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Investigation of Naringin's Antifungal Potential, Optimization and Characterization of Niosomal Gel Formulation

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Abstract - This research aims to assess the antifungal efficacy of Naringin and establish its minimum inhibitory concentration for the development and characterization of a niosomal gel formulation. A molecular docking study was conducted to elucidate the binding interactions between Naringin and Methionine synthase. *In-vitro* screening for antifungal activity against *T. mentagrophytes* was performed. To enhance drug stability, niosomal formulations were prepared using varying ratios of span 60 (a non-ionic surfactant) and cholesterol (a stable vesicle-forming agent) via the thin film hydration method. The niosomes were evaluated for entrapment efficiency, particle size, and polydispersity index. A niosomal gel was formulated using Carbopol 934, glycerol, and distilled water, and evaluated for physical appearance, pH, viscosity, spreadability, *in-vitro* drug release, *in-vitro* antifungal activity through an agar well diffusion method, and skin irritation for the optimized formulation. Naringin exhibited potent antifungal activity with a MIC of 50 µg/ml against *T. mentagrophytes*. The optimized niosomal gel formulation (Batch F3) demonstrated the highest entrapment efficiency at 75.88% and a 62.12% *in-vitro* release rate. The gel exhibited no skin irritation (score of 0) and a zone of inhibition of 4.6 cm in the *in-vitro* antifungal test. The findings suggest that Naringin possesses significant antifungal activity against *T. mentagrophytes*, and the newly developed niosomal gel formulation offers enhanced efficacy without causing skin toxicity.

Keywords**:** Naringin, Niosomes, Nanoformulation, dermatophytes, anti-fungal

1. Introduction

Fungal infections, particularly those caused by dermatophytes, have become a significant public health concern worldwide [1]. *Trichophyton mentagrophytes* is a common causative agent of various superficial fungal infections, including athlete's foot, jock itch, and ringworm. Conventional antifungal treatments often face challenges such as limited efficacy, drug resistance, and adverse effects [2]. Therefore, there is a pressing need to explore alternative therapeutic options, including natural compounds with antifungal properties.

Naringin, a flavonoid glycoside present in citrus fruits, has gained attention for its diverse pharmacological activities, including antimicrobial, anti-inflammatory, and antioxidant properties [3-6]. Previous studies have demonstrated the potential of Naringin as an antifungal agent against various fungal species [7-8]. However, its practical applications are limited due to low aqueous solubility, poor bioavailability, and stability issues [9,10].

Niosomal drug delivery systems have emerged as promising carriers for enhancing the solubility, stability, and targeted delivery of poorly soluble drugs. Niosomes are non-ionic surfactant-based vesicles that can encapsulate hydrophilic and hydrophobic drugs, improving their bioavailability and therapeutic efficacy [11-12]. Additionally, niosomal formulations can be incorporated into gel formulations for topical administration, offering advantages such as improved drug retention, controlled release, and increased patient compliance.

This research aims to investigate the antifungal potential of Naringin against T. mentagrophytes and optimize a niosomal gel formulation to enhance its efficacy and stability. The specific objectives are:

1.To evaluate the antifungal activity of Naringin against T. mentagrophytes and determine its minimum inhibitory concentration (MIC).

2.To develop and optimize a niosomal formulation for Naringin using different ratios of span 60 and cholesterol.

3.To characterize the optimized niosomal formulation for entrapment efficiency, particle size, and polydispersity index.

4.To formulate and characterize a niosomal gel containing the optimized niosomal formulation.

5.To assess the in-vitro drug release, antifungal activity, and skin irritation potential of the developed niosomal gel formulation.

2. Materials and Methods

2.1. Materials

The materials used in this study were of analytical grade. Cholesterol was obtained from Lipoid Pharma to stabilize the niosomal membrane. Methanol (Sujata Chemical) was chosen as the solvent due to its efficient solubility for the drug and stability in the UV region. Terbinafine gel (Sebifin) was used as the standard drug for comparison. Carbopol 934 (Oxford Lab Fine Chem LLP) was utilized for gel preparation. Span 60 (Suvidhinath Laboratories) was employed to achieve the desired hydrophilic-lipophilic balance. Chloroform (Loba Chemie Pvt. Ltd) facilitated the dissolution of other substances. Potassium dihydrogen orthophosphate and sodium hydroxide (Fisher Scientific, Mumbai) were used as buffer agents. Glycerin (Chiti-chem Corporation, Baroda) was incorporated as a moisturizer. Naringin (Yucca Enterprises, Batch: yucca/NHY/2022/11/05) was the antifungal drug under investigation. *Trichophyton mentagrophytes* strain (MCC 1888) was procured from the National Centre for Microbial Resource (NCMR).

2.2. *In Silico* Study

The 3D structure of *Candida albicans* Methionine Synthase in complex with Methionine was obtained from Protein data Bank (PDB ID 4L61). Ligands and targeted enzyme were prepared for docking using ChemDraw Ultra 12.0, ChemDraw 3D, 2D, and BIOVIA Discovery Studio Visualizer 4.5. Grids for docking were prepared, and the affinity values of the ligand molecules were estimated and compared to the endogenous ligand using AutoDock Vina 4.1 and BIOVIA Discovery Studio Visualizer 4.5.

2.3. *In-vitro* Antifungal Activity and MIC Determination

Various concentrations of naringin (1, 50, 100, 250 µg/mL) were added to the Dermatophyte Test Medium (DTM). The DTM plates were inoculated with *T. mentagrophytes* by spreading the fungal culture evenly on surface of agar plates using sterile cotton swab followed by incubation at 25°C for 7 days [13].

2.3. Preparation of Naringin-loaded Niosomes

Niosomes were prepared using the thin-film hydration technique [14]. Naringin, cholesterol, chloroform, and varying ratios of Span 60 and methanol were dissolved in a round-bottom flask. The organic solvents were evaporated at 40°C to form a thin surfactant film. The film was rehydrated with distilled water containing the herbal drug (5 mg) at 45°C for 20 minutes. The resulting niosomal suspension was sonicated for 15 minutes using a bath sonicator.

2.4. Evaluation of Niosomes

The entrapment efficiency of niosomes was determined by centrifugation at 10,000 rpm for 40 minutes at 4°C. The unentrapped drug concentration in the supernatant was measured using a UV-visible spectrophotometer at 285 nm. To construct the calibration curve, a standard stock solution of naringin (1000 µg/mL) was prepared in methanol. Serial dilutions were made to obtain concentrations ranging from 2 to 12 µg/mL. The absorbance was measured at 285 nm using a UV-visible spectrophotometer. The particle size distribution was analyzed using a Malvern Zeta Sizer. Transmission Electron Microscopy (TEM) and Fourier Transform Infrared Spectroscopy (FTIR) were employed for characterization [15].

2.5. Preparation of Naringin Niosomal Gel

The niosomal suspension (10 mL) containing naringin was incorporated into a gel base composed of Carbopol 934 (1 g), glycerol (10 mL), and distilled water (up to 15 mL) using a magnetic stirrer for 30-60 minutes [16].

2.6. Evaluation of Gel

The physical appearance, viscosity (Brookfield Viscometer), spreadability, pH (digital pH meter), and in vitro drug release (Franz diffusion cell with a semi-permeable dialysis membrane) of the niosomal gel were evaluated [16].

2.7. Acute Skin Irritation Study

Healthy albino Wistar rats (150-250 g) were used for the acute skin irritation study after obtaining approval from the Institutional Animal Ethics Committee (Protocol No. PIPH 04/22). The animals were housed under controlled conditions of temperature (22±2°C), humidity (55±5%), and a 12-hour light/dark cycle. The fur was removed 24 hours before the test using depilatory cream. The test gel was applied to 1cm^2 area of the shaved skin and covered with a gauze patch. The site

was observed 1 day after the removal of the test substance, and the observation was repeated for a period of 7 days. The scoring system was used to evaluate erythema, eschar formation, and edema formation [17].

2.8. Evaluation of Antifungal Activity by Agar Plate Method

The antifungal activity of the formulation was evaluated using the agar well diffusion method [18]. Agar plates were inoculated by spreading a volume of the fungal culture over the entire agar surface. Subsequently, a hole with a diameter of 6 to 8 mm was punched aseptically using a sterile cork borer, and a volume (1 g) of the antifungal gel was introduced into the well. The plates were incubated for 24-36 hours at 37°C. After 7 days, the zone of inhibition was quantified using a scale.

3. Results

3.1. Molecular Docking

Molecular docking studies revealed that the lead molecule (naringin) interacts with the amino acid residues ASN A:503, HIS A:707, GLY A:740, THR A:743, LYS A:742, CYS A:739, and GLU A:499 of the target protein. The binding affinity obtained was -9.7 (Figure 1).

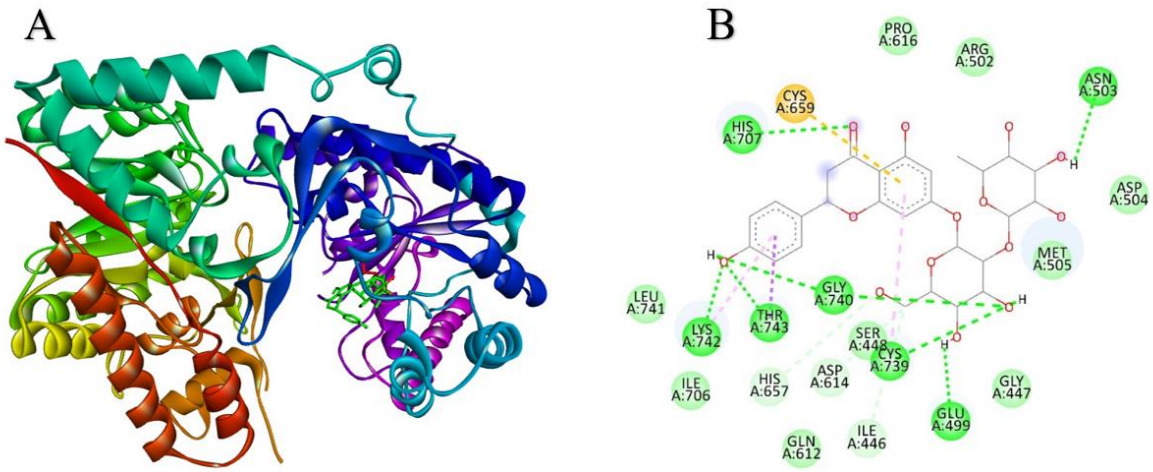


Fig. 1: Docking of Naringin with Methionine synthase
A: Target protein Met6p bound with Naringin (red molecule) and Methionine (green molecule);
B: Binding interactions of Naringin with target protein Met6p

3.2. Calibration Curve and UV Analysis

The calibration curve for naringin in methanol was constructed by measuring the absorbance at 285 nm for concentrations ranging from 2 to 12 µg/mL. The calibration curve exhibited a linear relationship with a regression equation of $y = 0.197x + 0.007$ and an R^2 value of 0.999, indicating that the drug concentration between 2-12 µg/mL follows the Beer-Lambert law (Figure 2A).

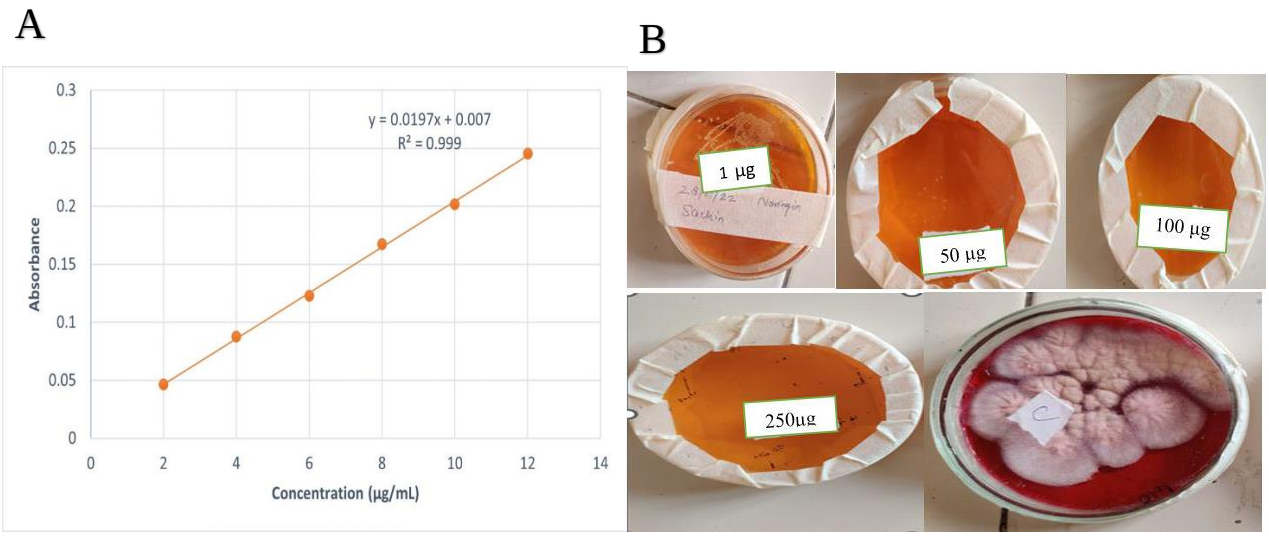


Fig. 2: Calibration Curve (A) and Minimum Inhibitory Concentration of Naringin Against *T. mentagrophytes*(B)

3.3. *In Vitro* Antifungal Studies

The minimum inhibitory concentration (MIC) of naringin against *T. mentagrophytes* was determined to be 50 µg/mL using the Dermatophyte Test Medium (DTM) agar. Concentrations of 1 µg/mL showed fungal growth, while concentrations of 50, 100, and 250 µg/mL exhibited no growth after 7 days of incubation (Figure 2B).

3.4. Niosome Preparation and Characterization

Eight batches of naringin-loaded niosomes (F1-F8) were prepared using the thin-film hydration technique. The particle size analysis revealed that batch F7 had the largest particle size (143.5 nm), while batch F1 had the smallest particle size (104.0 nm) (Table 1). Batch F3 exhibited the highest entrapment efficiency of 75.88% (Table 1).

The zeta potential of batch F3 was determined, and the corresponding zeta potential graph is shown in Figure 3. Transmission electron microscopy (TEM) analysis confirmed the spherical shape and well-defined wall structure of the niosomes, with sizes consistent with the particle size analysis. Fourier Transform Infrared Spectroscopy (FTIR) characterization of pure naringin revealed the presence of characteristic functional groups, including CH stretching (2921.57 cm^{-1}), C=C stretching (1513.81 cm^{-1}), and C-H (aldehyde) (1449.87 cm^{-1}), which matched the standard values.

Table 1: Particle size analysis and Entrapment efficiency of various batches of Naringin-loaded niosomes

Batches	Particle size (nm)	Entrapment efficiency (%)
F1	104.0	72.5%
F2	133.6	73.89%
F3	114.3	75.88%
F4	125.5	72.89%
F5	115.1	70.96%
F6	142.7	68.37%
F7	143.5	67.94%
F8	128.2	65.66%

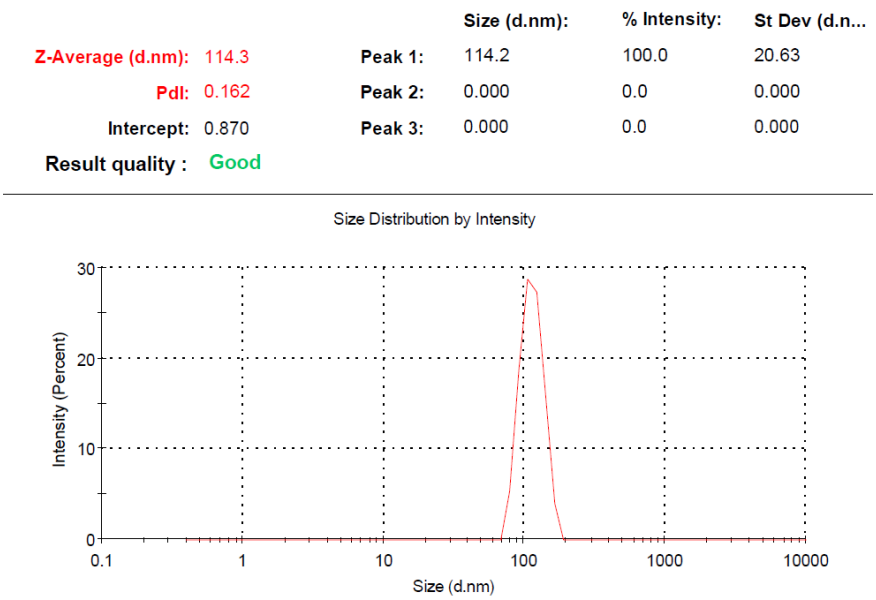


Fig. 3: Zeta Potential Distribution of Batch F3

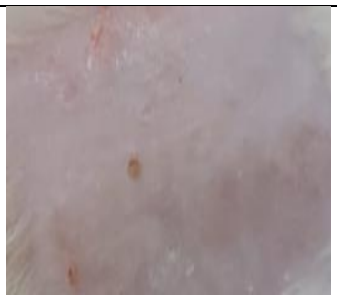
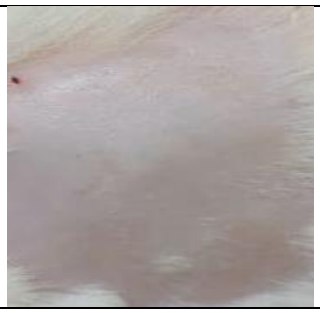
3.5. Niosomal Gel Evaluation

Based on the evaluation results, batch F3 was selected as the optimized formulation for the preparation of the naringin-loaded niosomal gel using Carbopol 934 and glycerol. The niosomal gel exhibited a white viscous appearance, free of particulate matter, with a pH range of 5.9 to 6.2. The spreadability of the gel was 5.2 cm/sec, and the viscosity was 9570 cps. *In vitro* drug release studies revealed a 62.12% cumulative drug release after 12 hours.

3.6. Skin Irritation Studies

The skin irritation studies performed on the niosomal gel formulation showed no signs of erythema, itching, or skin irritation, with a score of 0 on day 7 for all animals tested (Table 2).

Table 2. Representative images of skin irritation study performed on 3 Wistar rats on day 7

Animal Number	**1**	**2**	**3**
Skin Condition on Day 7			
Irritation Score	0 (No irritation)	0 (No irritation)	0 (No irritation)

3.7. Evaluation of Antifungal Activity

The antifungal activity of the niosomal gel was evaluated using the agar well diffusion method. The zone of inhibition for the niosomal gel (4.6 cm) was found to be larger than that of the standard terbinafine gel (4.3 cm) after 4 days of incubation, indicating the superior antifungal efficacy of the developed formulation (Figure 4).

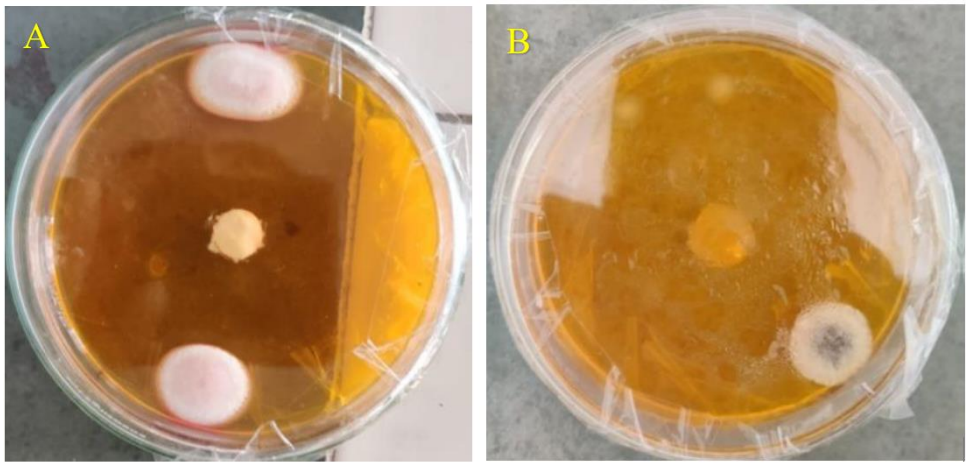


Fig. 4**:** Representative images of antifungal activity of Naringin niosomal gel formulation in comparison with Terbinafine gel post 4 days of incubation

(A) Zone of Inhibition in Agar Plate with terbinafine Gel (Marketed formulation)

(B) Zone of Inhibition in Agar Plate with Naringin niosomal gel formulation

4. Discussion

The present study delved into the exploration of Naringin as a potential antifungal agent targeting Methionine synthase. This enzyme plays a crucial role in fungal metabolism and virulence [19-21]. Inhibiting this enzyme disrupts methionine biosynthesis, leading to a metabolic imbalance that impairs cell energetics, growth, and virulence in fungi like Candida albicans [20, 22-24] and Aspergillus fumigatus [25]. The in silico inhibitory activity of naringin against methionine synthase suggests its potential as an antifungal agent by targeting this essential enzyme [23-24, 26] and perturbing sulfur assimilation pathways critical for fungal survival and pathogenicity [25, 27-28]. This finding corroborates previous research, which underscores Naringin's pharmacological potential, not only as an antifungal but also as an agent with diverse therapeutic properties [4-8].

Subsequent to molecular docking analysis, the antifungal efficacy of Naringin was experimentally validated through minimum inhibitory concentration (MIC) determination against T. mentagrophytes, yielding a MIC value of 50µg/ml after 7 days of incubation at 25°C. This MIC value aligns with prior studies that have identified Naringin's antimicrobial activity against various fungal strains [4-5].

To enhance the delivery of Naringin and optimize its therapeutic efficacy, niosomes were prepared using cholesterol as the film solvent and span 60 as a non-ionic surfactant via the thin film hydration method [14]. Niosomes offer a promising drug delivery system due to their ability to encapsulate hydrophilic and hydrophobic drugs, thereby improving bioavailability and therapeutic outcomes [29]. Our study revealed formulation F3 to exhibit optimal characteristics, including a notable entrapment efficiency of 75.88%, indicating the successful encapsulation of Naringin within the niosomal structure.

Further advancement in drug delivery was achieved through the incorporation of Naringin-loaded niosomes into a gel matrix composed of Carbopol 934 and glycerol. The resultant gel exhibited favorable characteristics such as acceptable pH, physical appearance, spreadability, and rheological properties. In vitro drug release studies demonstrated sustained release kinetics, with a maximum release of 62.18% over 12 hours. This sustained release profile is advantageous for prolonged therapeutic effects and minimization of dosing frequency, as emphasized in previous studies exploring controlled drug delivery systems [30].

Evaluation of the antifungal activity of Naringin-loaded niosomal gel was conducted using the agar well diffusion method, a standard technique in assessing the efficacy of antimicrobial agents [18]. Consistent with expectations, the gel exhibited significant antifungal activity, as evidenced by the formation of zones of inhibition against the fungal culture after 4 days. These findings are in line with established literature documenting the efficacy of Naringin against various fungal pathogens.

Furthermore, the safety profile of the Naringin-loaded niosomal gel was assessed through a skin irritation study, ensuring its suitability for topical application [17]. Notably, no adverse effects such as erythema, eschar formation, and edema were observed following application, suggesting its biocompatibility and potential for clinical translation.

In comparison with the standard terbinafine gel, our study underscores the comparable or superior efficacy of Naringin-loaded niosomal gel as an antifungal agent, thereby validating its potential as a promising alternative in the treatment of fungal infections [7-8].

In conclusion, the findings of this research elucidate the therapeutic potential of Naringin as an antifungal agent, facilitated by innovative drug delivery strategies such as niosomal encapsulation. The study highlights the importance of molecular docking studies in drug discovery, as well as the significance of formulation optimization and evaluation in enhancing therapeutic outcomes. Further preclinical and clinical investigations are warranted to fully elucidate the clinical utility of Naringin-loaded niosomal gel in the management of fungal infections.

5. Conclusion

The present study highlights the potent antifungal activity of Naringin against *T. mentagrophytes*, with a minimum inhibitory concentration of 50 μg/ml. The optimized niosomal gel formulation (Batch F3) exhibited excellent entrapment efficiency, controlled drug release, and significant *in-vitro* antifungal activity against the fungal strain. Furthermore, the niosomal gel did not cause any skin irritation, demonstrating its safety for topical application.

The findings suggest that the developed niosomal gel formulation can be a promising topical antifungal therapy, offering enhanced efficacy and stability of Naringin while ensuring patient compliance and minimizing adverse effects. However, further *in-vivo* studies are recommended to evaluate the clinical efficacy and pharmacokinetic profile of the formulation.

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