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Green Synthesis of Metal Nanoparticles for Development of Antifungal Topical Gel Formulation

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ABSTRACT

Fluconazole is the most widely used antifungal agent for the treatment of fungal topical infections. However, its utility is limited due to the development of resistance against *Candida* species. Therefore, there is a need to optimize and improve the antifungal efficacy of Fluconazole. Metal nanoparticles have been reported to exert antimicrobial properties. In the present study, fluconazole-loaded silver nanoparticles were prepared by green synthesized for topical gel formulation. Fourier transform infrared spectroscopy (FTIR) analysis confirms the compatibility of excipients with the drug. The prepared silver nanoparticles were reddish-brown with an average size of about 50nm. Nanoparticle gel was prepared using neem extract and carbopol 934p for achieving sustained release topical delivery (F1 to F4); the F2 formulation showed the maximum drug content and good spread-ability characteristics and was selected as the optimized formulation. *In vitro* antifungal activity of optimized topical gel formulation (F2) against *Candida albicans* showed a zone of inhibition of 13± 0.02mm which was greater than the zone observed for nanoparticle suspension, indicating the synergistic effect of fluconazole and neem extract.

1. INTRODUCTION

Fungal infection or mycosis, the most common dermatological problem is generally caused by various fungal species such as *Candida albicans, Aspergillus* species, *Cryptococcus neoformans,Rhizopus spp, Absidia spp, Mucor spp, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioidis immitis, Penicillium marneffei,* etc. Among these fungal species, *Candida albicans* is the most common pathogenic fungal organism causing mycosis in humans. Fungal skin infection is characterized by inflammation, redness, itching, swelling, scaly skin, blisters, lesions, etc(1). Numerous topical, as well as oral antifungal therapies, are available for the management of fungal skin infections. The major class of antifungal agents is Azoles and the most prescribed medication is Fluconazole. However, the application of Fluconazole is limited due to the development of resistance against *Candida* species. Therefore there is a need for the development of a dosage form that maximizes the antifungal activity and minimizes the chances of development of resistance(2).

Medicinal plant extracts have been assessed for their curative role due to ease of availability, low cost, non-toxicity, and effective therapeutic utilization. Neem (*Azadirachta indica*) a member of the Meliaceae family contains a phytoconstituent “nimbidin” that exhibits potent antifungal properties(3).

Nanotechnology including the green synthesis of metal nanoparticles in curbing infectious diseases is an exciting research area along with widespread applications. Although, there are several chemical methods available for the preparation of metal nanoparticles “green synthesis” of nanoparticles has attracted a lot of interest due to their outstanding advantages over the chemical methods(4,5). (4,5) Green synthesis of nanoparticles is a plant-based approach where extracts of plants, algae, fungus, viruses, or bacteria are utilized for the preparation of nanoparticles. It is an alternative approach for synthetic chemical-based nanoparticles as it offers many advantages such as simple processing, rapid synthesis, scalability, biocompatibility, and low cost(6). Several metals used for the preparation of nanoparticles include gold, silver, copper, iron, zinc, titanium, etc. Among all these metals silver is the most commonly used in therapeutic applications as it exerts antimicrobial properties(7). Silver nanoparticles synthesized from plant extracts that are rich in bioactive constituents have been documented to possess antimicrobial properties against pathogenic and resistant microbes(8). For the prophylaxis of dermatological problems, the selection of an effective drug delivery formulation depends on the penetration of the drug through the

targeted skin layer, in this regard topical gel is the preferred formulation. It provides localized drug administration, is convenient, easy to use, less greasy, easy to remove, and avoids first-pass metabolism(9).

In the present study, neem extract was used to prepare silver nanoparticles to avoid the development of resistance and improvement the efficacy of fluconazole. Fluconazole-loaded silver nanoparticles were prepared and were subsequently formulated into a gel matrix for achieving a sustained release profile for topical drug delivery.

2. MATERIALS AND METHODS

Fluconazole and Neem extract was received as a gift sample from Dayaram Pharma Company, Gujarat, India, and Ayush Herbs Himachal Pradesh, India respectively. Silver nitrate was purchased from Fischer Scientific Pvt. Ltd. All the chemicals were of analytical grade and used as received.

2.1 Drug-excipients compatibility studies

The compatibility of the drug with excipients was ascertained by FTIR (Bruker Alpha, Berlin, Germany) analysis. Drug and various excipients were mixed thoroughly in a ratio of 1:1 and samples were scanned by FTIR in the range of 400-4000 cm^{-1}.

2.2 Preparation of calibration curve

Standard stock solution (1mg/ml) was prepared by dissolving Fluconazole in methanol. A series of dilutions in the range of 20-140µg/ml were prepared by dilution of stock solution with methanol. The absorbance of the samples was recorded at 260nm using a UV-visible spectrophotometer (Shimadzu, Japan) and a standard curve was plotted.

2.3 Preparation of Silver Nanoparticles by Green Synthesis.

The solution of silver nitrate was prepared in a flask. Neem extract was added separately to silver nitrate solution in concentrations shown in Table No. 1. Then weighed amount of Fluconazole was added to it. The resultant solution was sonicated for a few minutes and thereafter incubated for 24h in a dark chamber to minimize photo-activation or degradation of silver nitrate. The reduction of Ag^{+} to Ag^{0} was confirmed by the color change of the solution from colorless to brown(10).

Table No. 1: Composition of Fluconazole loaded silver nanoparticles from green synthesis.

Sr.No	Formulation	Amount of Fluconazole(mg)	Amount of Silver nitrate (mM)	Neem extract(ml)
1	T1	10	0.5	1
2	T2	10	0.5	2
3	T3	10	0.5	3
4	T4	10	1	1
5	T5	10	1	2
6	T6	10	1	3
7	T7	10	2	1
8	T8	10	2	2
9	T9	10	2	3

2.4 Percentage Entrapment Efficiency

1ml sample was centrifuged at 14,000 rpm (Centurion, Scientific Ltd., UK) for 60 min at 4 °C. The supernatant was separated from the precipitated nanoparticles which were washed twice with methanol. Then, the clear fraction (supernatant) was collected and assayed for the free unentrapped drug. The amount of unentrapped drug was determined, in the collected supernatant, by UV-visible spectroscopy at λ_{max} 260 nm (Shimadzu-1700 UV, Japan)(11).

$$\% \, Entrapmenteff iciency = \frac{TotalDrugconcentration - concentrationof druginsupernatant}{Totaldrugconcentartion} X\ 100$$

2.5 Determination of particle size and zeta potential

Particle size and zeta potential were determined by photon correlation spectroscopy using a zeta sizer. Transmission electron microscopy was used for particle size analysis.

2.6 Formulation of gel containing fluconazole loaded silver nanoparticles

The optimized nanoparticle formulation (T1) was formulated into the gel. Carbopol 934P was used to prepare the gel base. 10ml of optimized formulation was taken & carbopol 934P was added to in a series of concentrations to get different percentage compositions of gel. The composition of each gel is shown in Table No.2. The system was placed at room temperature

for 24 hrs till the nanoparticles got completely incorporated into the gel. The pH of the solution was adjusted to 7 with 1M of NaOH. Thereafter neem oil, preservatives, and moistening agents were added to the formulation (Table No. 2)(12).

Table No. 2: Composition of different topical gel

Ingredients	**Formulation Code**			
	(F1)	(F2)	(F3)	(F4)
Fluconazole loaded silver nanoparticles formulation (ml)	10	10	10	10
Carbopol 934p (mg)	1	1.5	2	2.5
Glycerin (ml)	10	10	10	10
Methyl paraben (mg)	0.03	0.03	0.03	0.03
Propyle paraben (mg)	0.01	0.01	0.01	001
Neem oil (ml)	0.004	0.004	0.004	0.004
NaoH solution	q.s	q.s	q.s	q.s
Purified Water (ml)	100	100	100	100

2.7 Evaluation of optimized formulation

2.7.1 Physical Appearance: The appearances of the formulation were observed by visual observation. The observed appearances of the prepared systems were tested on color, and clarity(13).

2.7.2 pH determination

The pH of gel formulations was determined using a digital pH meter. One gram of gel was taken and the pH was recorded using a pH meter by bringing it in contact with the gel and allowing it to equilibrate for 1 min. pH was measured in triplicate and average values were calculated(14).

2.7.3 Viscosity: The viscosity of the sample was measured using a Brookfield viscometer, using an L-4 spindle at 10 & 50 pm. The measurements were carried out in triplicate(13).

2.7.4 Spreadability Study

The Spreadability of the gel was determined using the following technique: 0.5 g of gel was placed within a circle of 1 cm diameter premarked on a glass plate over which a second glass

plate was placed. A weight of 500 g was allowed to rest on the upper glass plate. The increase in the diameter due to the spreading of the gels was noted(15).

$$Spreadibility = weighttiedonupersideX \frac{lengthofglassslide}{timeinseconds}$$

2.7.5 Drug content

The drug content of the different preparations gel formulations was determined separately, about 500 mg of the gel was weighed in a 100 mL volumetric flask and dissolved in 50 mL of phosphate buffer of pH 7.4. The volumetric flask was kept for 2 h and shaken well in a shaker to mix it properly. The resulting solutions were filtered with a 0.45mL filter to obtain clear solutions. The drug content was measured spectrophotometrically at a wavelength of 260 nm(16).

2.7.6 *In vitro* drug release study

Franz diffusion cell was used to determine the release profile of the drug from nanoparticles. The cells consisted of donor and receptor chambers between which a cellophane diffusion membrane. The capacity of the receptor chamber was 18 ml. A magnetic bead was placed in the receptor chamber. The diffusion medium consisted of phosphate buffer pH 7.4. The whole assembly was put on a magnetic stirrer at 100rpm stirring speed and the temperature was maintained at 37.0 ± 0.5 C. 1g sample was kept over the membrane in the donor compartment and stirred. 1ml sample was withdrawn from the receptor compartment at predetermined time intervals, and the volume was replenished with the same volume of diffusion medium. The addition of a diffusion medium to the receptor compartment was performed with great care to avoid trapping air beneath the diffusion membrane. The samples were analyzed spectrophotometrically at 260nm after appropriate dilutions. The % drug release was calculated and a graph of % drug release vs time was plotted. For each formulation, the release studies were performed in triplicate(17).

2.7.7 Drug release kinetic studies

The drug release profile was fitted into different mathematical models, namely zero order, first order, Higuchi's Model, and the Korsmeyer-Peppas model. Graphs were plotted and the R^2 value was determined(18,19).

2.8 Anti-fungal studies

Weighed 16.25 gm of sabouraud dextrose agar was transferred in 500 ml of the conical flask and 250 ml of purified water and some amount of heat is applied to dissolve it completely. Sterilized for 15 min at 121°C at 15 lb pressure in an autoclave for about 20 min. The resultant solution was cooled to room temperature and the fungal strain(*Candida albicans*2 × 10^4 cells/ml) was dispersed in the medium under sterile conditions. The medium was poured into the Petri dish and allowed to solidify. Thereafter two wells were bored in each Petri dish with the help of a sterile steel bore of 6 mm and the calculated concentration of the sample was placed in the bores. The Petri plates were then incubated for 72 h at 25-30°C in incubators. Blank nanoparticle gel was used as the control. Optimized gel formulation(F2) was used as a test and was compared with drug-loaded nanoparticulate suspension (Formulation T2). The zone of inhibition was measured after incubation(20).

3. RESULTS AND DISCUSSION

3.1 Drug-excipients compatibility studies

Fig. No. 1 shows the FTIR spectra for fluconazole (Figure 1a), Carbopol 934p (Figure 1b), Neem extract (Figure 1c), and silver nitrate (Figure 1d). Figure 1e represents the FTIR spectra of the drug mixed with all the different excipients. It is clear from the figure that there is no new peak observed in the physical mixture in comparison to that of pure drugs and other excipients. This indicates the absence of any incompatibility between the different formulation components.

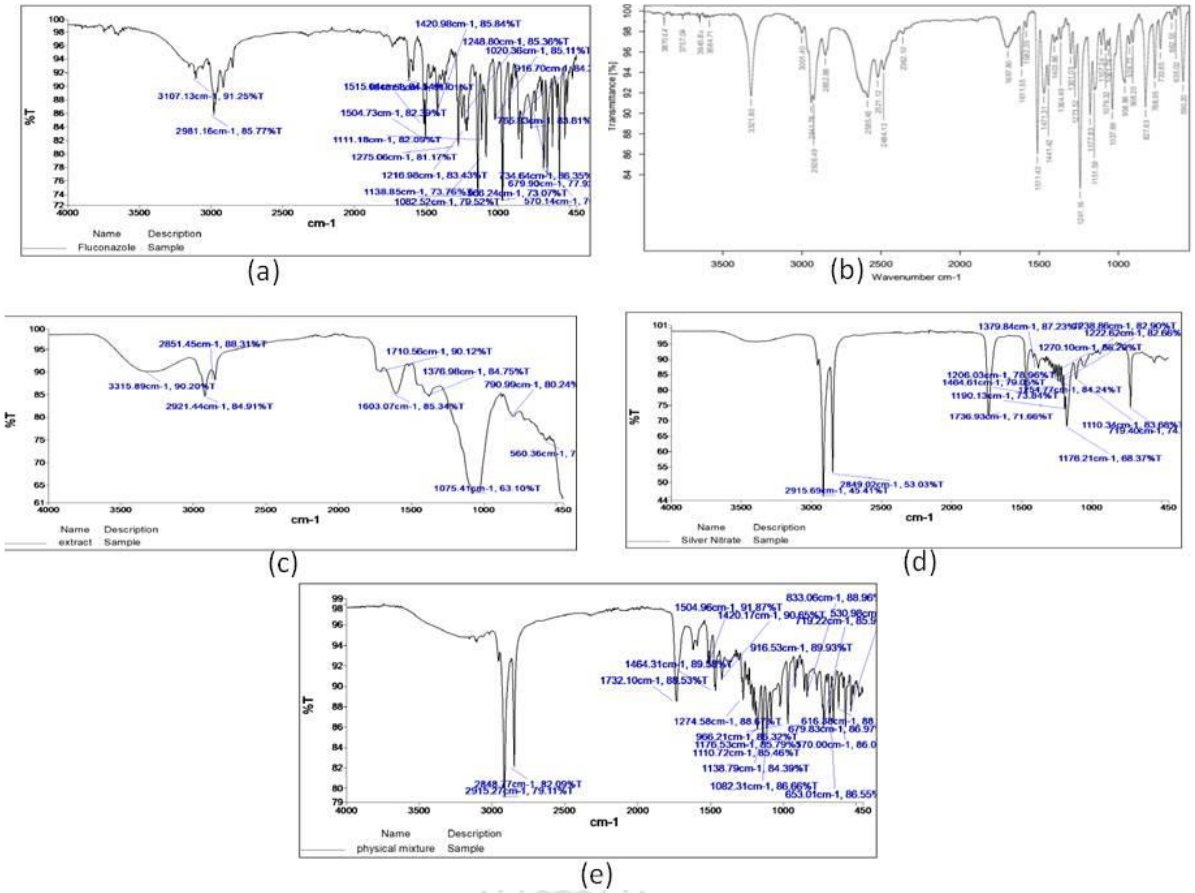
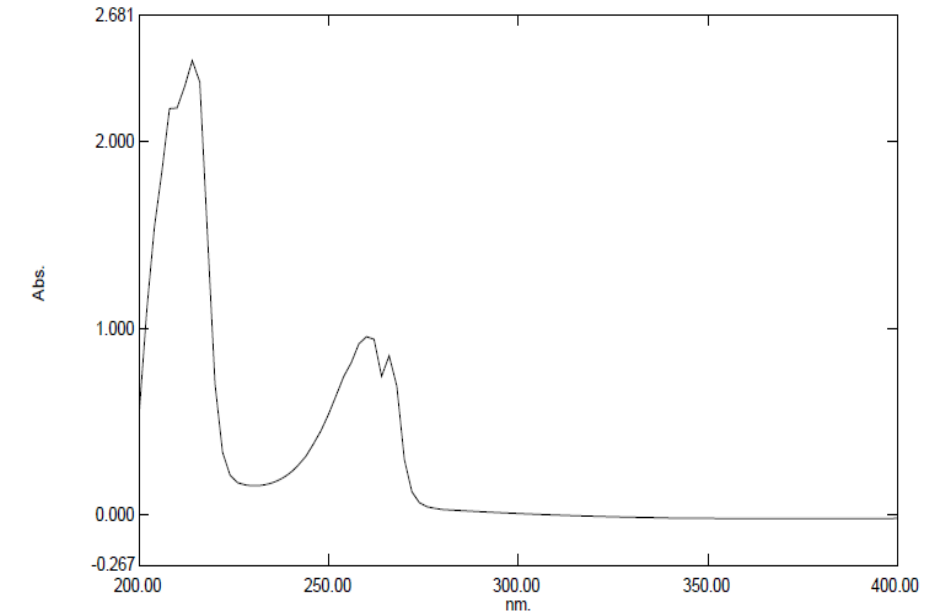


Fig. No. 1: FTIR spectra of Fluconazole (a), Carbopol 934p (b), Neem extract (c), Silver Nitrate (d), Physical mixture (e)

3.2 Calibration curve of Fluconazole

The absorption maxima for Fluconazole were obtained at 260 nm (Fig. No. 2), this was similar to that reported in the literature. The calibration curve obtained showed linearity in the range of 10-45 µg/ml, with $R^2 = 0.999$. The regression equation obtained was $y = 0.018x + 0.013$



[Measurement Properties]
Wavelength Range (nm.): 200.0 to 400.0
Scan Speed: Medium
Sampling Interval: 2.0

[Instrument Properties]
Instrument Type: UV-1800 Series
Measuring Mode: Absorbance
Slit Width: 1.0

No.	P/V	Wavelength	Abs.	Description
1	↑	266.00	0.853	
2	↑	260.00	0.954	
3	↑	214.00	2.436	
4	↓	264.00	0.742	
5	↓	230.00	0.156	

Fig. No. 2: UV spectra of Fluconzole

3.3 Evaluation of Fluconazole loaded silver nanoparticles

The fluconazole-loaded silver nanoparticles appeared reddish-brown and their particle size was 50nm. The yield was found to be in the range of 53% to 75%. T1 formulation had a maximum yield of 75%. Its zeta potential was observed to be -26mV, while particle size was in the range of 50±0.45nm. This formulation was further used for the development of nanoparticulate gel formulation.

3.4 Evaluation of nanoparticulate gel

Nanoparticulate gels (Fig. No. 3)were prepared with varying concentrations (Formulation F1-F4) of carbopol 934P. Uniform gels were formed for all formulations except F1. Therefore, further tests were not conducted on F1.

the pH of all the formulations F2-F4 was found to be in a range of 7.57±0.010to 7.6±0.017, while the viscosity was found to be in the range of 14080±10.00cpsto 22033±10.01cpsat 10rpm and 11234±10.06cps to 19532±10.408cps at 50rpm (Table No. 3).

Spreadability is an important property of topical formulation from a patient compliance point of view. The diameter was found to be in ranges of 7.84±0.017 to 6.47±0.012cm which is indicative of good spreadability.

The drug content gels were found to be 90.74±0.31%to 87.00±0.36% respectively. The percentage drug content of all formulations was found to be satisfactory. Hence, the method adopted for gel formulations was found to be suitable.

F2 formulation showed the maximum drug content and good spreadability characteristics, therefore it was selected as the optimized formulation.

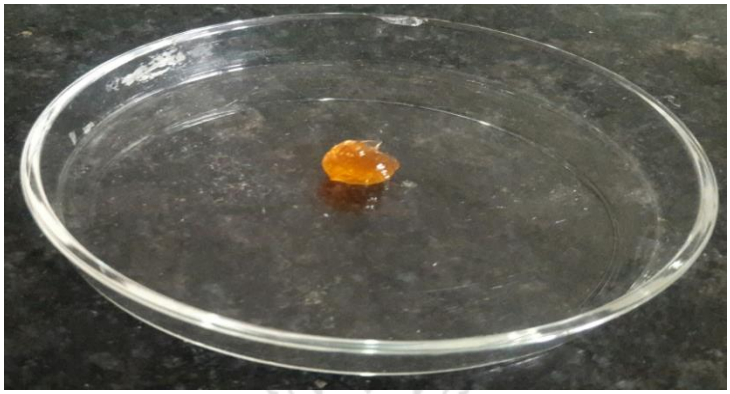


Fig. No. 3: Visual Appearance of Fluconazole loaded silver nanoparticles gel

Table No. 3: Evaluation of nanoparticulate gel formulations

Parameter	**Formulation F2**	**Formulation F3**	**Formulation F4**
Visual Appearance	Uniform gel formed	Uniform gel formed	Uniform gel formed
pH	7.57±0.010	7.44±0.026	7.6±0.017
Viscosity (cps) at 10rpm	14080±10.00	18068±9.50	22033±10.01
Viscosity (cps) at 50 rpm	11234±10.06	16285±8.021	19532±10.408
Spreadibility (cm)	7.84±0.017	7.43±0.026	6.47±0.012
% Drug Content	90.74±0.31	87.00±0.36	84.74±0.18

3.5 *In vitro* drug release studies

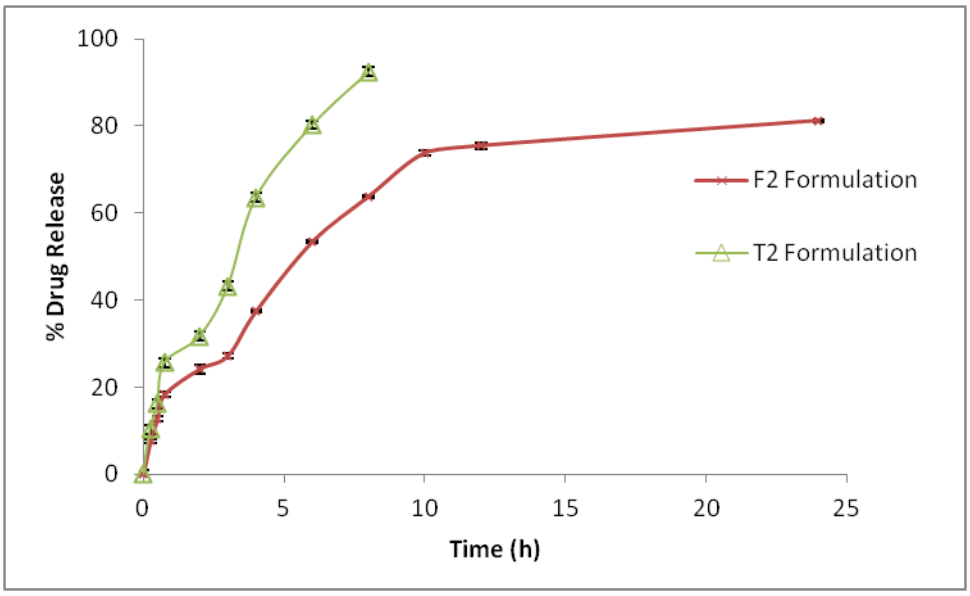


Fig. No. 4: *In-vitro*Fluconazole release from nanoparticle suspension (T2) and nanoparticle gel (F2)

The *in-vitro* drug release of F2 and T2 formulation was carried out to see the effect of the incorporation of nanoparticles into a gel. The gel formulation (F2) significantly prolonged the drug release when compared to the nanoparticulate formulation (T2). Drug release from Formulation T2 showed burst release characteristics with about 30% drug release in 2h and around 90% release within 8h. F2 formulation on the other hand demonstrated a sustained release profile with 81.2±0.31% drug release within 24 hr. The sustained release profile from gel formulation can be attributed to the development of polymer matrix by carbopol 934P, which delays the drug release from the gel matrix.

3.6 Drug release kinetic studies

Mathematical models were used to find the drug release mechanism from the optimized formulation (F2). Graphs were plotted between the % drug release vs time (zero-order), log percent drug remaining vs time (first-order), log percent drug release vs square root of time (Higuchi plot), and log of log % drug release vs. log time (Korsmeyer and Peppas Equation) (Fig. No. 5) and the value of R^2 value was calculated.

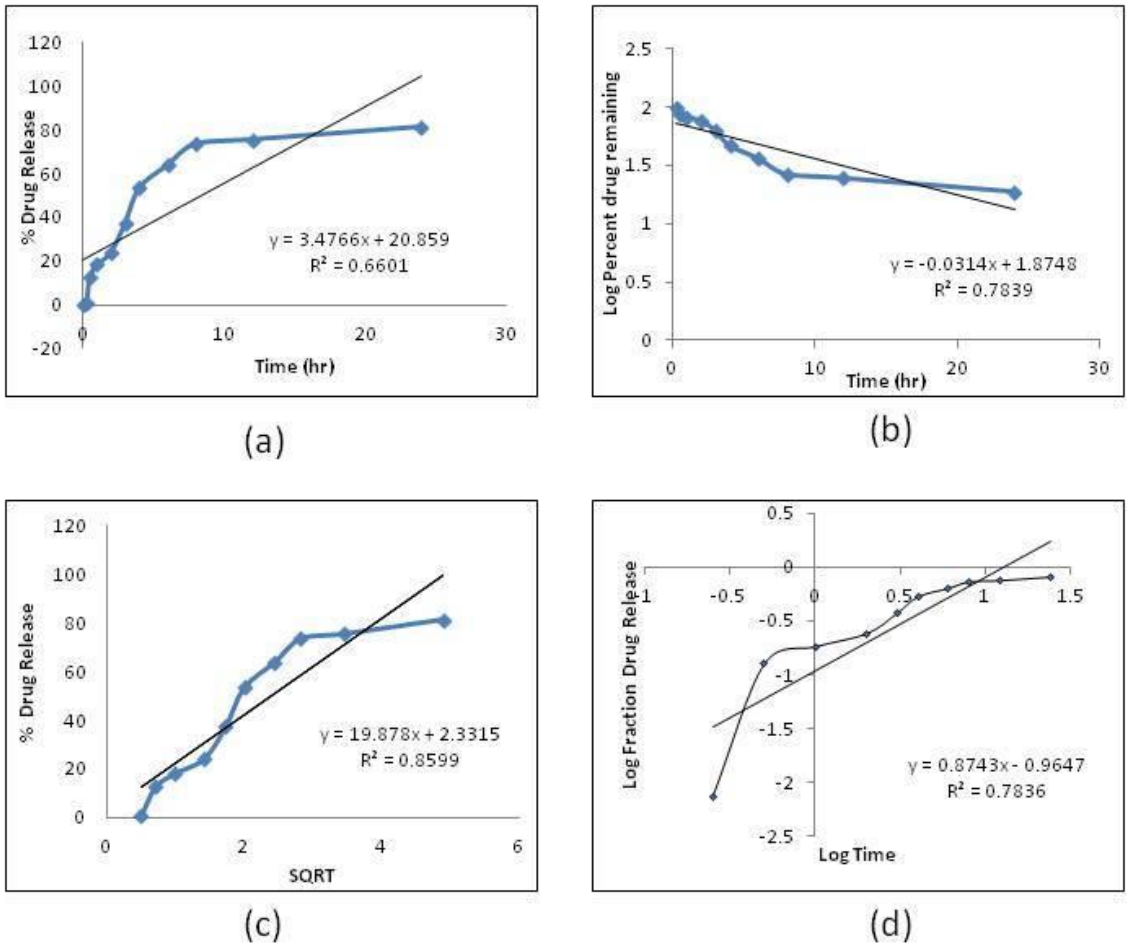


Fig. No. 5: Drug release data fitting into different kinetic models

From figure 5 it is clear that a maximum of R^2=0.859 was obtained for the Higuchi model, indicating that the data shows the best fit into the square root of time kinetics. Therefore, the drug release from fluconazole-loaded silver nanoparticles gel occurs by diffusion mechanism.

3.7 Antifungal activity

Candida albicans was used to study the antifungal activity of a gel. The zone of inhibition for formulation T2 was 4±0.02 mm and forF2 Formulation was 13± 0.02mm, while no zone was observed in the control group. The increase in the zone of inhibition obtained for F2 could be attributed to the presence of neem extract in the formulation, which has been reported to have significant anti-*candida* activity(21). Therefore, a synergistic antifungal effect of fluconazole and neem extract was observed.

4. CONCLUSION

Fluconazole is an imidazole derivative that was used to formulate topical gel for efficient delivery of drug across the skin. Silver nanoparticles were prepared by green synthesis. The nanoparticles were evaluated for physicochemical properties. T2 formulation showed maximum drug entrapment efficiency. From the optimized nanoparticle suspension, gel formulations (F1, F2, F3, F4) were developed using polymer (carbopol 934p) and evaluated for drug content, viscosity, spreadability, and *in vitro* diffusion study. Formulation F2 demonstrated maximum drug content and good spreadability characteristics. Anti-fungal studies showed synergistic antifungal activity of neem extract and fluconazole with a zone of inhibition of 13± 0.02mm. Moreover, the nanoparticulate gel formulations were able to sustain the drug release for 24h in comparison to drug-loaded nanoparticle suspension which showed burst release characteristics. Thus, the developed nanoparticulate formulation provided a sustained release fluconazole release. The release rate from nanoparticulate gel formulation showed the best fit to the Higuchi model.

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