The Cytotoxic Effect of Nasodren Nasal Spray (*Cyclamen Europaeum* Extract) in L929 Fibroblastic Cell Culture

Nasodren Nazal Sprey'in (*Cyclamen Europaeum* Ekstresi) L929 Fibroblastik Hücre Kültüründeki Sitotoksik Etkisi

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ABSTRACT Objective: Nasodren nasal spray (*Cyclamen europaeum* extract), is used in some countries as an alternative therapeutic agent for sinusitis. The aim of this study was to ivestigate the possible cytotoxic effects of the drug on cell morphology and viability in L929 mouse fibroblast cell culture model. **Material and Methods:** Nasodren solution was diluted to the dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400 (w/v), and these dilutions were grouped from I to V, respectively. The control group was prepared by using saline in a 1:1 ratio. L929 fibroblastic cell culture material was incubated in 96-cell incubation medium. Following a 12-hour incubation period, the material was taken into fresh culture medium and was stored for five days after treatment with various dilutions of the test material. Cell morphology was observed under microscope on 1st, 2nd, 3rd, 4th, and 5th days. Cell viability was evaluated by 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay method on the same days. **Results:** Cells maintained normal fibroblastic shape and morphology in Group V and the control group during the incubation period. Normal round ring shape was apparently lost in Groups I-IV. Cell viability decreased in Groups I-IV, whereas cell viability increased in Group V and the control group. **Conclusion:** Results of this study indicate that higher concentrations of *Cyclamen europaeum* have toxic effects on cell viability in vitro. There is need for in vivo animal studies before safely prescribing this drug as the first line treatment of sinusitis.

Key Words: Sinusitis; *Cyclamen Europaeum* Extract

ÖZET Amaç: Nasodren nazal sprey, (*Cyclamen europaeum* ekstresi) sinüzit tedavisinde alternatif bir tedavi ajanı olarak bazı ülkelerde kullanılmaktadır. Bu çalışmanın amacı, ilacın, hücre morfolojisi ve yaşayabilirliği üzerindeki olası sitotoksik etkilerini L929 fare fibroblast hücre kültür modelinde araştırmaktır. **Gereç ve Yöntemler:** Nasodren çözeltisi 1:25, 1:50, 1:100, 1:200 ve 1:400 (w/v) konsantrasyonlarında hazırlandı ve sırasıyla I'den V'e kadar gruplandırıldı. Kontrol grubu ise 1:1 serum fizyolojik kullanılarak hazırlandı. L929 fibroblastik hücre kültür materyali 96-hücreli saklama vasatında tutuldu. Çeşitli dilüsyonlardaki çözeltilerde 12 saatlik bekletilme süresinden sonra ilgili materyal taze kültür vasatına alınıp beş gün sure ile tutuldu. Hücre biçimi birinci günden beşinci güne kadar her gün mikroskopla gözlendi. Hücre yaşayabilirliği 3-(4.5-dimetiltiazol-2-yl)-2.5-difenilltetrazolyum bromid kolorimetrik yöntemiyle aynı günlerde değerlendirildi. **Bulgular:** Grup V ve kontrol grubundaki fibroblastlar normal hücre şekil ve biçimini sürdürdü. İlk dört gruptaki hücrelerin normal halka şekli önemli derecede bozuldu. Hücre yaşayabilirliği grup V ve kontrol grubunda artarken ilk dört grupta azaldı. **Sonuç:** Çalışmamız *Cyclamen europaeum*un yüksek konsantrasyonlarda hücre ölümüne sebep olduğunu göstermiştir. İlacın sinüzit tedavisinde güvenle kullanılabilmesi için invivo hayvan çalışmalarına ihtiyaç vardır.

Anahtar Kelimeler: Sinüzit; *Cyclamen Europaeum* Ekstresi

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Nasodren is a nasal spray prepared from *Cyclamen europaeum* extract (Hartington Pharmaceutical S.L. Barcelona, Spain) powder by adding sterile water. Its tubers contain saponins, such as cyclamin

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that has a triterpenoid structure accompanied by isocyclamin and methylcyclamin, and cyclamin is the predominant saponin. Aescin has a different chemical structure and is present at much smaller quantities. Tubers also contain small quantities of glucosides, such as arbutin and methylarbutin that release hydroquinone and hydroquinonemethylether upon hydrolysis. Both compounds exert weak antibacterial effects.

Nasodren nasal spray is used in the treatment of acute and chronic recurrent inflammation of the paranasal sinuses. Its solution at concentration of 1:1 (w/v) is sprayed into each nostril once a day for 6-8 days or every other day, for 12-16 days. Following intranasal administration, the saponins concentrate on mucosal surfaces due to their surfactant features and exert local irritation to mucous membranes of nasal cavity. Reflex secretion of nasal and paranasal mucous membranes is triggered by the irritation of parasympathetic fibers of trigeminal nerve. Immediately after the application, a prickling sensation, sneezing, and burning start in the nose. A few minutes after application, intense nasal secretion starts from intranasal and paranasal submucosal glands resulting in consequent prompt discharge, sudden dehydration, reduced edema, shrinkage of swollen mucosa, and opening of the swollen ostiomeatal unit. The stimulated secretion leads to an intense drainage and wash out of the nasal cavity.

Besides their therapeutic effects, saponins have multiple toxic effects on various human tissues. There is no study on the possible toxic effects of this medication which contains different saponin derivates. This in vitro study was conducted to investigate possible dose-dependent cytotoxic effects of topical application of Nasodren nasal spray on L929 fibroblastic cell lines.

MATERIAL AND METHODS

CELL CULTURE AND ASSESSMENT OF CELL MORPHOLOGY

L929 fibroblastic cells were placed in 96-well culture plates (Greiner bio-one, Germany) at an initial density of 20.000 cells/ml in six replicates and incubated in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany) in a humidified atmosphere of 95% air and 5% CO_2 for 12 hours at 37° C. Following incubation, the cells were treated with 1:25, 1:50, 1:100, 1:200, and 1:400 dilutions of the test material (Nasodren nasal spray) (Initial concentration of test material: 5 gr/5 ml culture medium) and cells were incubated for five days (Figures 1-6). The test material was diluted in cell culture medium corresponding to dilution I, dilution II, dilution III, dilution IV and dilution V groups respectively. Cells cultured without test material were used as the control group.

To identify morphological changes of fibroblasts, cultures were examined under an inverting microscope (IX70 Olympus, Japan). All groups within test series were compared with the control group separately for each evaluation period.

ASSESSMENT OF CELL VIABILITY

Cell viability was determined by MTT (tetrazolium salt 3-[4.5-dimethylthiazol-2-yl]-2.5-diphnyltetrazolium bromide) assay. Following 12 hours of incubation, viability of L929 cells were observed starting from post-incubation day 1 until day 7. At each evaluation period, the culture medium was removed and 100 ml DMEM/F12-without FBS containing 12.5 ml MTT was added into each well. Culture plates were covered with aluminum foil and cells were incubated in the dark for three hours. At the end of the incubation period, MTT solution was removed from the wells and 100 ml isopropyl alcohol was added. The absorbance at 560 nm was measured using an ultraviolet (UV) visible spectrophotometer (LPB Pharmacia, Bromma, Sweden).

Statistical analysis

The control and experimental groups were compared in terms of cell survival for each concentration (dilution) at each time point using Mann-Whitney Test. The comparison was made between control and 1:25; 1:50; 1:100; 1:200, and 1:400 dilution (i.e. dilution I, dilution II, dilution III, dilution IV and dilution V) groups of the test material (NasodrenTM). Significance of differences was declared at $p< 0.05$.

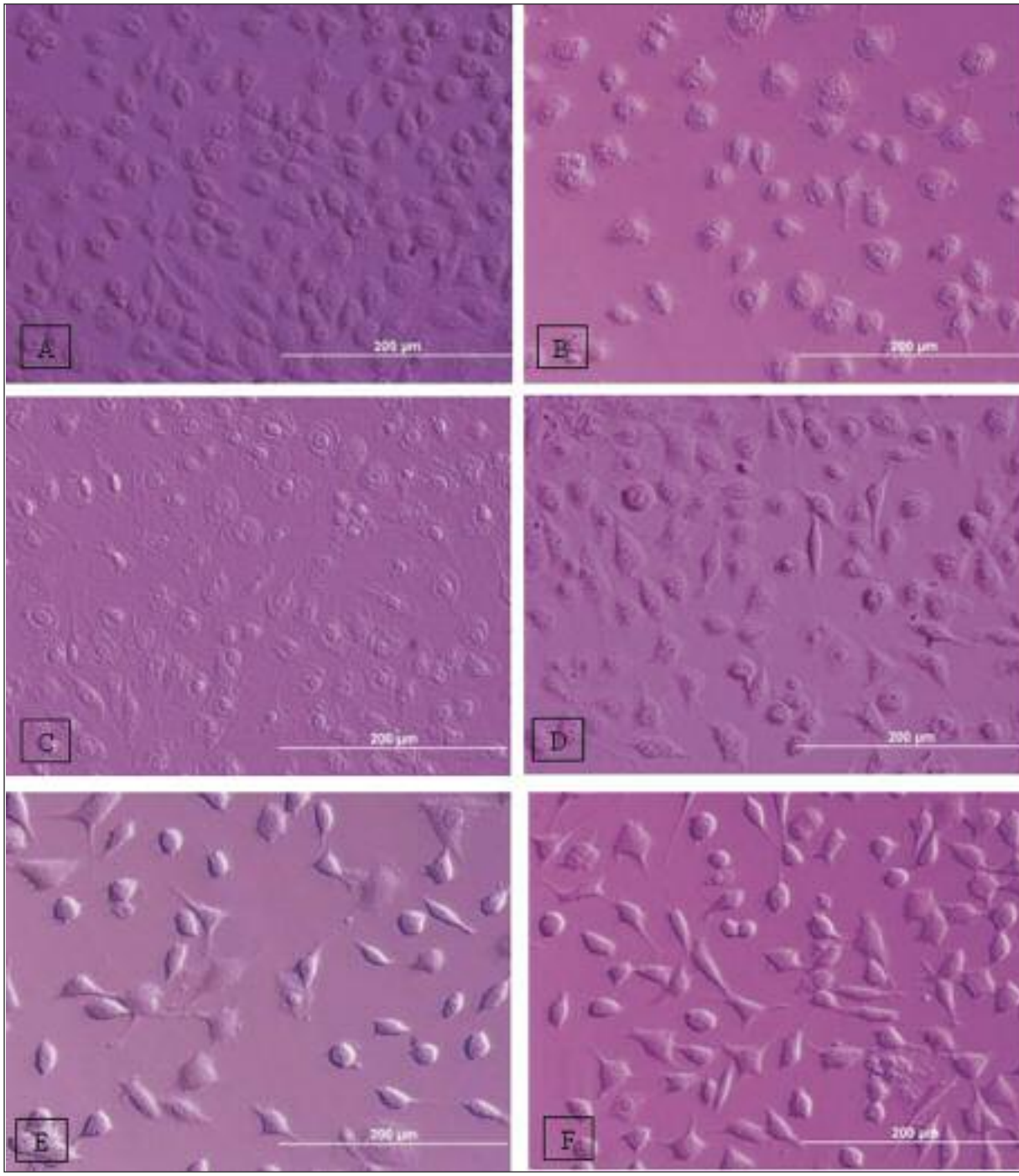


FIGURE 1: Cell morpology after 2 h incubation in different dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

RESULTS

CELL MORPHOLOGY

Two hours after incubation, cells were in normal fibroblastic shape and morphology in dilution V (1:400) and in the control group (Figure 1; e and f). However, cells possessed round shape and exhibited divergence from normal fibroblastic morphology in dilutions I (1:25), II (1:50), III (1:100), and IV (1:200) (Figure 1; a, b, c, and d). A marked cel-

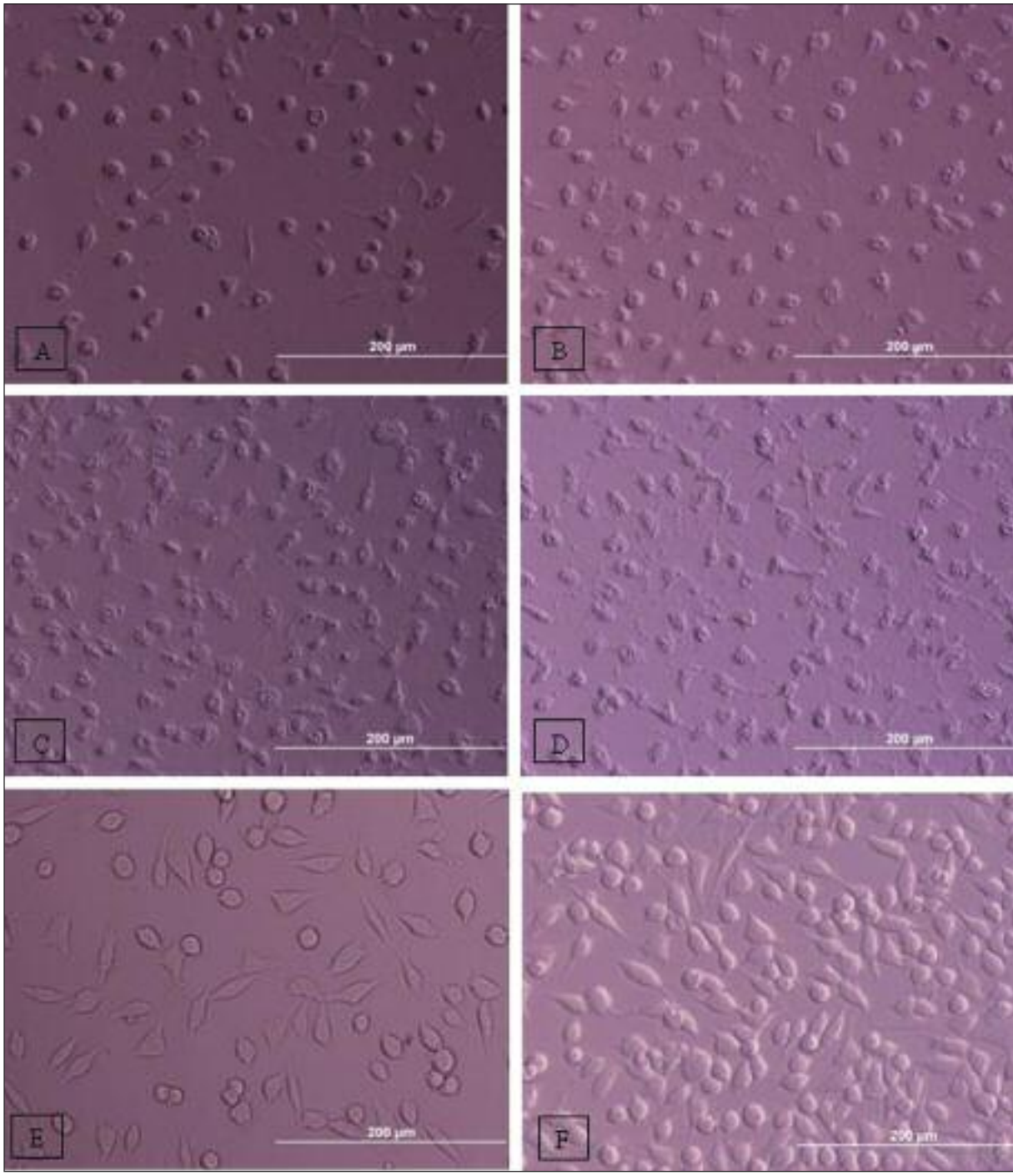


FIGURE 2: Cell morpology on day 1 in different dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

lular degeneration and nuclear condensation were observed in dilution I (1:25), dilution II (1:50), dilution III (1:100) and dilution IV (1:200).

On day 1 (Figure 2; a, b, c, and d) and day 2 (Figure 3; a, b, c, and d), cells displayed less density in dilutions I (1:25), II (1:50), III (1:100), and IV (1:200) when compared to dilution V (1:400) and the control groups (Figure 2; a and b, and Figure 3; a and b). Cellular degeneration and nuclear condensation were observed in the initial four dilutions (I, II, III, IV). All of the cells in these dilutions exhibited similar degenerative morphologic characteristics. How-

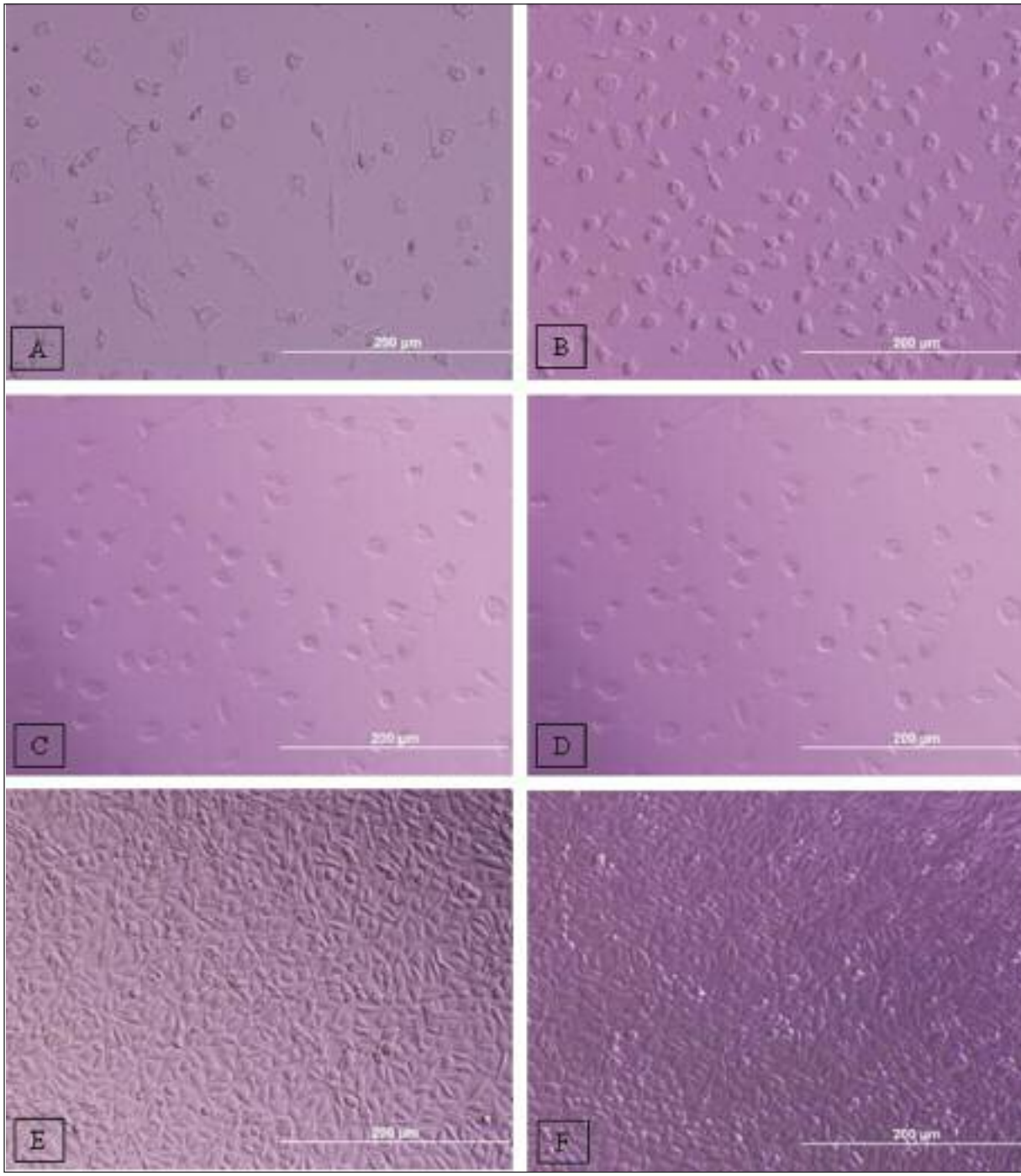


FIGURE 3: Cell morpology at day 2 with respect to dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

ever, in dilution V and control group, degenerative changes of this nature were not observed. Cells maintained their normal fibroblastic morphology in dilution V (1:400) and the control group on the 1st and 5th days of the incubation period.

CELL VIABILITY

Cell viability for each evaluation period is presented in Graphic 1. Except for dilution V (1:400) and the control groups, all dilution groups demonstrated low cell viability (cell proliferation) during the

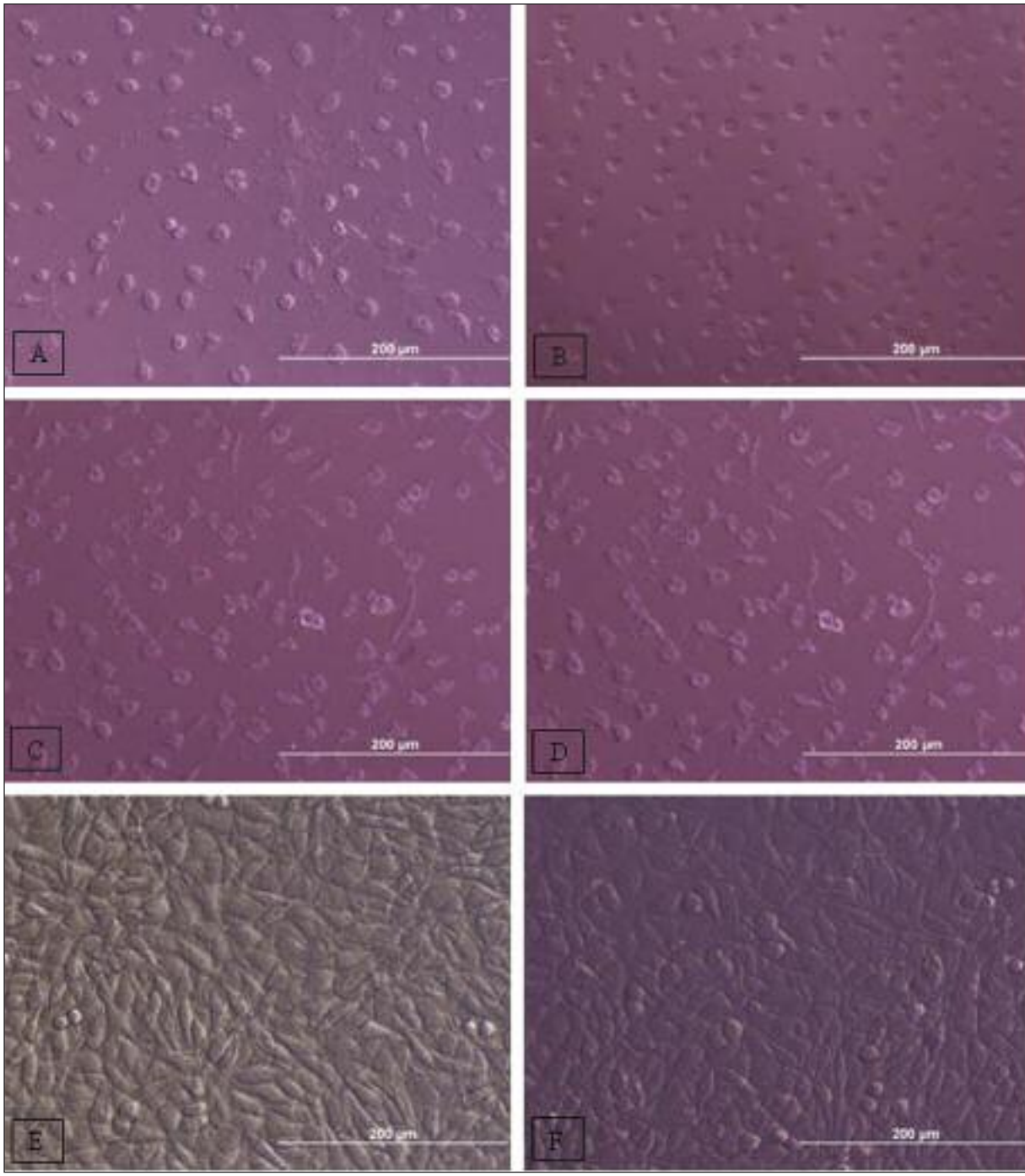


FIGURE 4: Cell morpology at day 3 with respect to dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

incubation period. Regardless of the incubation time, cell viability was different in each group except for the cell viability in dilution V and the control group ($p > 0.05$).

Despite the overall low cell viability in dilutions I (1:25), II (1:50), III (1:100) and IV (1:200), dilution V (1:400) and the control group demonstrated high cell viability in all evaluation periods (Graphic 1).

DISCUSSION

Acute rhinosinusitis is defined as an inflammatory condition of the nose and paranasal sinuses persist-

FIGURE 5: Cell morpology at day 4 in different dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

ing up to four weeks.[1] Viruses and bacteria are the most common causes of acute rhinosinusitis; they both grow due to, as well as cause mucosal thickening and trapped mucous secretions. Chronic rhinosinusitis, defined as symptoms lasting longer than 12 weeks, is a condition different from acute rhinosinusitis in its etiology and pathophysiology. Chronic rhinosinusitis appears to be a primarily inflammatory condition, with intermittent acute exacerbations primarily caused by bacteria. The treatments of uncomplicated acute rhinosinusitis and of acute exacerbation of chronic rhinosinusitis

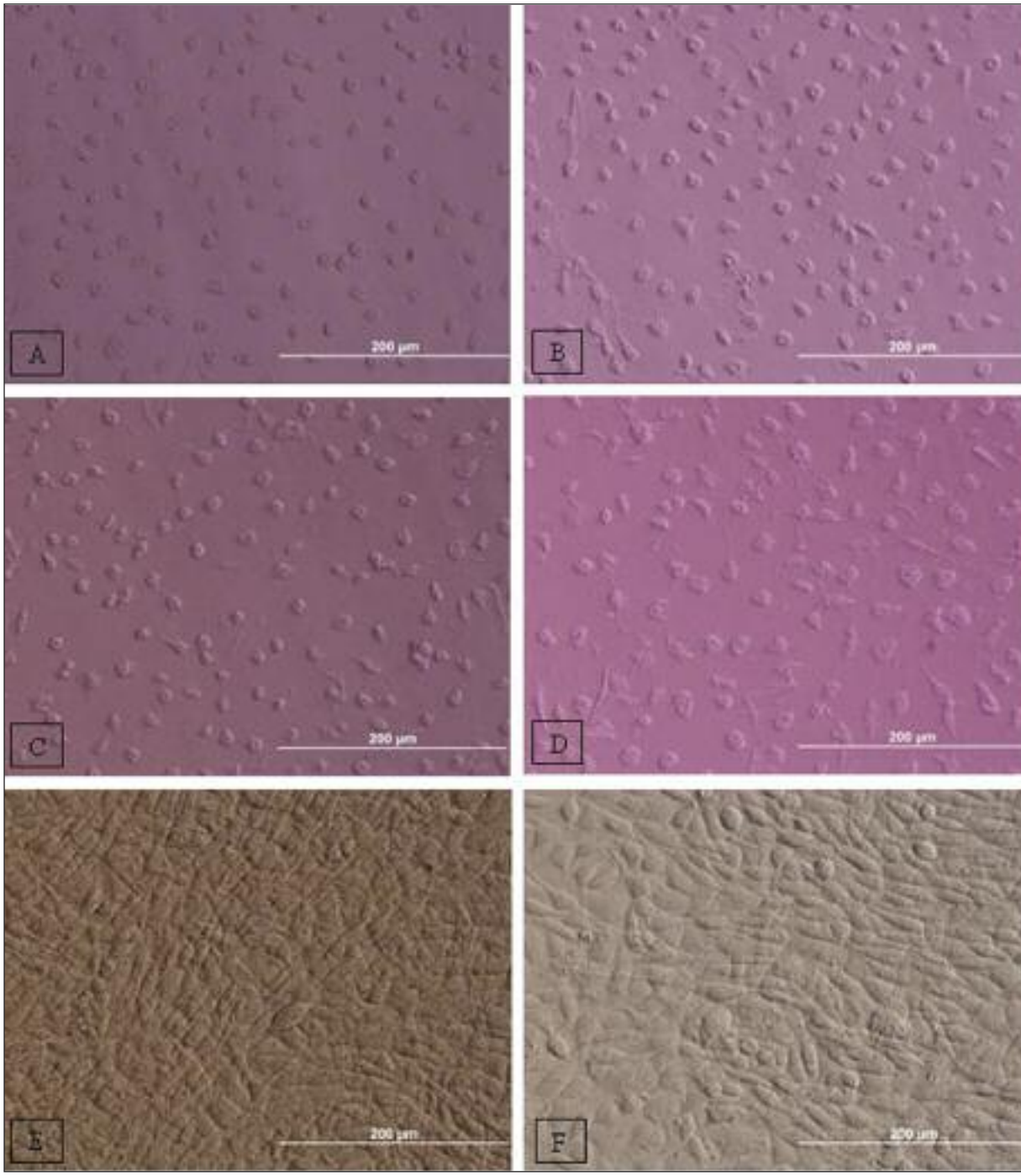
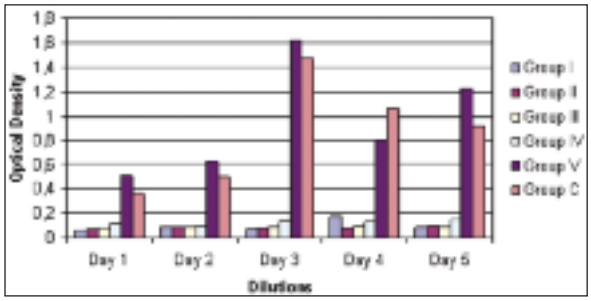


FIGURE 6: Cell morpology on day 5 in different dilutions of Nasodren. **A, B, C, D, E** and **F** represent 1:25, 1:50, 1:100, 1:200, 1:400 dilutions of Nasodren and control, respectively (10x20).

both emphasize antimicrobials as the primary modality. Medications to treat the underlying chronic inflammatory condition play a much more important and sustained role in acute exacerbations of chronic rhinosinusitis.

Viruses are thought to play a significant role in the pathogenesis of acute bacterial rhinosinusitis (ABRS), since viral upper respiratory tract infections (URIs) commonly preceede the episodes of ABRS. Nevertheless, ABRS is an uncommon sequ-



GRAPHIC 1: Time-dependent changes in the viability of fibroblasts incubated with nasodren in different dilutions [Dilution I (1:25), dilution II (1:50), dilution III (1:100), dilution IV (1:200), dilution V (1:400)] and in the control group. There is a significant difference between dilutions I- IV, and dilution V and the control group regarding cell viability.

ela of viral URIs, complicating less than 2% of them.[2] The exact mechanism by which a viral infection can lead to a bacterial one is not entirely clear, although impaired mucociliary clearance through inflammation with colonization from nasal and nasopharyngeal sources is the possible pathogenesis. Ciliary transport of secreted mucus can be impaired by viral mucosal injury directly, especially in adenoviral and influenza virus infections.

Mucous secretion increases and becomes viscous during inflammation of the respiratory mucosa, further inhibiting the mucus transport. Edema may narrow or completely obstruct the sinus drainage pathways, resulting in mucus stasis and favoring bacterial growth.[3] Treatment of acute rhinosinusitis, therefore, is targeted at reducing inflammation and its associated mucociliary transport impairment, in addition to eliminating causative bacteria.

Today, mucolytics, antibiotics, anticholinergics, corticosteroids, decongestants and antihistamines are used in the treatment of acute rhinosinusitis. Despite all treatment modalities, it is difficult to provide a definitive and permanent treatment for all patients. In recent years, a new product in the form of nasal spray (Nasodren nasal sprey) obtained from plant extracts (*Cyclamen europaeum*) has been developed. It acts on mucous membranes of nasal and paranasal sinuses, activating physiological mechanisms clearing nasal mucosa and facilitating drainage of the accumulated secretions.[4,5] This drainage triggers an intense natural clearing of the paranasal sinuses demonstrating high effectiveness of this new plant extract-based product.

Literature reveals different adverse effects of different species of cyclamen. Cyclamen species contain different toxic saponins which cause different adverse effects such as respiratory allergic adverse effects (i.e. of Nasodren nasal spray)[4,5] and plant poisonings.[6,7] On the other hand, they exert some useful effects such as tumor inhibition[5,8] and analgesic-anti-inflammatory effect.[6,9] In vivo side effects are local and temporary because the product is not absorbed or enters the bloodstream.[10-12]

In this study, in vitro cytotoxic effect of a new commercial plant extract (*Cyclamen europaeum*) (Nasodren nasal spray) was investigated in L929 fibroblast culture. The in vitro toxic effect of the drug seemed to be dose dependent and irreversible. It was found that there were significant differences between the groups. Most marked differences were those between group V and groups I- IV ($p< 0.05$). The difference between group V and the control group was not statistically significant ($p> 0.05$). At lower concentrations (1:400), *Cyclamen europaeum* extract maintained cell viability in vitro. On the other hand, at higher concentrations, this extract had potential a toxic effect on cell viability and cell morphology of L929 cells. The toxicity threshold was noted in dilution IV and higher dilutions. (1:200). It is logical to expect a decrease in the possible toxic effects of this material as the dilution factor increases (Graphic 1).

Consequently, results of this study indicate that high concentrations of *Cyclamen europaeum* have toxic effects on cell viability in vitro. In vivo animal studies are needed before safely prescribing this drug as the first line treatment of sinusitis.

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