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Research Article

Comprehensive Assessment of Transcorneal Permeation, Antimicrobial, and Antifungal Activities of Andrographolide-Loaded Nanosuspension: In *vitro* and *In vivo* Studies

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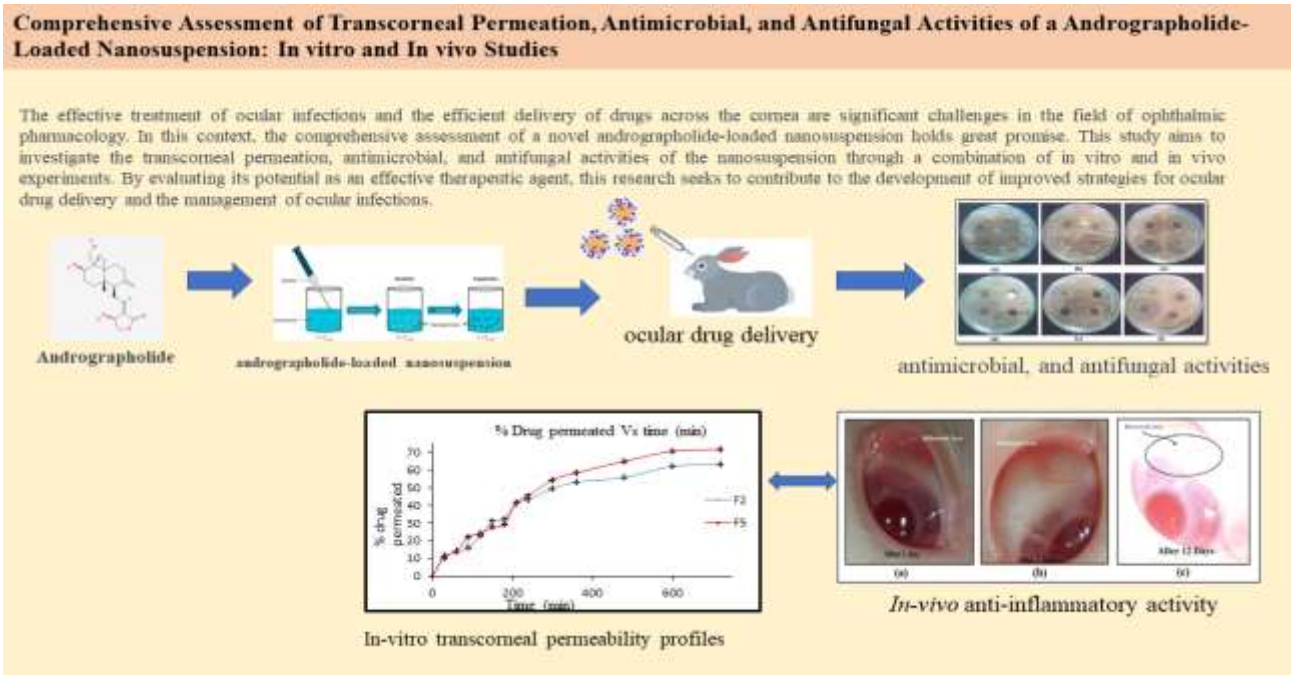
Abstract

This study aimed to comprehensively assess the transcorneal permeation, antimicrobial, and antifungal activities of a nanosuspension loaded with Andrographolide, a promising herbal compound. Through a combination of in vitro and in vivo studies, the efficacy, and potential applications of the nanosuspension in ocular drug delivery were investigated. In the in vitro phase, transcorneal permeation studies were conducted using Franz diffusion cells with excised rabbit corneas. The nanosuspension demonstrated significantly enhanced permeation compared to a control formulation, indicating its ability to effectively deliver Andrographolide through the cornea and into the ocular tissues. Additionally, the nanosuspension exhibited potent antimicrobial and antifungal activities against various ocular pathogens, as determined by agar diffusion and broth microdilution assays. Building upon the promising in vitro results, in vivo studies were performed using a rabbit model. Ocular tolerability of the nanosuspension was assessed through eye observations, with no observed signs of irritation or adverse effects. Furthermore, the nanosuspension's in vivo antimicrobial and antifungal activities were evaluated by instilling the formulation into the rabbits' eyes and monitoring conjunctival congestion and inflammation. The results demonstrated a significant reduction in conjunctival congestion, highlighting the nanosuspension's potential for combating ocular infections and inflammation. The comprehensive assessment presented in this study establishes the transcorneal permeation capability of the Andrographolide-loaded nanosuspension and its remarkable antimicrobial and antifungal activities. These findings underscore the potential of the nanosuspension as an effective ocular drug delivery system for various ocular infections and inflammatory conditions. The study contributes to the development of novel therapeutic approaches in ophthalmology, aiming to improve patient outcomes and provide alternative treatment options for ocular diseases.

Keywords: Transcorneal permeation; Nanosuspension; Andrographolide; Antimicrobial activity; Antifungal activity; Ocular drug delivery

Graphical Abstract



1. INTRODUCTION

The effective delivery of therapeutic agents to the eye poses unique challenges due to the protective barriers of the ocular tissues. Transcorneal permeation, the process by which drugs cross the cornea, plays a pivotal role in ocular drug delivery strategies. Understanding the transcorneal permeation mechanism and evaluating the antimicrobial and antifungal activities of ocular formulations are crucial in developing efficient treatments for ocular infections and diseases [1,2].

In recent years, nanosuspensions have emerged as promising carriers for ocular drug delivery. Their small particle size and high drug-loading capacity offer advantages in enhancing drug bioavailability and therapeutic efficacy [3]. Andrographolide, a natural compound derived from Andrographis paniculata, has gained attention due to its potent antimicrobial and antifungal properties. Loading Andrographolide into a nanosuspension presents a promising approach to harness its therapeutic potential for ocular applications[4].

This comprehensive assessment aims to investigate the transcorneal permeation, antimicrobial, and antifungal activities of an Andrographolide-loaded nanosuspension [5]. Through a combination of in vitro and in vivo studies, we aim to gain insights into the formulation's effectiveness and potential therapeutic applications [6,7].

By evaluating the transcorneal permeation of the nanosuspension, we can assess its ability to overcome the barriers of the cornea and efficiently deliver Andrographolide to the target tissues [8,9]. Furthermore, exploring the antimicrobial and antifungal activities of the nanosuspension will provide valuable information on its potential for combating ocular infections and diseases [10,11].

This research contributes to the growing body of knowledge on ocular drug delivery systems and their therapeutic applications. The findings have the potential to advance the development of effective treatments for ocular conditions, addressing the limitations of current therapies and improving patient outcomes [5].

Overall, this study presents a comprehensive assessment of the transcorneal permeation, antimicrobial, and antifungal activities of an Andrographolide-loaded nanosuspension. The results obtained from this investigation will aid in enhancing our understanding of ocular drug delivery strategies and pave the way for the development of novel and effective ocular therapeutics.

2. MATERIAL AND METHODS

Materials

The andrographolide-loaded nanosuspension was prepared using a high-pressure homogenization method. Andrographolide, obtained with known purity, was formulated into a nanosuspension using appropriate solvents, surfactants, and stabilizers. Optimization of andrographolide concentration was performed, and the nanosuspension formulation was characterized for particle size, zeta potential, and morphology using Malvern Zetasizer. For the transcorneal permeation studies, corneal tissue was employed, following ethical approval and institutional guidelines. Transcorneal permeation experiments were conducted by exposing the corneal tissue to the andrographolide-loaded nanosuspension in a donor-receiver compartment setup. The receptor fluid was maintained at specific pH and temperature. The permeation studies utilized a concentration of the andrographolide-loaded nanosuspension. Sample collection was performed at regular intervals, and subsequent analysis was carried out. Antimicrobial activity assays were performed using Gm +ve and Gm -ve bacteria obtained. The minimum inhibitory concentration (MIC) was determined using the microdilution or agar diffusion method, with a range of concentrations of the andrographolide-loaded nanosuspension. Bactericidal activity was evaluated through time-kill assays, while antifungal activity was assessed via fungal susceptibility testing. Statistical analysis was conducted to interpret the data obtained from all experiments.

Preparation of nanosuspension:

The nanosuspension formulation containing andrographolide (AG) was prepared using the quassi emulsification solvent diffusion method [12,13]. A total of 20 mg of AG was utilized in the process, with varying drug-to-polymer weight ratios as specified in Table No. 1. The drug and polymer (Eudragit RS 100/Eudragit RL 100) were co-dissolved in 5 mL of methanol, which served as an organic water miscible solvent. The resulting solution was slowly injected into 20 mL of water (nonsolvent) containing 0.5% Poloxamer 407, a hydrophilic surfactant, under moderate magnetic stirring [14]. Continuous stirring at a speed of 1500-2000 rpm was maintained for approximately 6-7 hours to facilitate the evaporation of the organic solvents. This process led to the formation of nanosuspensions, wherein AG was successfully encapsulated within a polymer matrix [14,15].

Table 1: Details about formulation contents of AG loaded polymeric nanosuspension batches.

Batch	Drug (mg)	Polymer (mg)		Surfactant Poloxamer 407 (%)	Distilled water (mL)
		Eudragit RS100	Eudragit RL100		
F1	20	80	-	0.5	20
F2	20	100	-	0.5	20
F3	20	120	-	0.5	20
F4	20	-	80	0.5	20
F5	20	-	100	0.5	20
F6	20	-	120	0.5	20

3. In-vitro study

3.1 Trans corneal permeation studies

The trans corneal permeability of andrographolide created formulation was examined in goat corneas [16,17]. Goat eyeballs that were still intact and fresh were purchased from a nearby at a low temperature. Then, surrounding the corneas was carefully removed, and they were then carefully kept in freshly made simulated tear fluid conducted in a that had been modified. In the upper chamber, an equivalent quantity of the drug (2 mg) solution or formulation under research was inserted as a donor compartment [18,19]. The Franz diffusion cell's clamped donor and receptor compartments were used to position the excised goat cornea so Freshly made artificial tear fluid was fed into the lower chamber, which was employed as a receiver compartment. The overall system was kept at 37 0.5 °C. andrographolide perfusate at 227 nm at regular intervals for up to 12 hours [19–21].

3.2 In-vitro antimicrobial and antifungal activity

Measure in-vitro antibacterial activity and antifungal activity [21,22]. Improved commercially available (such as Ciprofloxacin) against Gram +ve and Gram -ve microorganisms was determined by the microbiological studies[23]. a layer of nutritional agar (20 mL) inoculated using the pour plate method with the test microorganism (0.2 mL). It was permitted to set up in the Petri dish [24]. create cups on the hardened agar layer. After that, two cups each received a volume of the formulations (marketed eye drops and optimised formulations) containing an equal amount of medication. The entire treatment was carried out in an aseptic room. Petri plates were The inhibitory zones were discovered [25,26]. A comparison was made between (which lacked any additions or formulations or commercially available drugs). Readings were taken three times. Identical steps were taken to test antifungal activity, although Sabouraud's agar medium was utilised as the medium (20 mL). For comparison, the commercially available formulation (like Fluconazol) was employed [26].

4. In-vivo studies

a) Ocular tolerability study

Using a slit-lamp and a modified Draize test, compounds' potential for causing ocular irritation and/or harm was assessed [27,28]. In the experiment, approved all study procedures and the housing of the animals. Every 30 minutes for six hours, a 0 to 3, 0 to 4, and 0 to 3, the severity of the congested, swollen, and reddened areas was evaluated. On a scale of 0 to 4, the degree of corneal opacity was assessed. 8.5 Table was used to interpret the data [29].

b) Anti-inflammatory activity in rabbits

In this study, two drops of a 0.4% solution of xylocain were injected as a local anaesthetic into each rabbit's eye of a group of two male rabbits weighing 1.5-2 kg [30,31]. A heat app roach was used to create four inflammatory regions (ulcers) in each eye's epithelium of the cornea, distant from the pupil, one to two minutes after instillation. The lesions were 2 mm in diameter and extended deep into the ocular epithelium. An intense red colour appears in the inflamed areas. Hence, the absence of a vivid red colour was considered a sign that an ulcer had healed. Each eye received for each rabbit [32,33]. Throughout the observation period, one drop of ciprofloxacin solution was administered as the control treatment each morning. During the 12-day consisted administering every morning, andrographolide nanosuspension. Using Table No. 2, the data on the healing of ocular inflammation was interpreted [33–35].

Table 2: Various inflammation scales of Eye- inflammation/ disease [27].

1	**Discharge**	No discharge	0
		Minimal discharge	1
		Moderate discharge	2
		Sticking of the eyelid with discharge	3
		Hair around the eye wetted with discharge and surrounding skin area inflammation	4
2	**Lid edema**	No lid edema	0
		Minimal edema	1
		Moderate edema	2
		Swelling on both eye lids	3
		Puffy swelling of eyelids	4
3	**Conjunctival congestion**	No congestion	0
		Minimal congestion	1
		Moderate congestion	2
		Bright red conjunctiva	3
		Beefy red conjunctiva	4
4	**Conjunctival necrosis**	No conjunctival necrosis	0
		Minimal conjunctival necrosis	1
		Minimal conjunctival necrosis	2
		Severe conjunctival necrosis	3

5. Short Term Stability studies

The manufactured F5 selected Nano suspension's short-term stability research. Two months and stored at 40^0C [36]. After two months, the sample was visually examined for any signs of sedimentation, and it was then analyzed for a variety of factors [37,38].

6. RESULT AND DISCUSSION

6.1 *In-vitro* transcorneal permeation study

Studies on in-vitro transcorneal permeation of formulation F5 after 4 hours reveal increased permeation over the goat cornea (45.44 0.17%) compared to that of formulation F2 (43.39 0.10%). Due to the higher permeability of STF in Eudragit RL100 polymer than Eudragit RS100 after 4 hours, AG permeation through the cornea of the eye increased from F5 formulation compared to F2 mentioned in Table 3 and figure 1.

Table 3: *In-vitro* transcorneal permeation study for andrographolide by using goat cornea.

Time (min)	Percent (%) permeated AG*	
	Batch F2	Batch F5
0	0	0
30	10.46 ± 0.07	11.25 ± 0.03
60	13.83 ± 0.17	14.12 ± 0.00
90	16.23 ± 0.06	22.19 ± 0.15
120	23.08 ± 0.10	24.18 ± 0.04
150	31.25 ± 0.5	27.70 ± 0.21
180	32.27 ± 0.14	29.17 ± 0.05
210	41.31 ± 0.21	41.61 ± 0.12
240	43.36 ± 0.22	45.44 ± 0.17
300	49.36 ± 0.10	54.28 ± 0.12
360	53.13 ± 0.08	58.28 ± 0.05
480	55.81 ± 0.05	64.88 ± 0.07
600	62.13 ± 0.00	70.49 ± 0.28
720	63.13 ± 0.11	71.70 ± 0.22

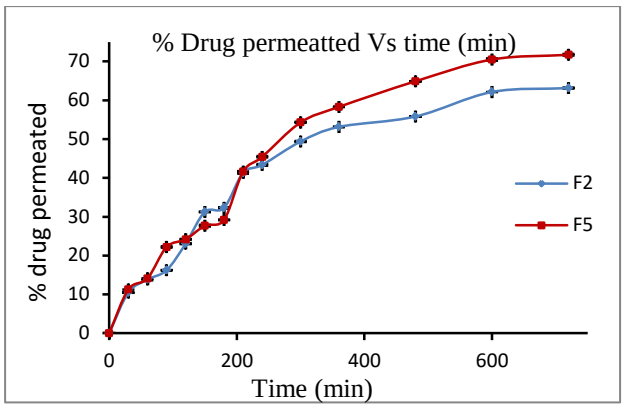


Figure 1: In-vitro transcorneal permeability profiles of formulations F2 and F5 in comparison.

6.2 *In-vitro* antimicrobial and antifungal activity

The agar plate (cup-plate) approach was used to test the microbiology of the F2 and F5 polymeric nanosuspension formulations. Zones of inhibition were clearly visible (Fig. No 2). Fig. No. 3 and Table No. 9.12 both for both used Gm +ve and Gm -ve bacteria, (Ciprofloxacin-100 g/ml) was close to 12.330.471 mm. The zone of inhibition for the employed Gm +ve bacteria and Gm -ve bacteria, respectively, was around 8.660.471 mm and 9.000.0 mm for the comparable F2 and F5 formulations (concentration-500 g/ml), with the exception of the S. aureus bacterium (no zone of inhibition observed). With the exception of S. aureus microorganism, the zone of inhibition for used Gm +ve bacteria and Gm -ve bacteria rose as the concentration of formulation F2 and F5 increased (no zone of inhibition observed).

The antifungal activity of the commercial formulation (fluconazole, 100 g/ml) was demonstrated on the Aspergilus niger fungus (zone of inhibition, 10.33 mm x 0.321 mm). Nevertheless, F2 and F5 formulations failed to exhibit any zone of inhibition for the A. niger fungus at varied concentrations (500 g/ml, 750 g/ml, and 100 g/ml).

Hence, the AG polymeric nanosuspension formulations F2 and F5 were shown to be less powerful than the marketed formulation (Ciprofloxacin and Fluconazole).

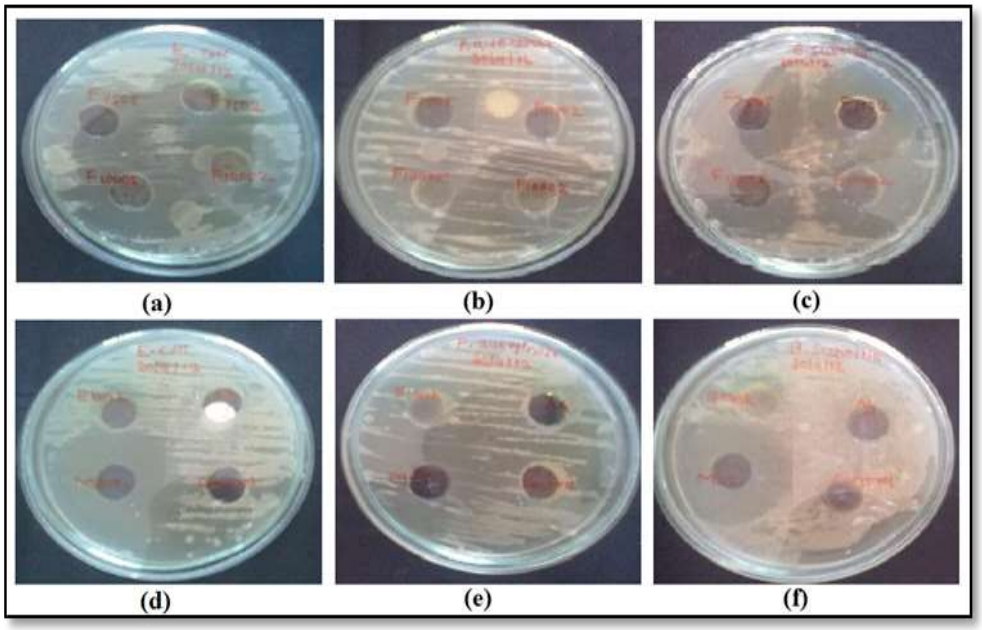


Figure 2: Antimicrobial activity of AG loaded nanosuspension formulation

Table 4: Comparison of antimicrobial and antifungal activity of AG loaded nanosuspension (batch F2 and F5) with marketed formulations ciprofloxacin and fluconazole.

Sample		Zone of inhibition diameter (mm)				
		Gm +ve bacteria		Gm -ve bacteria		Fungus
	Conc. (µg/ml)	Bacillus subtilis	Staphylococcus aureus	Escherichia coli	Pseudomonas aeruginosa	Aspergilus niger
AG	1000	2.33±0.471	-[b]	2.33±0.471	-[b]	-[b]
Batch F2*	500	2.33±1.69	-[b]	3.00±2.16	6.33±0.471	-[b]
	750	7.33±0.471	-[b]	7.00±0.816	13.00±0.0	-[b]
	1000	8.66±0.471	-[b]	7.66±0.471	12.33±0.471	-[b]
Batch F5*	500	3.33±0.471	-[b]	4.33±0.471	12.33±0.471	-[b]
	750	8.66±0.942	-[b]	7.66±0.471	13.33±0.471	-[b]
	1000	9.00±0.0	-[b]	8.66±0.471	13.66±0.471	-[b]
Ref	100	Ciprofloxacin 12.33±0.471	Ciprofloxacin 12.00±0.00	Ciprofloxacin 14.33±1.247	Ciprofloxacin 13.66±0.471	Fluconazole 10.33±0.321

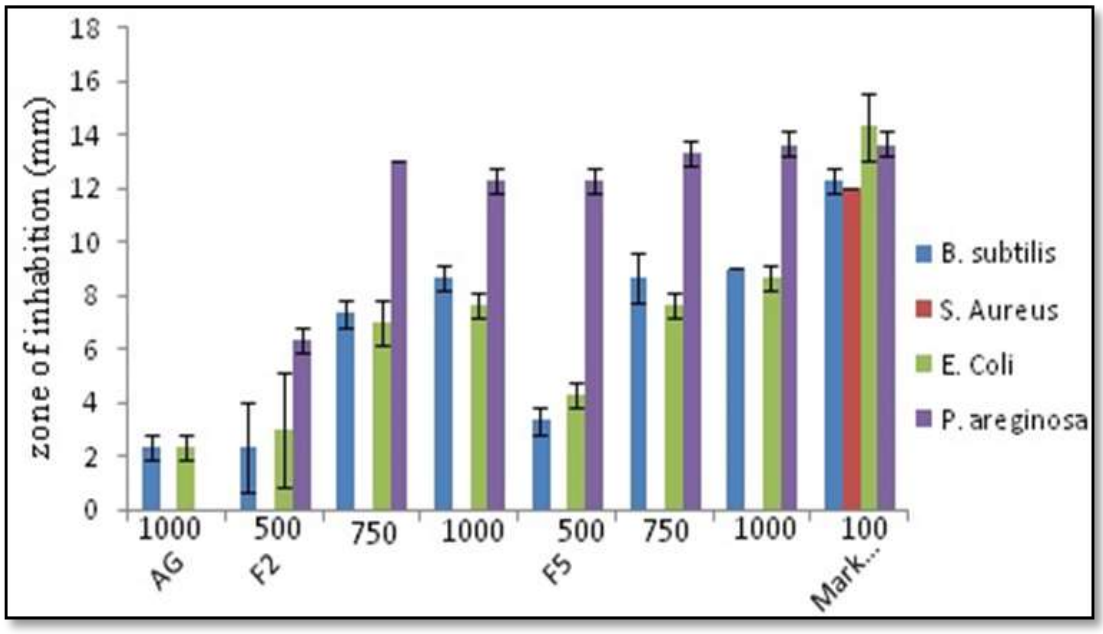


Figure 3: Comparison antibacterial activity of pure AG, AG loaded polymeric nanosuspension batches F2, F5 and marketed formulation (ciprofloxacin).

6.3 In-*vivo* studies

From of F2 and F5 polymeric nanosuspension formulations, F5 formulation was shown better result in *in-vitro* drug release study (Data analysis r^2= 0.903), antimicrobial activity and *in-vitro* trans-corneal permeation study than F2 formulation.

Ocular tolerability study:

In order for recommended critical to evaluate both the ocular tolerability and. Thus, using a modified Draize test technique, RS100 and Eudragit RL100 nanosuspension was assessed. In-vivo findings revealed no evidence of received a score of zero during all observations mentioned in table 5. Thus, it was better to employ the Eudragit RS100 and Eudragit RL100 loaded nanosuspension for ophthalmic preparation in-vivo.

Table 5: Data interpretation of *in-vivo* ocular tolerability study of formulation F5 by using rabbits.

Volunteers (Rabbits)	Eye observation after Dose instillation Time (min)	Eye observation				
		Right eye (Test formulation F5)				Left eye (distilled water/control)
		congestion	Swelling	Redness	corneal opacity	Congestion, swelling, redness and corneal opacity
1st	10	0	0	0	0	0
	360	0	0	0	0	0
	1440	0	0	0	0	0
2nd	10	0	0	0	0	0
	360	0	0	0	0	0
	1440	0	0	0	0	0
3rd	10	0	0	0	0	0
	360	0	0	0	0	0
	1440	0	0	1	0	0
4th	10	0	0	0	0	0
	360	0	0	0	0	0
	1440	0	0	0	0	0

***In-vivo* anti-inflammatory activity:**

The prepared polymeric nanosuspension (batch F5) was cure the inflammatory areas within 12 days, which produced by the thermal techniques. AG loaded polymeric nanosuspension was shown anti-inflammatory activity due to inhibition or supperation of inflammatory mediators like cyclo-oxygenase-2, interleukin-2, leucotrine and inhibit the production of reactive oxygen species. So, the AG loaded polymeric nanosuspension may be used in surgical trauma mentioned in figure 4 and table 6.

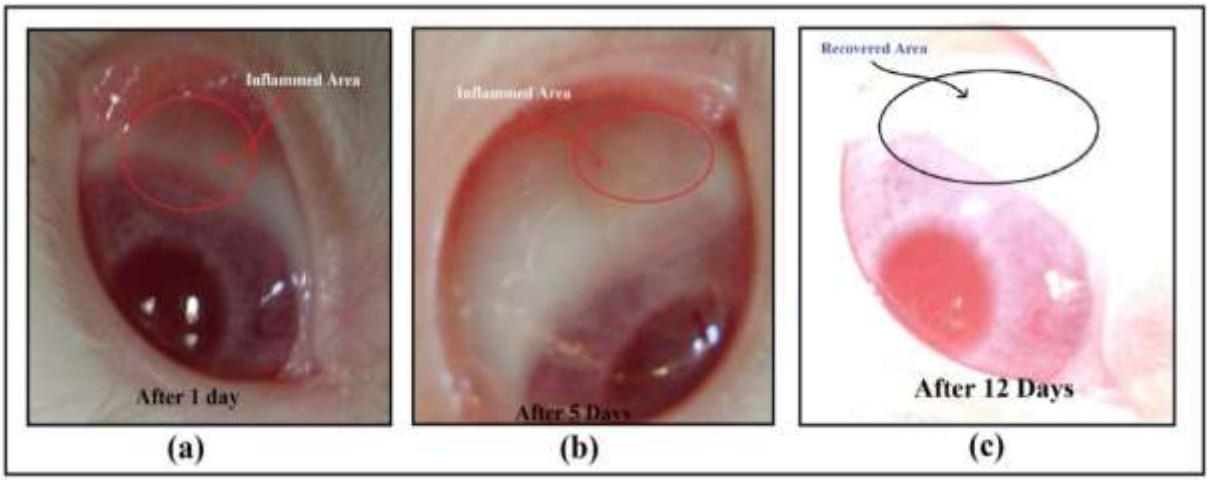


Figure 4: *In-vivo* anti-inflammatory activity of F5 formulation, After day 1(a); After day 5 (b); After day 12(c).

Table 6: Data interpretation of *in-vivo* anti-inflammatory activity.

Volunteers (Rabbits)	Eye observation after daily dose instillation (days)	Eyes observation (Conjunctival congestion)	
		Right eye (Test formulation F5)	Left eye (control)
1st	Initial condition	0	0
	After thermal tech.	3	3
	After day 1	3	3
	After day 5	2	3
	After day 12	0	3
2nd	Initial condition	0	0
	After thermal tech.	3	3
	After day 1	3	3
	After day 5	2	3
	After day 12	0	3

No congestion- 0, Minimal congestion- 1, Moderate congestion- 2, Bright red conjunctiva- 3.

6.4. Short Term Stability studies

No obvious variations in the amount of drug present and the amount of entrapment efficiency of the polymeric nanosuspension during the two months of stability testing were found (batch F5). It demonstrates that there was finished polymeric nanosuspension formed sediment after storage simple with and required more than 4 hours to sediment once more. Macroscopic characteristics did not alter. As compared to the initial values, the average size slightly increased, most likely as a result of particle aggregation. At 6 hours, the formulation (F5) likewise saw a rise in the rate of drug release. This might possibly be a result of the medicine that has been adsorbed onto the polymeric nanosupension surface. The polymeric nanosupension demonstrated excellent stability at 4°C and room temperature. As a result, it can be anticipated to be stable, secure, and functional even after extended storage mentioned table 7.

Table 7: Short term stability studies data interpretation.

days	% drug content*	% entrapment efficiency*	Sedimentation time (hrs)	Particle size* nm	pH*	% *in-vitro* release* (after 6 hrs)
Before storage						
0	76.18±0.30	97.80±0.88	>4	237±0.12	5.45±0.04	93.60±0.13
After storage						
30	75.80±0.12	96.89±0.45	>4	-	5.42±0.03	94.41±024
60	74.84±0.43	96.04±032	>4	-	5.39±0.04	96.45±0.25

7. CONCLUSIONS

In conclusion, this comprehensive assessment investigated the transcorneal permeation, antimicrobial, and antifungal activities of an Andrographolide-loaded nanosuspension through a combination of in vitro and in vivo studies. The findings demonstrated the potential of the nanosuspension formulation in efficiently delivering Andrographolide to ocular tissues by overcoming the barriers of the cornea. Moreover, the nanosuspension exhibited promising antimicrobial and antifungal activities, highlighting its potential for combating ocular infections and diseases. These results contribute to the growing body of knowledge on ocular drug delivery systems and emphasize the therapeutic applications of Andrographolide in ocular conditions. The comprehensive assessment of the transcorneal permeation, antimicrobial, and antifungal activities of the Andrographolide-loaded nanosuspension provides valuable insights for the development of novel and effective ocular therapeutics, addressing the limitations of current treatments and improving patient outcomes in ocular health.

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