; gelling triggered by temperature change: poloxamers or Pluronics (Miller and Donovan, 1982; Desai and Blanchard, 1998), a class of block copolymers of poly (oxyethylene) and poly(oxypropylene), tetronics (Spancake, 1991), ethyl(hydroxyethyl) cellulose (Lindell and Engström, 1993), methyl cellulose and Smart Hydrogel™ exhibit thermoreversible gelation (Chen et al., 2006), gelling triggered by ionic strength change like: Gelrite (Rozier et al., 1989, Sanzgiri et al., 1993, Paulsson et al., 1999), and alginate gel (Séchoy et al.,2000, Cohen et al., 1997), in the presence of mono or divalent cations (Ding, 1998).

In present study the in situ gel was formed by using the thermosesitive polymer pluronic F127 and chitosan as a mucoadhesive polymer. The formulation was in liquid form at room temperature while get converted in to gel form at temperature 35±0.5ºC.

Rheological study

In case of ophthalmic preparations specially *in situ* gels, viscosity play important role, because too viscous solution is very difficult to instilled in eyes while if the viscosity of solution is too less then it gel drain out from eyes within 3-4 min decreasing the contact time of drug with eye. The G1 batch has very low viscosity even at 35^0C; that it did not improve the contact time of drug with the eye. The batch containing 20% of PF 127 and 0.1 or 0.3% chitosan showed very high viscosity at the 4^0 and 35^0 C that it forms very stiff gel which may leads to the difficulty for instillation and also retards the release of drug, batch G5 and G6 forms very stiff gel even at room temperature. Batch G4 showed good results with better viscosity at 4^0 and 35^0C. As from the rheological study the concentration of pluronic affects the viscosity of formulation.

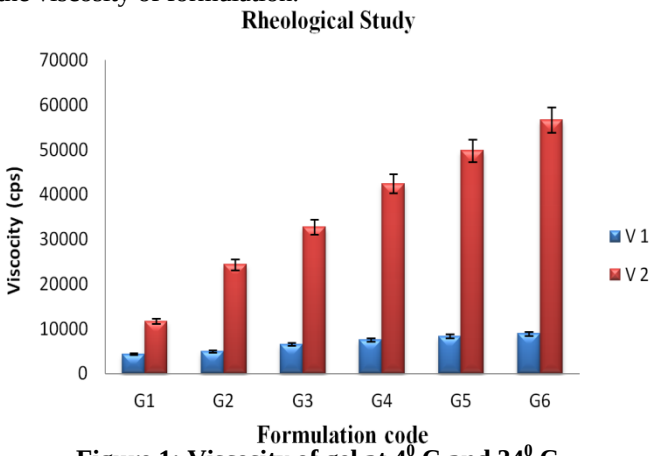

Figure 1: Viscosity of gel at 4^0 C and 34^0 C

V1: Viscosity of formulation at 4^0 C, **V2:** Viscosity of formulation at 34^0 C

Gelation temperature

Gelation temperature, the temperature at which the liquid phase changes to a gel, is an important parameter for *in situ* gel-forming systems. The ideal sol to gel transition should be between the average ambient temperature 25^0 C and 35^0 C, the eye temperature. Pluronic shows the gelation within the range of 25-35^0 C, so Pluronic was chosen as the gelling agent. Also, Pluronic F 127 was reported to be the less toxic than the commercially available poloxamers. It was found that increase in concentration of pluronic decreases the phase transition temperature. Pluronic concentrations used for the ophthalmic formulation were in the range of 14 – 20%. In order to obtain an *in situ* gel having increased mucoadhessiveness, chitosan was added into the formulation. The effect of 0.1% and 0.3% chitosan concentration on the gelation was measured. It is found that the chitosan did not affect the gelation temperature of formulation significantly; in addition it forms the viscous solution.

Table 3: Gelation temperature of *in situ* gels

Formulation Code	Gelation temperature(°C) before dilution	Gelation temperature(°C) after dilution
G1	43.6 ± 0.5	45.2 ± 0.3
G2	40.4 ± 0.2	42.1 ± 0.4
G3	36.3 ± 0.3	38.2 ± 0.4
G4	34.8 ± 0.5	35.2 ± 0.5
G5	29.7 ± 0.3	31.6 ± 0.6
G6	26.5 ± 0.5	28.4 ± 0.5

In vitro antibacterial study of in situ gel

In vitro antibacterial activity of the formulation was determined using the bacteria *P.aeruginosa, S. aureus, E. coli* and fungus *C. albicans* which may cause the various ocular infections like bacterial conjunctivitis, keratitis etc. Antibacterial activity was determined with the help of agar well diffusion method. Zone of inhibition produced by the optimised batch, i.e., G4 was measured and compared with the zone of inhibition produced by marketed eye drops. Gel formulation showed the minimum activity producing ZOI of diameter 13.21±3 on *C. albicans* and maximum activity producing ZOI of diameter of 19.54 ± 1 was on *P. aeruginosa.*

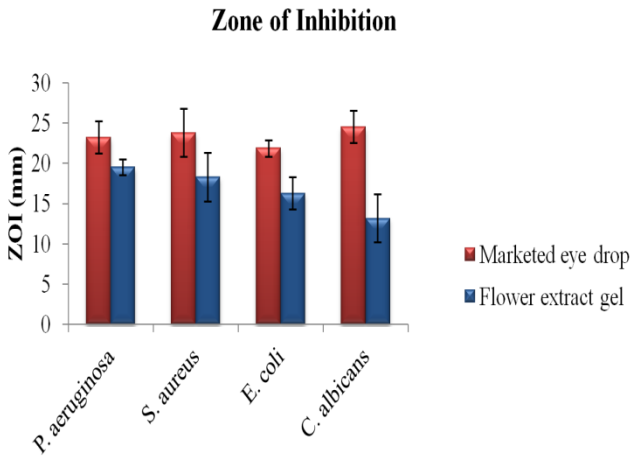

Figure 2: ZOI of *in situ* gel and marketed eye drop

In vitro diffusion study

From the obtained results, it was observed that, the formulation batch G1 and G2 having 14% of PF127 and 0.1% and 0.3% of chitosan, failed to satisfy the evaluation parameter of ocular *in situ* gels as the gelation temperature of these formulations were in the range of 40 – 45^0C, much greater than the desired value. G1 and G2 could not be considered as good ocular *in situ* gel formulations as they exhibited drug release 78.31% and 77.53% in very short time 2 hrs and 3hrs respectively due to lack of gel forming ability at 35^0C, so these batches were not taken in to consideration. Batch G3 and G4 having 17 % PF 127 each and 0.1% and 0.3% of chitosan respectively, exhibited 73.55% and 79.64% drug release in 5 hrs and 8 hrs respectively. Drug release study for G5 and G6 (19% of PF 127 each, 0.1 % and 0.3 chitosan respectively) was carried out up to 8 hrs only as the gel formed of these batches was very stiff and the drug release was very slow.

Sterility testing

Above all tested batches; among them we select the optimised batch G4 which gives the satisfactory results. So batch G4 was subjected for the ocular safety studies. The sterility testing was done using two media namely, Alternative Thioglycolate Medium (ATGM) and Soya bean-Casein Digest (SBCD) medium to investigate the presence or absence of aerobic, anaerobic bacteria and fungi. The formulation was inoculated separately with AGTM and SBCD medium and was incubated at 37^0C and 20^0 - 25^0C respectively for 7 days, a control was also kept in same way without formulation. The samples were observed for the turbidity every day. The formulation did not show turbidity or microbial growth.

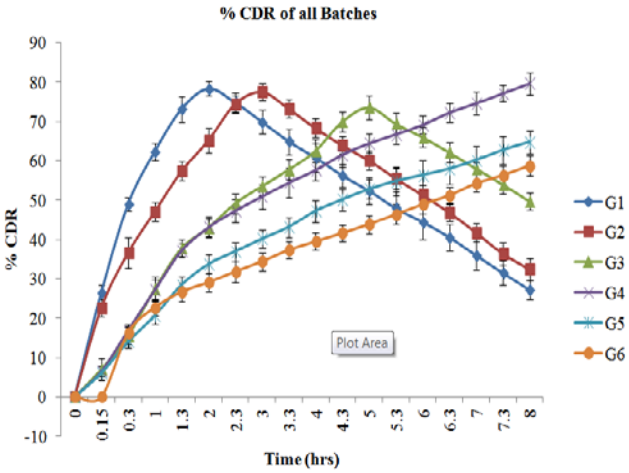

Figure 3 Drug release

CONCLUSION

From the results obtained, the G4 batch of *in situ* gel of the *Sesbania grandiflora* flower extract was active against the microorganisms *P. aeruginosa, S. aureus, E. coli,* and fungus *C. albicans* which cause the bacterial conjunctivitis and provide the sustained release of drug up to 8 hrs. The drug release of the formulation was mainly depends on the concentration of the Pluronic F 127 and chitosan. So the G4 batch could be a promising in situ gelling formulation for long acting ocular delivery.

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