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Development and characterization of green tea loaded microemulsion for vaginal infections

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ABSTRACT

Natural products are important sources for new drug development. Though antibiotic therapies are first line of treatment for vaginal infections but prolonged use results in various side effects, hence there is a need to develop alternative therapies based upon natural products. Green tea powder (GTP) has been reported to exhibit anti-microbial activity and has synergistic effect with some antibiotics. Conversely, the role of GTP against vaginal infections has not been explored extensively. GTP is safe even at high doses but exhibits poor oral bioavailability. Encapsulation of GTP in a microemulsion (ME) vehicle would protect it against degradation. Aim of this study is to formulate GTP loaded MEs for vaginal application and to assess its efficacy against various vaginal pathogens. UV analysis of the GTP was done and solubility studies in various oils, surfactants and co-surfactants were carried out to select various phases of MEs. Titrations were carried out with various oil:Smix ratios and the data obtained was used to plot pseudo-ternary phase diagrams. The emulsion regions which corresponded to particle less, transparent regions and within GRAS limits were subjected to thermodynamic stability studies. Droplet size of thermodynamically stable MEs was found to be in nanometre range. *Escherichia coli* and *Staphylococcus epidermidis* were found to be sensitive to GTP and its ME via disc diffusion assay.

Keywords: Disc diffusion assay; encapsulation; green tea powder; microemulsion; vaginal infection.

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Introduction

Vaginal infection or vaginitis is an inflammation of the vagina that results in discharge, odor, irritation, or itching **[1].** Infections of the vagina are very common, affecting about one third of women during their lifetimes **[2].** First line treatments prescribed for vaginal infections involve the use of antibiotics. Antibiotics exhibit strong antimicrobial activity but have unavoidable side effects and limited specificity **[3].** Various vaginal formulations such as tablets, pessaries and vaginal suppositories, semisolid systems like creams, gels, ointments, liquid systems like vaginal douching with liquids containing antimicrobial drugs and vaginal foams are available commercially **[4].** But these conventional dosage forms suffer from certain limitations such as poor residence time and frequent dosing. To surmount these limitations, several aesthetic and functional qualities must be incorporated into vaginal drug delivery system **[5].**

Recently, natural products with antimicrobial effects have been identified with increasing interest by clinical pharmacologists. One of the natural products which have engrossed attention is green tea. Green tea has also been known to possess various other therapeutic properties like anti-tumor, anti-inflammatory, anti-ageing and anti-oxidative **[6].** These medicinal properties of green tea can be attributed to the higher content of catechins present in its leaves. Catechins comprise the major polyphenol component of green tea. There are four types of catechins namely: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Out of these, EGCG comprises the major portion of the total catechin content **[7].**

MEs are fine sized emulsions ranging from 10-200nm that can be developed as efficient drug carrier systems. MEs are attractive as pharmaceutical formulations because they form spontaneously, are thermodynamically stable, and optically transparent. MEs provide much longer oil-water contact area due to the nanosize droplet compared to classical emulsions, which facilitates drug release from the dispersed droplets and protects the drug **[8].**

The objective is to develop and characterize an intravaginal GTP loaded ME displaying high residence time, high drug carrying capacity, site specific delivery resulting in less frequent doses.

Experimental

Materials

Green Tea Powder and Labrasol were kindly gifted by Lakshayam Herbs Pvt. Ltd., Delhi (India) and Gatefosse (India), respectively. Cremophor EL was obtained from Sigma-Aldrich (India). All the chemicals used in the study were of analytical grade.

Establishment of analytical methodology

A UV/VIS Absorbance module containing the NanoDrop 1000 Spectrophotometer has been used to measure the GTP absorbance from 220nm to 750nm. Absorption spectra of GTP (dissolved in distilled water) of different concentrations were used to determine λmax.

Selection of excipients

To find out the suitable oil which can be used as oil phase in MEs, the solubility of GTP in various oils (sesame oil, olive oil, clove oil, linseed oil, coconut oil, corn oil, canola oil, IPM, labrasol, soybean oil and almond oil) was checked. The solubility of GTP in various surfactants (tween 20, span 80, cremophor EL) and co-surfactants (plurol oleic, capryol 90, transcutol P, glycerol, isopropanol) was also determined. An excess amount of GTP was added in 2ml of the selected oil, surfactant and co-surfactant in stoppered vials and then preliminary mixing was carried out over magnetic stirrer for few minutes. Later on, these vials were kept in mechanical bath shaker for 48h at 37^{o}C. The equilibrated samples were centrifuged at 10,000rpm for 10min. The supernatant was separated, filtered and after appropriate dilution with distilled water, solubility was determined by UV method at 275nm (λmax).

Construction of pseudo-ternary phase diagram

In order to optimize surfactant and co-surfactant, phase diagrams for each surfactant and co-surfactant combinations were constructed by using aqueous titration method. The ratio of each of selected surfactant to co-surfactant (Smix) was kept constant (1:1) while oil to Smix ratio was taken 1:9. Surfactant and co-surfactant were selected on the basis of number of ME points demonstrated by phase diagrams. After selection of surfactant and co-surfactant, their optimum concentration ranges were determined by detailed study of phase diagrams using different ratios of Smix (1:0, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1). For each Smix ratio, oil:Smix ratio was varied. Total sixteen different combinations of oil and Smix (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 1:2.3, 1:2, 1:1.5, 1:1, 1:0.7, 1:0.43, 1:0.25, and 1:0.1) were made so that maximum ratios were covered for the study to delineate the boundaries of phases precisely formed in the phase diagrams. Slow titration with aqueous phase was performed to each oil:Smix ratio and transparency, flowability and physical state of ME was observed **[9].** For preparation of GTP loaded ME, GTP (20mg/ml) was added to the oil phase of the ME.

Characterization of MEs

Thermodynamic Stability of MEs: To assess the thermodynamic stability of GTP loaded ME, clarity and phase separation were evaluated before and after subjecting to the following stress tests:

a) Heating cooling cycle: MEs were subjected to six cycles between refrigerator temperature (4°C) and 45°C (storage not less than 48h at each temperature).

b) Centrifugation: Formulations were centrifuged at 3500rpm for 30min and the samples that did not show any phase separation were taken for the freeze thaw stress test.

c) Freeze thaw cycle*:* Formulations were subjected to three freeze thaw cycles between -21°C and +25°C (storage not less than 48h at each temperature).

d) Dispersibility Test*:* The dispersibility test is carried out by mixing 1ml of ME with 10ml of water at room temperature followed by gentle vortexing. Formulations that passed dispersibility testing Grade A [rapidly forming (within 1min) ME having a clear or bluish appearance] and Grade B (rapidly forming, slightly less clear ME having bluish white appearance) were selected for further studies.

Droplet size and size distribution

Droplet size was determined by photon correlation spectroscopy that analyzed the fluctuations in light scattering due to brownian motion of the particles **[10],** using a Zetasizer (100 HS, Malvern Instruments, UK). The formulation (0.1ml) was dispersed in 50ml of water in a volumetric flask, mixed thoroughly with vigorous shaking and light scattering was monitored at 25°C at a 90° angle. Polydispersity index (PI), for the formulation was determined.

Determination of antimicrobial activity

Disc Diffusion Assay: The absorbance of the bacterial culture was taken and concentration was equilibrated according to 0.5 McFarland standards (The absorbance at 625nm should be 0.08 to 0.13 for 1-2 X 10^8cfu/ml). The obtained concentration was diluted to 10^5cfu/ml and plated on nutrient agar **[11]**. The sterile discs (Whatman Filter Paper) were impregnated with 20µl of aqueous GTP solution, GTP loaded ME, and corresponding placebo. Gentamicin (4µg/ml) was used as positive control and exhibited a zone of inhibition of 26mm. The discs were applied on agar plates and incubated at 37 ºC for 16hrs. Zone of inhibitions in the plates were measured and the strains showing a zone of inhibition greater than 7mm were reported **[12].**

Results and discussion

Screening of Oil

GTP being a hydrophilic compound, it was very important to find out its solubility in appropriate solvent because only the dissolved GTP can permeate though the vaginal walls. In order to screen appropriate excipients for the preparation of ME, the solubility of GTP in various oils, surfactants and co-surfactants was measured.

Solubility of GTP in labrasol was found to be best amongst the oils investigated. In addition, labrasol is a non-ionic oily carrier and, thus was selected as an oil phase in the present study.

Screening of surfactants/co-surfactants

Cremophor EL and glycerol were selected as surfactant and co-surfactant, respectively after preliminary solubility tests for aqueous titrations. Sixteen possible combinations were made and in each combination a constant surfactant to co-surfactant ratio (6:1) was taken since higher concentration of surfactant is favorable for ME formation, while oil to Smix ratio was varied.

Each combination was titrated with water and the resultant physical state of ME was marked on a pseudo-ternary phase diagram with one axis representing the water, second representing oil and the third representing a Smix.

Phase diagram studies

Physical appearance of all ME formulations showed no distinct conversion boundaries from w/o to o/w at all Smix ratios. The rest of the region on the phase diagram represents the turbid and conventional emulsions. Significant difference was seen in ternary phase diagrams of ME constructed with different Smix ratios.

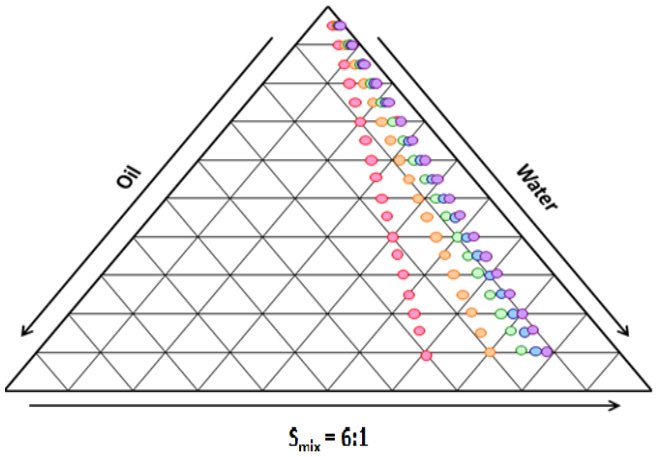

Fig. 1. Pseudo-ternary phase diagram of microemulsion regions of existence (represented by dots) with S_{mix} ratio (6:1) composed of labrasol (oil), cremophor EL (surfactant), glycerol (co-surfactant) and water (% w/w).

It was observed when cremophor EL was used alone without glycerol (Smix=1:0), very low amount of oil could be solubilized. This could be due to the fact that addition of a co-surfactant is required to attain negative interfacial tension, necessary for the formation of MEs.

At equal amounts of cremophor EL and glycerol (Smix=1:1), negligible ME region was observed in the phase diagram and same pattern was observed for four other Smix ratios (2:1, 3:1, 4:1, 5:1). The decrease in ME area is possibly due to presence of low concentration of cremophor EL which decreases the solubilization capacity of ME **[13].** However, when the concentration of cremophor EL was increased with respect to glycerol (Smix=6:1), the ME area was increased, possibly because cremophor EL is a non-ionic solvent that forms clear solution in water, so the area of o/w ME was increased (**Fig. 1**).

Selection of formulations from phase diagrams

Following criteria were chosen for the selection of formulations: The oil concentration should be such that it solubilizes the single dose of the drug. Formulations with Smix ratio 1:0 and 1:1 were not selected because the formulations were unstable and showed phase separation. Furthermore, at Smix 2:1, none of the formulations could solubilize the amount of oil required to carry the single dose of GTP. Formulations with Smix ratio 3:1, 4:1 and 5:1 were not selected because area of ME isotropic region was small as compared to Smix 6:1. Therefore, MEs in Smix 6:1 were subjected to thermodynamic stability studies.

Table 1. Composition of selected Green Tea Powder loaded microemulsion formulations.

S_{mix} Ratio	Formulation	Percent w/w of		
		Oil	Water	S_{mix}
6:1	GTP 1	13.33	60.00	26.67
	GTP 2	8.89	60.00	31.11
	GTP 3	5.8	65.22	28.99
	GTP 4	5	70.00	25.00
	GTP 5	5	65.00	30.00
	GTP 6	3.57	75.00	21.43
	GTP 7	3.13	75.00	21.88

Based on phase diagrams, one Smix ratio 6:1 was optimized. From the selected Smix ratio, GTP 1, GTP 2, GTP 3, GTP 4, GTP 5, GTP 6 and GTP 7 were selected from the region of existence (**Table 1**).

Characterization of MEs

Thermodynamic Stability studies of GTP loaded MEs: Stress test including heating cooling cycle, centrifugation and freeze thaw cycles showed that out of sixty four formulations only seven had a good physical stability. After three months, GTP loaded MEs were found to be stable. Thus, it can be concluded that the ME formulations physically stable.

Droplet size and size distribution

In this work, the influence of ME components and GTP on the characteristics of ME was studied. Malvern Zetasizer revealed that MEs droplets (placebo and GTP loaded) had nanometer size range (<250nm).

Values of polydispersity index (PI), which is a measure of uniformity of droplet size within the formulation, were also calculated. All the ME formulations exhibited a narrow size distribution (PI < 0.452) (**Table 2**). The mean droplet size of placebo ranges from 29.16 to 174.5nm, which was lower as compared to the GTP loaded MEs (41.98 to 238.5nm) (**Table 2**). It is hypothetically described that at the optimum Smix ratio (6:1), cremophor EL and glycerol lowered the interfacial tension between the oil and water molecules thereby stabilizing MEs and resulting in smallest droplet diameters (29.16nm) with lowest PI value (0.128). In addition, particle size analysis revealed that the higher concentration of Smix may be required to solubilize oil phase in the aqueous phase resulting an increase in size **[14].**

Disc diffusion assay

In this study, disc diffusion assay was carried out for susceptibility testing of GTP dissolved in distilled water; GTP loaded ME and the corresponding placebo. The absence of growth around the discs is an indirect measure of the ability of that compound to inhibit that organism. *S. epidermidis* was found to be more sensitive to GTP loaded ME as compared to *E. coli* while its corresponding placebo was found to be inert against both the pathogens (**Table 3**).

Table 2. Particle Size and PDI of microemulsions and corresponding placebos.

Formulation	Droplet size (d.nm)		PDI	
	Placebo	ME	Placebo	ME
GTP 1	174.5	238.5	0.338	0.452
GTP 2	147.5	181.9	0.291	0.351
GTP 3	92.86	103	0.236	0.211
GTP 4	80.74	82.44	0.172	0.21
GTP 5	77.28	79.13	0.197	0.202
GTP 6	43.85	53.81	0.15	0.144
GTP 7	29.16	41.98	0.128	0.148

Table 3. Zone of inhibition for different formulations against two vaginal pathogens.

Formulations	Zone of Inhibition (mm) for *S. epidermidis*	Zone of Inhibition (mm) for *E. coli*
Aqueous GTP	12	11
Placebo	-	-
ME	14	12

Conclusion

GTP loaded thermodynamically stable oil-in-water MEs were optimized based upon optimum droplet size, minimum polydispersity, lower viscosity, lower surfactant concentration and solubility. The optimized formulation contained labrasol as oil phase, cremophor EL as surfactant, glycerol as co-surfactant and water as aqueous phase. The mean droplet size of GTP loaded MEs was found to be less than 250 nm. Furthermore, particle size analysis revealed that increased amount of oil (w/w) resulted into the larger droplet size. The optimized

formulation showed better antimicrobial activity as compared to green tea aqueous extract via disc diffusion assay against *S. epidermidis* and *E. coli*, the potential vaginal pathogens. Our study indicates that GTP loaded ME can be used as a potential anti microbial agent for vaginal infections. Further characterization and release studies of GTP loaded MEs in simulated vaginal fluid needs to be carried out. Cytotoxicity assay of ME in mammalian cell line will help in understanding its safety profile.

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