THE EFFECT OF ALOE VERA ON HUMAN GINGIVAL FIBROBLAST: AN *IN VITRO* STUDY

Dissertation

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In the partial fulfilment of the requirements for the degree

Of

MASTER OF DENTAL SURGERY

In

PERIODONTICS

By

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Under the guidance of

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BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES,

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(Faculty of Babu Banarasi Das University)

BATCH: 2014-2017

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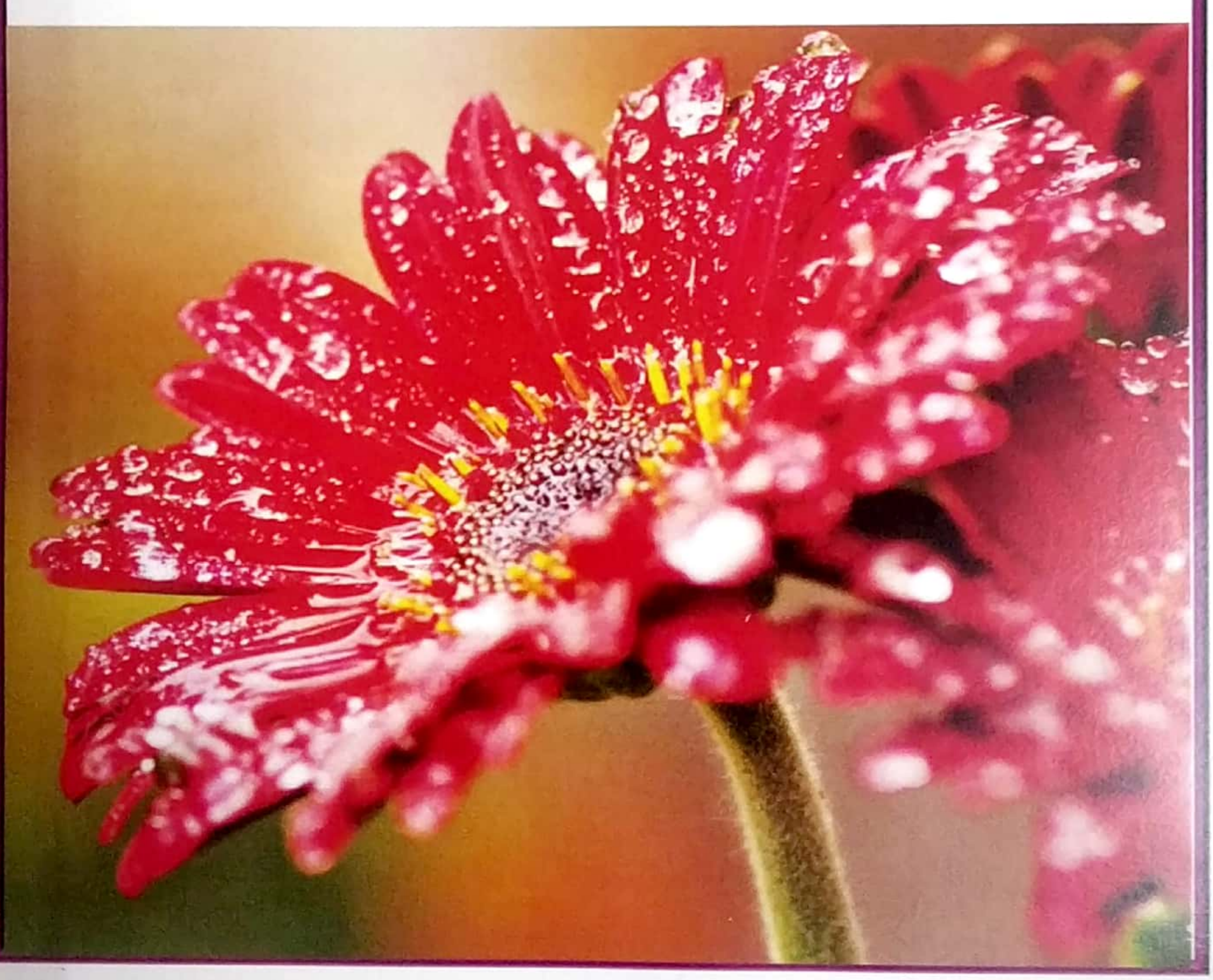

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"The battle of life is, in most cases, fought uphill; and to win it without a struggle were perhaps to win it without honor. If there were no difficulties there would be no success; if there were nothing to struggle for, there would be nothing to be achieved."

-- Samuel Smiles

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Dr. Nida Ansari

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ABBREVIATIONS

AVE	*Aloe vera* extract
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CHX	Chlorhexidine
CEJ	Cemento Enamel Junction
CHX	Chlorhexidine Gluconate
DMEM	Dulbecco's Modified Eagle Medium
EO	Essential Oils
EDTA	Ethylenediaminetetraaceticacid
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
HCV	Hepatitis C virus
hGF	Human Gingival Fibroblasts
H_2O_2	Hydrogen Peroxide
KCL	Pottasium Chloride
LPO	Lipid Peroxidation
LPS	Lipopolysaccharide
L. acidophilus	*Lactobacillus Acidophilus*
MTCC	Microbial Type Culture Collection and Gene Bank
MS	*Mutans Streptococci*

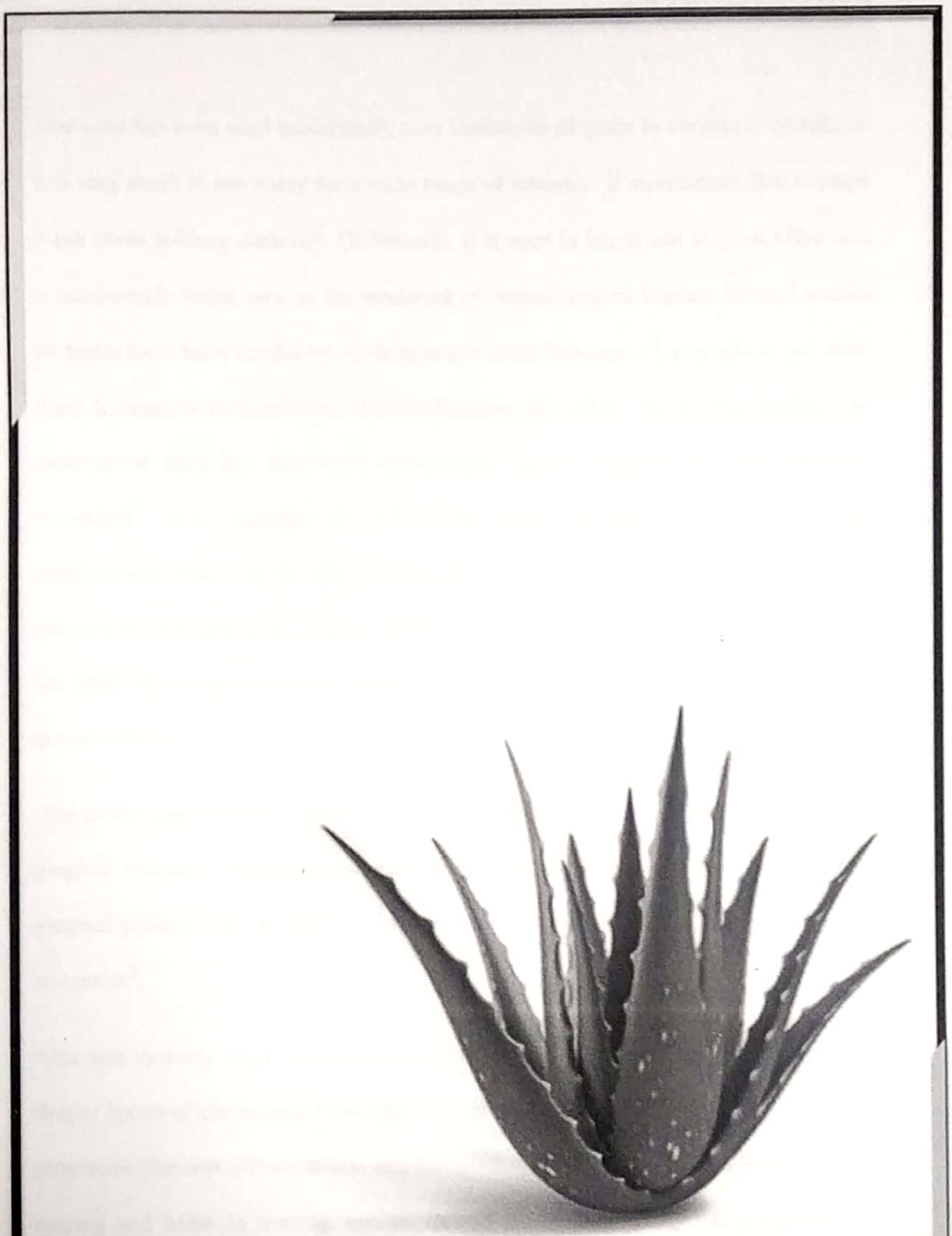
NDEA	N-nitrosodiethylamine
OPD	Outpatient Department
OD	Optical Density
P.gingivalis	Porphyromonas Gingivalis
PDL	Periodontal Ligament
PBS	Phosphate Buffered Saline
S-G	Smulow-Glickman S-G
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
S. mutans	Streptococcus Mutans
SRB assay	Sulforhodamine-B Assay
TFC	Total Flavonoid Contents
TPC	Total Phenolic Contents
TCA	Tetrachloroacetic Acid
NaOCl	Sodium Hypochlorite NaOCl
SRP	Scaling and Root Planing
T.cordifolia	TinosporaCordifolia
Viz.	Namely
vis-à-vis	Compared with
w/v	Weight/Volume
°C	Degree Celsius

Abstract

ABSTRACT

Most of the diseases with which dentists must deal with are related to microbial plaque and bacterial infection. Many chemical agents delivered as mouthwashes are helpful in plaque removal. Chlorhexidine (CHX) was introduced as an antiseptic agent with activity against different organisms such as bacteria, viruses and different types of fungi. Different studies on chlorhexidine have demonstrated its potential to prevent oral complications, such as the occurrence of chronic or opportunistic infections. Unfortunately, the toxic qualities of CHX do not seem to be reserved entirely for bacteria. In addition, we know that CHX application directly to surgical wounds in the oral cavity can delay and alter wound healing. However, herbal preparations (herbal oral mouth rinses) are not toxic and hence are better as compared to chemical mouthwashes like chlorhexidine and are effective in plaque removal and control as well. Owing to these reasons, Aloe Vera (AVE) has been used as an oral applicant and mouth rinse for inhibition/control of gingivitis and periodontitis. This study attempts to assess and evaluate the influence and effect of CHX and AVE on Cultured hGF. Experiments have been conducted using hGF cell line routinely maintained. The effects of CHx and AVE were evaluated on cultured Human Gingival Fibroblasts (hGF) through morphological tests, cytotoxicity and biochemical assays: FACS analysis, Suforhodamine B assay (SRB), wound healing assay, cellular morphology. Cytotoxicity and analysis of AVE displays marked safety as compared with CHx. CHx beyond 1% concentration exhibits toxic effect on hGF at 1 minute time exposure. However, AVE does not adversely affect the fibroblasts even up to 100% concentration showing no toxic effect in comparison with CHx on these cells. FACS analysis showed: The % cell cycle in G0/G1 phase lowered in both the treatment group at all three concentrations as compared to control. The % cell cycle in

S phase was found higher in both chlorhexidine and *Aloe vera* as compared to control. The cell cycle in G2/M phase was found higher in all concentrations of AVE while only 50% in chlorhexidine as compared to control. The in vitro model for wound healing ensures that AVE significantly enhanced wound filling faster than CHX treated hGF cells over 48 hours.



Introduction

INTRODUCTION

Aloe vera has been used medicinally over thousands of years in various civilizations. It is very much in use today for a wide range of ailments. It moisturizes skin because it has water holding capacity[1]. Orthodoxly, it is used in burns unit to great effect and is increasingly being used in the treatment of dermatological lesions. Several studies on burns have been conducted in comparison trials between Aloe products and non-Aloe. It seems to promote more rapid healing and pain relief. There are more than 300 varieties of Aloe but *Aloe vera barbadensis* variety exhibits the best medicinal properties[2]. It is a member of the Liliaceae family and grows in hot climate. The problem with *Aloe vera* has always been that of maintaining the therapeutic function because it is unstable and oxidizes rapidly like any cut leaf or fruit. It has been due to the relatively recent successful stabilization process that has so greatly increased its use worldwide.

The dental uses of *Aloe vera* are multiple. It is extremely helpful in the treatment of gingival diseases, reduces the bleeding of the gingiva, is powerfully antiseptic in gingival pockets and its Antifungal properties help greatly in the problem of denture stomatitis[3].

Aloe acts in many ways. It is particularly known for its penetrating capacity to reach deeper layers of the dermis when applied topically[4]. *Aloe vera* has various medicinal properties like anti-inflammatory, anti-bacterial, anti-tumor which accelerates wound healing and helps in treating various lesions of the oral cavity[5]. It promotes cell growth. It is neurologically calming and also acts as a detoxifying agent. If we consider its constituents, it will be easier to understand its range of action.

The various forms of *Aloe vera* used in dentistry are – the toothpaste, the jelly for healing promotion, which can also be used for burns, stings, insect bites and many skin lesions; the Aloe & Propolis hand cream which helps to counteract frequent hand washing and the wearing of latex gloves; the Aloe activator spray which is excellent for throat infections, painful erupting wisdom teeth and joint pains; lastly, the juice which is taken as a drink and has been shown to have various beneficial effects on the body[6]. This is used in conjunction with any topical treatment for skin lesions, joint lesions and, in itself, for ameliorating in irritable bowel syndrome. The juice is also a strong detoxifying agent and if used in conjunction with the planned removal of mercury amalgams, acts as a scavenging agent for mercury, a neuro-sedative and immune enhancer. As a routine general tonic it generally makes people feel better who are otherwise not well enough but not frankly ill. Because of the many properties and functions of Aloe, it is a powerful nutritional supplement and anti-oxidant. It protects and promotes healing can be used following extractions[7].

We shall attempt to investigate and establish the efficacy of this herbal agent on the proliferation, morphology and toxicity, if any on the Human Gingival Fibroblasts (hGF). A detailed study will be conducted that will enable us to comprehend the clinical use of *Aloe vera* as an adjunct to periodontal therapy by analyzing its morphological and proliferative effects on hGF.

Aims & Objectives

AIM AND OBJECTIVES

The ***Aim*** of the present study was:

To evaluate the effects of *Aloe vera* on hGF.

The ***Objectives*** of the present study were:

1. To evaluate the effects of *Aloe vera* on human gingival fibroblasts (hGF) morphology, proliferation, cytotoxic effect and regulation of cell cycle.
2. To compare the changes induced by CHX in hGF versus *Aloe vera.*
3. To evolve strategy for the eventual development of *Aloe vera* derived mouthwash.

Review of Literature

REVIEW OF LITERATURE

Wright CS (1937)[8] described remarkable improvement obtained in two cases of X-Ray ulceration with the use of whole leaf aloe vera gel. Case I was a long standing case of x-ray ulceration with a duration greater than 10 years while case II had X-ray ulceration of a relatively short duration of 3 years. The gelatinous constituents of aloe vera were scraped out and mixed with an equal quantity of aquaphor and massaged into the affected area every night by the patients themselves. In case I the symptoms gradually improved in 3 months while in case II a complete resolution of the symptoms occurred in 3 weeks.

Yagi A, Harada N, Yamada H, Iwadare S, Nishioka I (1982)[9] studied a material, having anti-bradykinin (pain reduction) activity on isolated guinea pig ileum, partially purified from the non-dialysate of the pulp of Aloe saponaria by repetition of gel chromatography. The antibradykinin-active material was estimated to be a glycoprotein. It was found that this material catalyzes the hydrolysis of bradykinin at pH 7.4.

Greenstein G, Berman C, Jaffin R (1986)[10] reviewed the use of chlorhexidine as an adjunct to periodontal therapy. At low concentrations, low molecular weight substances leak out of the effected cells. At high concentrations, precipitation of cytoplasmic contents causes cell death. Approximately 30% of the drug has been shown to be retained in oral cavity followed a rinse with 10ml of 0.2% solution for minute. The drug is released over next 8 to 12 hours and prolonging the bactericidal effect. Systemic absorption of the drug was poor and the side effects were minimal.

Grindlay D, Reynolds T. (1986)[11] conducted a review to evaluate the effects of Aloe vera Modern clinical use of the gel began in the 1930s, with reports of successful treatment of X-ray and radium burns, which led to further experimental studies using laboratory animals in the following decades. Chemical analysis have showed the gel to contain various carbohydrate polymers, notably either glucomannans or pectic acid, along with a range of other organic and inorganic components. Although many physiological properties of the gel have been described, there is no certain correlation between these and the identified gel components.

Gribel' NV, Pashinskiĭ VG (1986)[12] carried out an evaluation of antimetastatic properties of succus Aloës using three types of experimental tumors of mice and rats. It was found that succus Aloës treatment contributes to reduction of tumor mass, metastatic foci and metastasis frequency at different stages of tumor progress without affecting major tumour growth. They concluded that Succus Aloës potentiates the antitumor effect of 5-fluorouracil and cyclophosphamide as components of combination chemotherapy.

Womble D, Helderman JH (1988)[13] reported that acemannan, the active ingredient of the aloe vera plant, is an important immune-enhancer in that it increases lymphocyte response to alloantigen. It was suggested that the mechanism involves enhancement of monocyte release of IL-I under the aegis of alloantigen. This mechanism may explain in part the recently observed capacity of acemannan to abrogate viral infections in animal and man.

Pucher JJ, Daniel JC (1992)[14] studied utilized human fibroblasts derived from skin and oral tissues to test the effects of chlorhexidine on viability, growth, collagen gel

contractions, and total protein synthesis. Results indicate that a 0.002% concentration of the drug shows minimal cytotoxicity, but is able to suppress cell division almost completely. Total protein synthesis was suppressed by chlorhexidine in collagen gel culture. The data support the hypothesis that chlorhexidine is highly cytotoxic to cells in vitro

***van Wyk BE, van Rheede van oudtshoorn MCB, Smith GF* (1995)**[15] investigated the Geographical variation in fresh Aloe ferox leaf exudate of which the dried product is commercially known as Cape Aloes, throughout the natural distribution range of the species. The composition of the major compounds is remarkably invariable, with aloeresin A, aloesin, and aloin (both epimers A and B) contributing between 70% and 97% of total dry weight, in a ratio of approximately 4:3:2, respectively.

Lacroix JM, Walker CB (1996)[16] collected subgingival plaque samples from 68 patients with a history of moderate to severe adult periodontitis and enumerated on Trypticase-soy blood agar plates, with and without tetracycline at 4 micrograms/ml. Approximately 12% of the total cultivable flora was resistant to tetracycline, and the percentage of the tetracycline-resistant flora with thetet (Q) gene varied greatly from one patient to another with a range from 0.0 to 67%.

Williams MS et al (1996)[17] conducted two studies; the first one was double blinded, utilized a placebo gel, and involved 194 women receiving breast or chest wall irradiation. The second trial randomized 108 such patients to aloe vera gel vs. no treatment. Skin dermatitis was scored weekly during both trials both by patients and by health care providers. Skin dermatitis scores were virtually identical on both

treatment arms during both of the trials. The only toxicity from the gel was rare contact dermatitis.

***Chithra P, Sajithlal GB, Chandrakasan G* (1998)**[18] studied the influence of Aloe vera on collagen characteristics in healing dermal wounds in rats. In this work, they reported the influence of Aloe vera on the collagen content and its characteristics in a healing wound. Aloe vera increased the collagen content of the granulation tissue as well as its degree of cross linking. Wounds were treated either by topical application or oral administration of Aloe vera to rats and both treatments were found to result in similar effects.

Hayes SM (1999)[19] described the first case of treatment of Oral lichen planus using Aloe vera juice and Aloe vera gel for 3 months .He described a case of successful treatment of lichen planus.

***Reynolds T, Dweck AC* (1999)**[20] reviewed the various properties of Aloe. Although many physiological properties of the gel have been described, there is no certain correlation between these and the identified gel components. A common theme running though much recent research is the immunomodulatory properties of the gel polysaccharides, especially the acetylated mannans from Aloe vera, which are now a proprietary substance covered by many patents. There have also been, however, persistent reports of active glycoprotein fractions from both Aloe vera and Aloe arborescens. There are also cautionary investigations warning of possible allergic effects on some patients. Reports also describe anti-diabetic, anticancer and antibiotic activities, so we may expect to see a widening use of aloe gel.

Vogler BK, Ernst E (1999)[21] suggested that oral administration of aloe vera might be a useful adjunct for lowering blood glucose in diabetic patients as well as for reducing blood lipid levels in patients with hyperlipidaemia. Topical application of aloe vera is not an effective preventative for radiation-induced injuries. It might be effective for genital herpes and psoriasis. They concluded that whether it promotes wound healing is unclear.

Mariotti AJ, Rumpf DA. (1999)[22] examined the effects of chlorhexidine on gingival fibroblast proliferation as well as collagen and non-collagen protein production in cell culture. The results indicated that chlorhexidine will induce a dose dependent reduction in cellular proliferation and that concentrations of chlorhexidine that have little effect on cellular proliferation can significantly reduce both collagen and noncollagen protein production of human gingival fibroblasts in vitro. Hence, the introduction of commercially available concentrations (0.12%) or diluted commercial concentrations (as low as 0.00009%) of chlorhexidine to surgical sites for short periods of time prior to wound closure can conceivably have serious toxic effects on gingival fibroblasts and may negatively affect wound healing.

Fine DH et al (2000)[23] determined the effect of 2x-daily rinsing with an essential oil-containing antiseptic mouthrinse (Listerine Antiseptic) on levels of recoverable S. mutans and total streptococci in supragingival interproximal plaque and in saliva. Additionally, a follow-up in vitro study is reported which determined whether a differential susceptibility to the antiseptic mouthrinse exists among different strains of streptococci. The essential oil mouthrinse produced respective reductions of 69.9%

and 75.4% in total recoverable streptococci and in S. mutans in plaque, and corresponding reductions of 50.8% and 39.2% in saliva.

Pugh N, Ross SA, El Sohly MA, ElSohly MA, Pasco DS (2001)[24] characterized a new immunostimulatory polysaccharide called Aloeride from commercial aloe vera (Aloe barbadensis) juice. Acemannan, the major carbohydrate component from aloe, used at 200 microg/mL in the macrophage assay resulted in negligible NF-kappa B activation.

Poor MR, Hall JE, Poor AS (2002)[25] conducted a study to evaluate the reduction in the incidence of alveolar osteitis in patients treated with the SaliCept patch, containing Acemannan hydrogel. A retrospective evaluation was performed of the records of 587 patients (1,031 sockets), whose extraction sites had been treated with Clindamycin-soaked gelfoam. The study results suggested that the SaliCept Patch significantly reduces the incidence of AO compared with Clindamycin soaked gelfoam.

Heggers JP et al (2002)[26] investigated the antibacterial activity of aloe vera at varying time intervals and concentration levels and tissue toxicity in an effort to determine a concentration that was both microbicidal and nontoxic and in what period of time.

Agarry O.O, Olaleye MT (2005)[27] studied the comparative antimicrobial activities of the gel and leaf of Aloe vera against staphylococcus aureus, Pseudomonas aeruginosa, Trichophyton mentagraphytes, T.schoeleinii, Microsporium canis and candida albicans. Antimicrobial effect was measured by the appearance of zones of inhibition.

The results of this study tend to give credence to the popular use of both Aloe vera gel and leaf.

Boudreau MD, Beland FA (2006)[28] reported that ingestion of Aloe vera is associated with diarrhea, electrolyte imbalance, kidney dysfunction, and conventional drug interactions; episodes of contact dermatitis, erythema, and phototoxicity have been reported from topical applications. The also examined the botany, physical and chemical properties, and biological activities of the Aloe vera plant.

Nigam M, Ranjan V, Srivastava S, Sharma R, Balapure A.K (2008)[29] studied isolation of Human Gingival Fibroblasts (hgF) and their employment for research with anti-breast cancer compounds (Life sciences). More, these cells have been utilized for delineating the apoptotic potential of curcumin, an essential component of Haldi, a commonly used spice in cooking.

George D, Bhat S.S, Antony B (2009)[30] compared the antimicrobial effectiveness of Aloe vera tooth gel with two popular, commercially available dentifrices. The preliminary results showed that Aloe vera tooth gel and the toothpastes were equally effective against Candida albicans ,Streptococcus mutans ,Lactobacillus acidophilus, Enterococcus ,Prevotella intermedia and Peptostreptococcusanaerobius. Aloe vera tooth gel demonstrated enhanced antibacterial effect against S.mitis.

Rajabalian S, Mohammadi M, Mozaffari B (2009)[31] evaluated the cytotoxic effects of Persica™ and chlorhexidine (CHX) mouthwashes on cultured human and mouse cell lines.The results indicated that both Persica™ and CHX mouthwashes are toxic to

macrophage, epithelial, fibroblast, and osteoblast cells in a concentration-dependent manner.

Tello CG, Ford P, Iacopino AM (2010)[32] evaluated Acemannan denture adhesive formulations for pH changes, cytotoxicity to human gingival fibroblasts and adhesive strength in both dry and wet conditions. Acemannan, a complex mannose carbohydrate derived from the Aloe vera plant, has an inherent stickiness/viscosity. Acemannan formulation 150:1 should be adjusted to contain the preservative concentration of formulation 4 and have an initial pH value of 6.0 or higher.

Lessa FC et al (2010)[33] examined toxicity of chlorhexidine on odontoblast-like cells. The result suggested that CHX concentrations had a high direct cytotoxic effect to cultured MDPC-23 cells.

Pandey R, Mishra A (2010)[34] tested the antibacterial activity of Aloe barbadensis on clinically isolated bacterial pathogens i.e. Enterococcus bovis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Morganella morganii, and Klebsiella pneumoniae causing infection in human beings. Ethanolic and aqueous extracts were used for the antibacterial effect. Among gram negative bacteria, highest inhibitory effect was observed with P. aeruginosa, followed by M. morganii, P. mirabilis, and P. Vulgaris.

Brecx M, Netusehil L, Reiehert B, Schreil G (2011)[35] compared the anti-plaque, anti-gingivitis and anti-microbial efficacies of a phenolic compound (Listerine)® and a stannous fluoride mouthwash (Meridol)®, using a placebo preparation as negative control and a chlorhexidine solution as positive control in a double-blind study. The

study demonstrated that chlorhexidine was superior to Listerine and Meridol in its ability to maintain low plaque scores and gingival health during this 3-week period of no mechanical oral hygiene.

Eick S, Goltz S, Nietzsche S, Jentsch H, Pfister W (2011)[36] studied the in vitro action of chlorhexidine digluconate and different commercially available mouth rinses on oral microorganisms. The results indicated that most of the chlorhexidine digluconate formulations as well as essential oil were active against oral microbes studied i.e, Streptococci, Enterobacteria, Candida albicans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum.

Bhat G, Kudva P, Dodwad V (2011)[37] evaluated total number of 15 subjects were evaluated for clinical parameters like plaque index, gingival index, probing pocket depth at baseline, followed by scaling and root planing (SRP). Test site comprised of SRP followed by intra-pocket placement of Aloe vera gel, which was compared with the control site in which only SRP was done, and clinical parameters were compared between the two sites at one month and three months from baseline. Results exhibited encouraging findings in clinical parameters of the role of Aloe vera gel as a drug for local delivery.

Singh N et al (2012)[38] studied Human Gingival Fibroblasts (hGF), where the study included (1) analysis of insulin-mediated mitogenesis on CUR-treated hGF cells and (2) development of an in vitro model of wound healing. Apoptotic rate in CUR-treated hGF cells with and without insulin was observed by AnnexinV/PI staining. The in vitro model for gingival wound healing establishes that insulin significantly

enhanced wound filling faster. The cells employed for this purpose were developed per Nigam et al (2008).

Verma UP and Dixit J (2012)[39] attempted to study the influence of Chlorhexidine and Neem extract on cultured Human Gingival Fibroblasts (hGF) through morphological and biochemical assays. Neem did not adversely affect the Fibroblasts even up to 50 % concentration showing less toxic effect in comparison with Chlorhexidine on these cells. The cytoprotective, oral friendly quality of Neem emphasize the superiority of Neem over Chlorhexidine.

Athiban PP, Borthakur BJ, Ganesan S, Swathika B (2012)[40] evaluated the antimicrobial efficacy of Aloe vera and to determine its effectiveness in decontaminating gutta percha cones. A concentrated extract of Aloe vera was used to check for the antimicrobial efficacy using the agar well diffusion method. Presence of zones' of diffusion was identified against three common GP contaminants namely, E.coli, E.faecalis and Staph. Aureus.

Woźniak A, Paduch R (2012)[41] reported no toxicity of ethanol, ethyl acetate and heptane extracts of Aloe vera on human corneal cells. No ROS reducing activity by heptane extract and trace action by ethanol (only at high concentration 125 µg/ml) extract of Aloe vera was observed. Only ethyl acetate extract expressed distinct free radical scavenging effect.

Moniruzzaman M et al (2012)[42] conducted a study to measure the total phenolic and flavonoid contents, the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging ability and the ferric reducing power (FRAP) of Aloe vera to determine the

antioxidant activity of this species. The in vivo experimental antioxidant parameter MDA is strongly correlated with the in vitro antioxidant parameters of flavonoids and polyphenols, namely the DPPH and FRAP values (r = 0.94, 0.92, 0.93, 0.90), thus confirming the antioxidant potential of the Aloe vera extracts.

Chandrahas B et al (2012)[43] reported a statistically significant decrease in PI, MGI, and BI scores upon rinsing with an Aloe Vera containing mouthwash. Mouth wash containing Aloe vera showed significant reduction of plaque and gingivitis but when compared with chlorhexidine the effect was less significant.

Calderini A, Pantaleo G, Rossi A, Gazzolo D, Polizzi E (2013)[44] evaluated the clinical and microbiological effects of a single session of mechanical and manual scaling and root planing (SRP) combined with the use of two different chlorhexidine formulations in the treatment for generalized chronic periodontitis. The study resulted in clinical and microbiological benefits in the treatment for generalized chronic periodontitis.

Abdelrawaf SS, Al-Saady HA, Attitalla IH (2013)[45] evaluated the potential cytotoxic and genotoxic effects of ethanol mucilaginous tissue of leave of Aloe vera extract by observing cytological parameters such as mitotic index and chromosome aberration, including chromatid gap, chromatid break, chromosome fusion, ring chromosome, dicentric chromosomes, chromatid deletion, stickiness chromosomes in bone marrow of Swiss albino mice.

***Ehsani M, Marashi MA, Zabihi E, Issazadeh M, Khafri S* (2013)**[46] compared the antibacterial activities of three different propolis extracts (alcohol concentrations: 0,

15, 40%) and Aloe vera gel on E. faecalis using three methods: disk diffusion, microdilution and direct contact test. In addition to the above bacterium, the Aloe vera gel effect on Staphylococcus aureus and Streptococcus mutans was evaluated. The hydroalcoholic extracts of propolis and Aloe vera gel had antibacterial effects on E. faecalis, however, propolis is more potent than Aloe vera. The antibacterial effect of Aloe vera on S. aureus and S. mutans is low (MIC $\geq$ 2250 µg/ml).

Garnick JJ, Singh B, Winkely G (2014)[47] evaluated the effectiveness of a gel containing silicon dioxide , Aloe vera and allantoin in the healing of recurrent aphthous ulcers. The subjects were patients with histories of developing multiple ulcers on the oral mucosa during a 3 to 4 month period. A consistent pattern was not present, which indicated a lack of effect of the gel on aphthous ulcers.

Kaur H, Jain S, Kaur A (2014)[48] compared the antiplaque efficacy of green tea catechin mouthwash with chlorhexidine gluconate mouthwash. The result showed that both the groups, that is green tea catechin mouthwash (0.25%) and chlorhexidine mouthwash (0.12%) have comparable results in plaque reduction.

Gupta D et al (2014)[49] assessed the effectiveness of Ocimum sanctum on dental plaque, gingival inflammation and compared it with gold standard chlorhexidine and normal saline (placebo). The results showed that Ocimum sanctum mouthrinse was equally effective in reducing plaque and gingivitis as Chlorhexidine. The results also demonstrated a significant reduction in gingival bleeding and plaque indices in both groups over a period of 15 and 30 days as compared to control group.

Karim B et al (2014)[50] showed that Aloe vera mouth rinse is equally effective in reducing periodontal indices as Chlorhexidine. There was a significant reduction on plaque and gingivitis in Aloe vera and chlorhexidine groups and no statistically significant difference was observed among them ($p>0.05$). Aloe vera mouthwash showed no side effects as seen with chlorhexidine.

Herrera AH, Ospina LF, Fang L, Caballero AD (2014)[51] evaluated the antibacterial effect of M. americana extract against Porphyromonas gingivalis and Streptococcus mutans. For this, an experimental study was conducted. Ethanolic extract was obtained from seeds of M. americana (one oil phase and one ethanolic phase). The strains of Porphyromonas gingivalis ATCC 33277 and Streptococcus mutans ATCC 25175 were exposed to this extract to evaluate its antibacterial effect. Bactericidal and bacteriostatic activity was detected against S. mutans, depending on the concentration of the extract, while M. americana extract presented only bacteriostatic activity against P. Gingivalis.

Mansour G, Ouda S, Shaker A, Abdallah HM (2014)[52] evaluated the clinical efficacy, and safety of newly customized natural oral mucoadhesive gels, containing either aloe vera or myrrh as active ingredients, in the management of minor recurrent aphthous stomatitis (MiRAS). The new formulated aloe- and myrrh-based gels proved to be effective in topical management of MiRAS. Aloe was superior in decreasing ulcer size, erythema, and exudation; whereas myrrh resulted in more pain reduction.

Badakhsh S, Eskandarian T, Esmaeilpour T **(2014)**[53] investigated the use of Aloe vera as a storage media for avulsed tooth The purpose of this study was to assess the effectiveness of different concentrations of Aloe Vera extract compared to DMEM

(cell culture medium) and egg white. The results indicated that culture media and Aloe Vera extract (10, 30, and 50% concentration) were statistically similar and significantly preserved more PDL cells compared to other experimental storage media.

Chandu AN, Kumar SC, Bhattacharjee C, Debnath S (2016)[54] performed a test on herbal extracts of Aloe vera to kill the cancerous cells and to protect the normal cells from the cytotoxic effect of anticancer drugs. Here the culture performed for cancer cell, the molecular involvement and potency of chemotherapeutic drug in cancer cell was well presented. Evaluation of in-vitro antitumor activity revealed that Aloe vera exhibits good antitumor activity. The best antitumor activity by Aloe vera was shown at 300 µg/ml concentration.

Materials & Methods

MATERIALS AND METHODS

A comparative*in vitro* studyevaluating the effects of *Aloe vera*on hGF was conducted by the Department of Periodontology, Babu Banarasi Das College of Dental Sciences, Lucknow, Uttar Pradesh in collaboration with Cell and tissue Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI).The approval for the study was taken from ethics committee, Babu Banarasi Das College of Dental Sciences, Lucknow. **(Appendix I)**

MATERIALS

The effect of the *Aloe vera* extract and Chlorhexidine (Control Group) were evaluated on cultured hGF cells. The hGF cells were cultured at CSIR-CDRI. Commercially available Chlorhexidine 0.2 % w/vwas procured from the local market, by KAYVEE Aeropharm pvt. Ltd, Kadi, Ahmedabad, Gujarat (Batch number: ND15-11).All the reagents, Sterile Plastic ware,flasks, multiwelled tissue culture plates, filter ware etc.were mainly procured from Sigma Chemicals Co. St. Louis, MO (USA).

SUBJECTS:

Inclusion and exclusion criteria

As the study is completely *in vitro* therefore no human subjects will be involved

STUDY DESIGN:

Four tests were conducted in the study:

1. Cellular morphology assessment.

Principle: Cellular morphology assessment is done by a Trypan blue dye exclusion test[55]. A dye exclusion test is done which is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not.

2. Cell growth and Cytotoxicity assessment/Sulforhodamine B (SRB) dye Assay.

Principle:Sulphorhodamine is a bright pink aminoxanthine dye with two sulphonic groups. It is one of the family of related dyes, such a napthol yellow S and comassie brilliant blue that are used widely as protein stains in biochemistry and molecular biology. Under mild acidic conditions, SRB binds to basic amino acid residue of TCA fixed proteins. It provides a sensitive index of culture cell protein that is linear with cell number over a cell density range of more than hundred folds.

3. Cell cycle analysis/Fluorescent activated cell sorter analysis (FACS).

Principle:Cell cycle analysis was done by using flow cytometer. The cytometer is capable of analyzing cells treated with a fluorescent stain. The fluorescence intensity is directly proportional to the DNA content of each cell.

4. Wound healing assay.

Principle: In a typical wound healing assay, a "wound gap" in a cell monolayer is created by scratching, and the "healing" of this gap by cell

migration and growth towards the centre of the gap is monitored and often quantitated. Factors that alter the motility and/or growth of the cells can lead to increased or decreased rate of "healing" of the gap.

PLANT:

The present study was undertaken to evaluate the effectsof medicinal plant extract (*Aloe vera*) on Human Gingival Fibroblasts (hGF):

Aloe vera **(PLATE: I)**

Scientific Name: ***Aloe vera barbadensis***

Family: Asphodelaceae

Broad circumscribed family: Lilyaceae[56]

Common Name: Aloe, India Aloe, True Aloe, Chinese Aloe, Gheekuvar (Hindi)

Part Used: Leaf gel**(PLATE: I)**

CHEMICAL COMPOSITION OF *ALOE VERA*: *Aloe vera*is composed of Anthraquinones/anthrones, carbohydrates, chromones, enzymes, inorganic compounds, proteins, sachharides, vitamins , hormones, organic compounds and lipids[56].

COLLECTION AND VALIDATION OF PLANT MATERIAL:

Aloe vera(leaf) was collected, corroborated and validated by Cell andTissue Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI).

PREPARATION OF EXTRACT:

Aqueous solution of *Aloe vera*was prepared from the fresh leaf of the plant,extracted with 10% w/v of chilled 1.15% Potassium chloride(KCL) by grinding with a pestle and mortar and then centrifuged the extract at 1000 × g for 5 minutes at 4° C to get rid of debris and finallyit is sterilizedby filtering through 45µm syringe filter at the Cell and Tissue Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI).**(PLATE: II-IV)**

ISOLATION AND CULTURE OF HUMAN GINGIVAL FIBROBLASTS

Experiments was conducted with the hGF cell line routinely maintained at Cell and Tissue and Culture Laboratory, Biochemistry Division, CSIR- Central Drug Research Institute For experimental utilization of the cells, they were propagated in DMEM (Dulbecco's modified eagle medium) supplemented with 10%Fetal Bovine Serum (FBS) in a humidified CO_2 incubator at 37°C[57].

METHODOLOGY:

CYTOMORPHOLOGICAL STUDY

A trypan blue dye exclusion test of cells for morphological studies was performed according to Nigam et al (2008)[29]. A confluent flask of hGF was trypsinized {using 0.5% trypsin in Ethylenediaminetetraacetic acid (EDTA)}, the cells counted by trypan blue dye exclusion test.0.2x106 cells were plated onto a 6 well plate and cultured in DMEM supplemented with penicillin(100 µg/ml), streptomycin (200 µg/ml), gentamycin (50 µg/ml) and sodium bicarbonate (2.2g/l)and incubated at 37° C in a humidified 5% CO_2 incubator. The cells were treated with AVE extract and chlorhexidine respectively for 1 minute , after 48 hours of culture period the cells were observed for the dose response of exposure of hGF cells with AVE (1%, 10%, 25%, 50% and 100%) and with Chlorhexidine (1%, 10%, 25%, 50% and 100%).Followed by washing thrice with DMEM and fresh medium was added and cultured in a humidified 5% CO_2 incubator for the next 48 h at 37° C. At the end of the experiment the cells were photographed by Nikon™ ECLIPSE Ti Phase Contrast Microscopelater **(PLATE: V-VI)**.

SULFORHODAMINE-B (SRB) DYE ASSAY

Sulforhodamine-B (SRB) assay[58]is an accurate and reproducible assay based upon the quantitative staining of cellular proteins by sulforhodamine B (SRB)[59] .It was performed to ascertain cell density determination, based on measurement of cellular protein content and the cytostatic/cytotoxic/proliferative effects of cells upon treatment with AVE/CHX at various concentrations according to Shagufta et al,

2006[60].Briefly, 10^4cells/well plated in 96 welled micro titre plate. After 24 hours of culture the cells were treated for 1 minute with different doses of AVE (1%,10%,25%,50%,75%,100%) and CHX (1%,10%,25%,50%,75%,100%) followed by its washing with DMEM and cultured for next 48 hours .At the end of experiment, the cells were fixed with chilled 10% Trichloroacetic acid (TCA) and incubated at 4°C for 1 hour then washed thrice with distilled water followed by air drying.0.4% (w/v)SRB solution dissolved in 1% acetic acid(100µl/well) was added and incubated for 30 minutes at room temperature.Lastly, the SRB boundto the cellular protein was stabilized with 10 mM Tris (pH 10.5) optical density was determined at 560 nm with Spectrophotometer (SpectraMax M2: Molecular Devices).[29,58].**(PLATE: VII-VIII)**

FLUORESCENCE ACTIVATED CELL SORTER (FACS) ANALYSIS

Cell cycle analysis[61] was done by using flow cytometer. The cytometer is capable of analysing cells treated with a fluorescent stain. To analyse cell cycle kinetics/apoptosis, human gingival fibroblasts (0.2×10^{6}) were plated in a 6-welled plate and cultured for 24 hours in DMEM following treatment with *Aloe vera*/CHX as described above and further cultured for next 48 hours. At the end of experiment the cells were collected by trypsinization andwashing with chilled Phosphate buffered saline (PBS, pH 7.4) cells suspension was permeabilized with chilled 70% ethanol for 1 hour at 4°C[61]. Cells collected by trypsinization were washed with chilled Phosphate Buffered Saline (PBS) and centrifuged at 100g for 10 minutes at 4° C,the pellet was resuspended in Propidium Iodide (PI) (40 µg/ml) containing PBS (500 µl) and Ribonucease (RNase) (100 µg/ml)And analysed by Flow cytometry for cell cycle

studieson a Beckton-Dickinson Fluorescence-Activated Cell Sorter (FACS) employing the Cell Quest Software[62,63,29,]. **(PLATE: IX-XII)**

WOUND HEALING ASSAY

In vitro cellular response concerning alignment pattern of human gingival fibroblasts was assessed. Human gingival fibroblasts (0.2×10^{6}) in a 6-welled plate in DMEMsupplemented with penicillin (100 µg/ml), streptomycin (200 µg/ml), gentamicin (50 µg/ml) and 0.2% FBS were cultured at 37°C.Monolayer formation happens after the cells werecultured, one wound/well was created with a sterile 200 µl pipette tip.The monolayer with the wound was washed with growth medium, pre-incubated for 24 hours which is then exposed to AVE and CHX. After 48 hours we observed and noted wound repair using Nikon Eclipse Ti E200 fluorescence microscope and photographed[64]. **(PLATE: XIII)**

STATISTICAL ANALYSIS

Data were summarised as Mean ± SD (standard deviation). Groups were compared by Student's t test. Groups were also compared by one way analysis of variance (ANOVA) and the significance of mean difference between the groups was done by Tukey's post hoc test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variance between groups by Levene's test. A two-tailed $p<0.05$ was considered statistically significant. Analyses were performed on STATISTICA software (Windows version 7.1, StatSoft, Inc., USA). **(APPENDIX II)**

Aloe Vera Plant

LEAF

PLATE NO. I

PREPARATION OF EXTRACT

Weighing of *Aloe Vera*

PLATE NO. II

PREPARATION OF EXTRACT

Extracted with kcl

CENTRIFUGED AT 1000 × G FOR 5 MINUTES
AT 4° C

PLATE NO. III

ALOE VERA EXTRACT AT 4°C

FILTERED THROUGH 45µM SYRINGE FILTER

PLATE NO. IV

CELLULAR MORPHOLOGICAL STUDY

CELLS IN 6 WELL PLATE

PLATE NO. V

CELLULAR MORPHOLOGICAL STUDY

NIKON™ ECLIPSE TI PHASE CONTRAST MICROSCOPE

PLATE NO. VI

CELL GROWTH/CYTOTOXICITY ASSESSMENT

CO_2 INCUBATOR

PLATE NO.VII

CELL GROWTH/CYTOTOXICITY ASSESSMENT

MICROPLATE READER

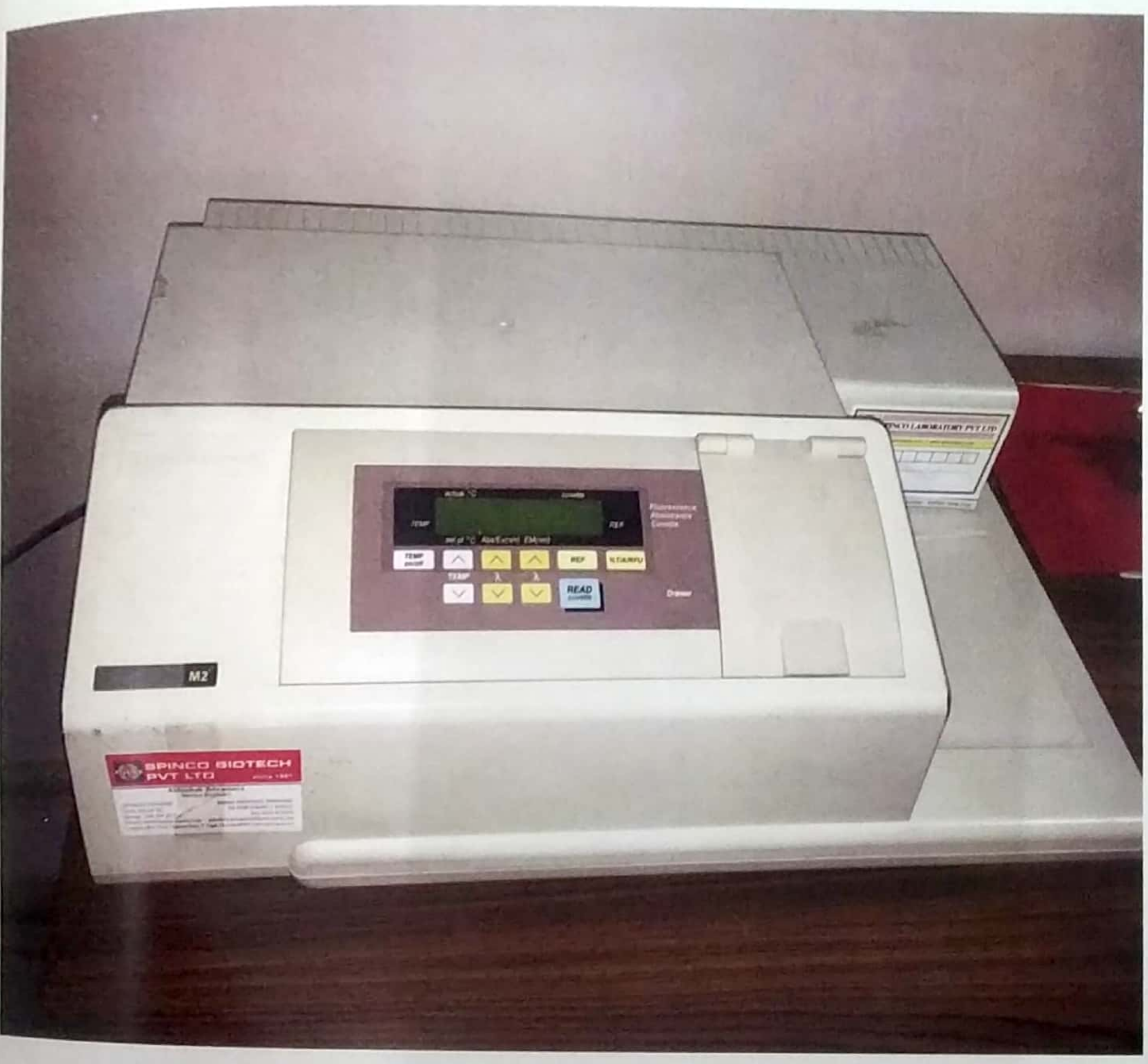


PLATE NO.VIII

CELL CYCLE ANALYSIS

TREATED CELLS WITH DIFFERENT CONCENTRATIONS

PLATE NO. IX

CELL CYCLE ANALYSIS

Cells cultured at 37°C in CO_2 INCUBATOR

PLATE NO. X

CELLS WAS RESUSPENDED IN 40 MG/ML OF PI CONTAINING PBS (500 ML) AND RNASE (100 MG/ML) FOR FLOWCYTOMETRY

PLATE NO. XI

BECKTON-DICKINSON FLUORESCENCE-ACTIVATED CELL SORTER UNIT

PLATE NO. XII

WOUND HEALING ASSAY

NIKON ECLIPSE TI E200 FLUORESCENCE MICROSCOPE

PLATE NO.XIII

Observations & Results

OBSERVATIONS AND RESULTS

The present *in vitro* study evaluates the effect of *Aloe vera* on human gingival fibroblasts (hGF). The effect of different concentrations of *Aloe vera* was observed on cell cycle kinetics (G0/G1, S and G2/M), cell survival/cytotoxicity which was evaluated using FACS analysis and SRB Assay and compared with chlorhexidine (control group). The cells were treated with various concentrations of *Aloe vera* extract and Chlorhexidine for 1 minute, washed with medium and incubated at 37°C humidified CO_2 incubator in DMEM for next 48 h. The cells were examined daily under phase contrast microscope to evaluate their cytomorphology. In addition SRB assay was performed to evaluate cell survival cytotoxicity. To analyze the cell cycle kinetics (G0/G1,S & G2M), FACS analysis was also performed.

A. Cytomorphological study

The cytomorphology of cultured hGF was observed after treatment with consecutive concentrations of *Aloe vera* extract (AVE), CHx and the cells were further cultured for next 48 hours. At the end of experiment the cells were observed under Nikon phase contrast microscope and photographed to study their cytomorphology.

I. Control :

Figure 1 represents the control hGF (without any treatment). It has been observed that the cells appeared abundant, healthy and depicted typically fibroblastic morphology. **(PLATE XIV)**

CYTOMORPHOLOGY OF hGF AFTER TREATMENT WITH CHX AND ALOE VERA (NIKON™ ECLIPSE TI PHASE CONTRAST MICROSCOPE)

Control hGF (100x)

FIGURE: 1

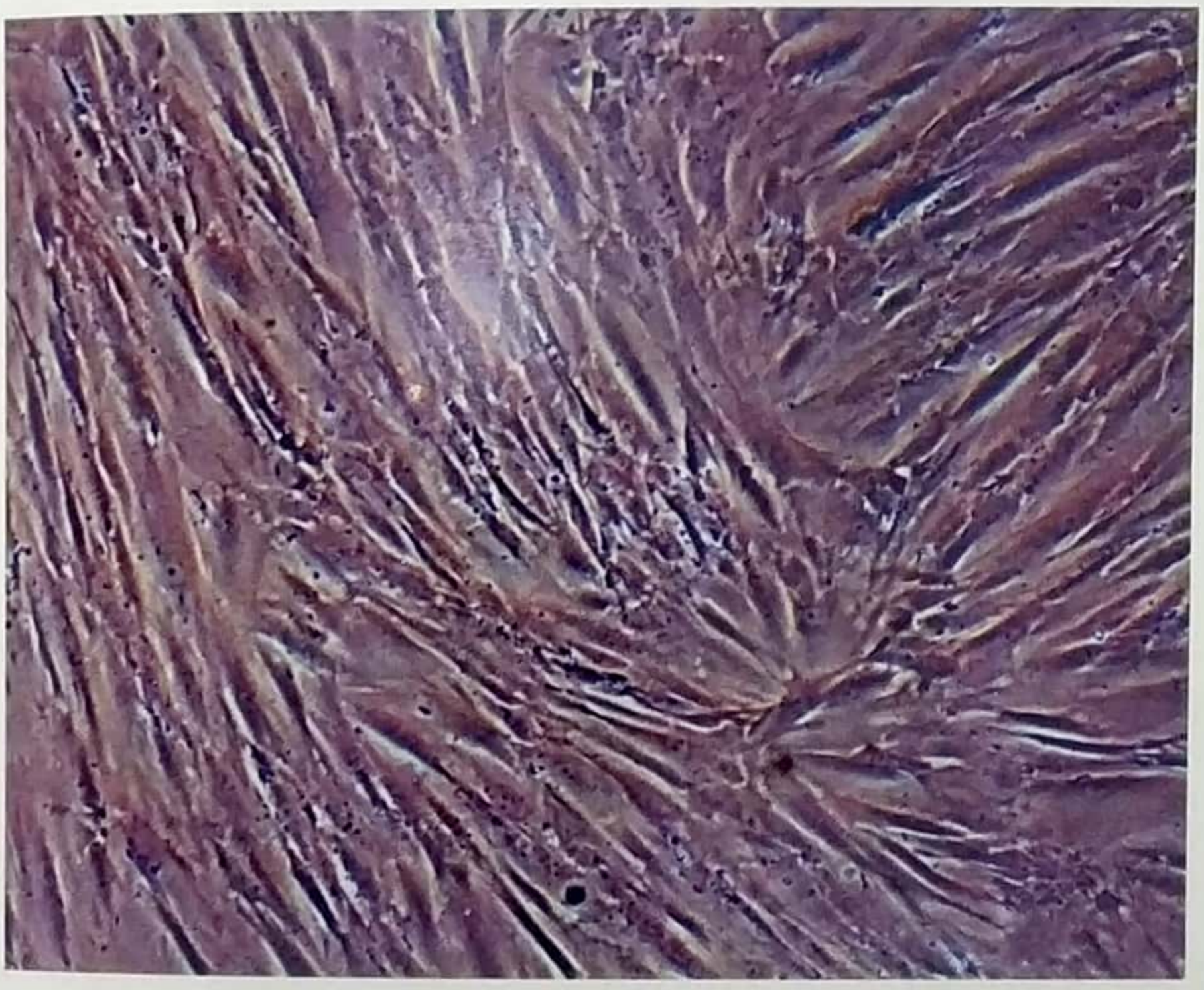


PLATE NO. XIV

Exposure of CHX on hGF (100x)

FIGURE: 2 CHX 1%

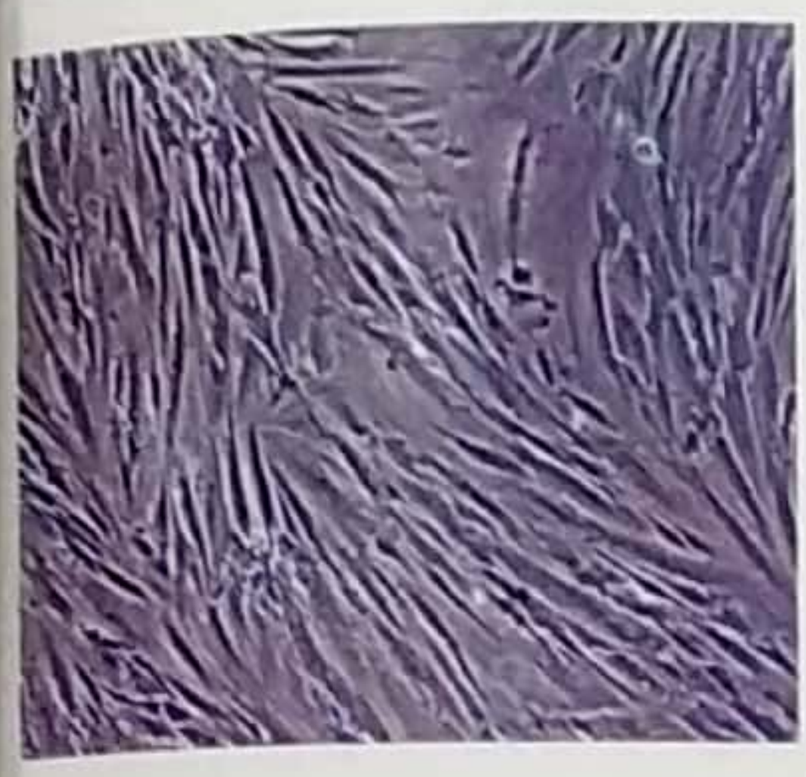


FIGURE: 3 CHX 10%

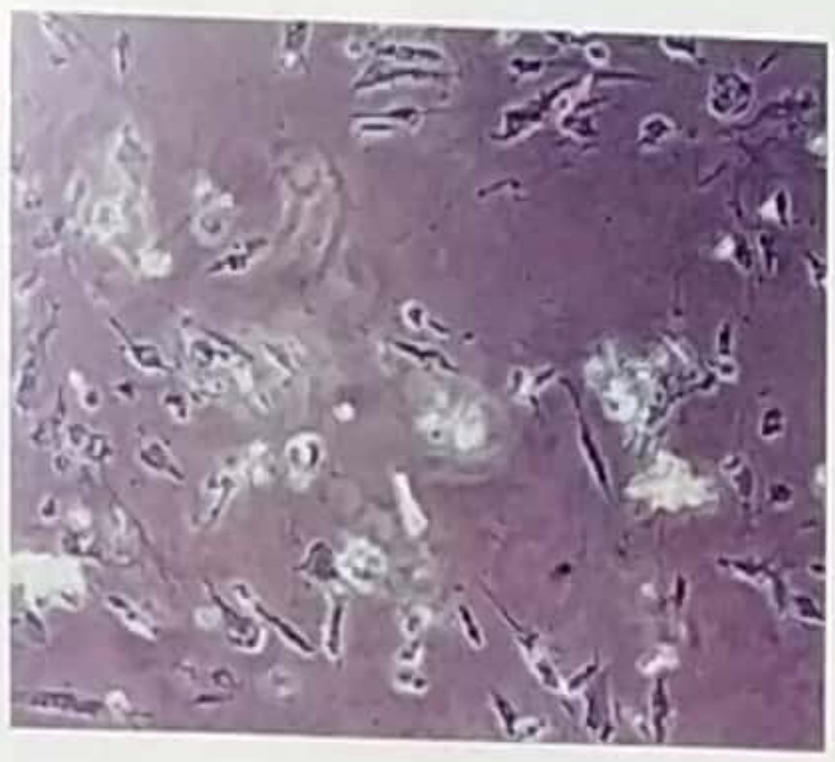


FIGURE: 4 CHX 25%

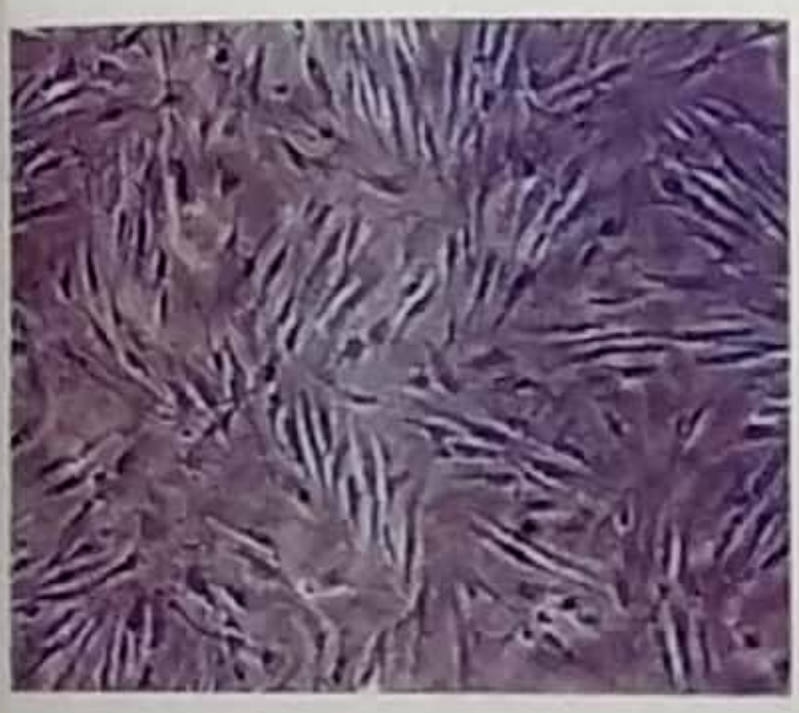


FIGURE: 5 CHX 50%

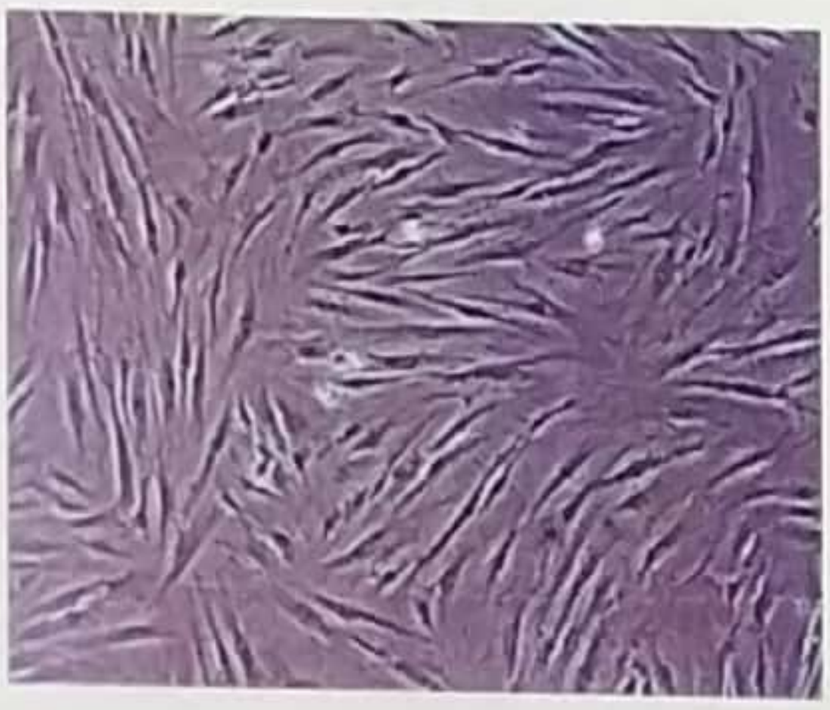


FIGURE: 6 CHX 75%

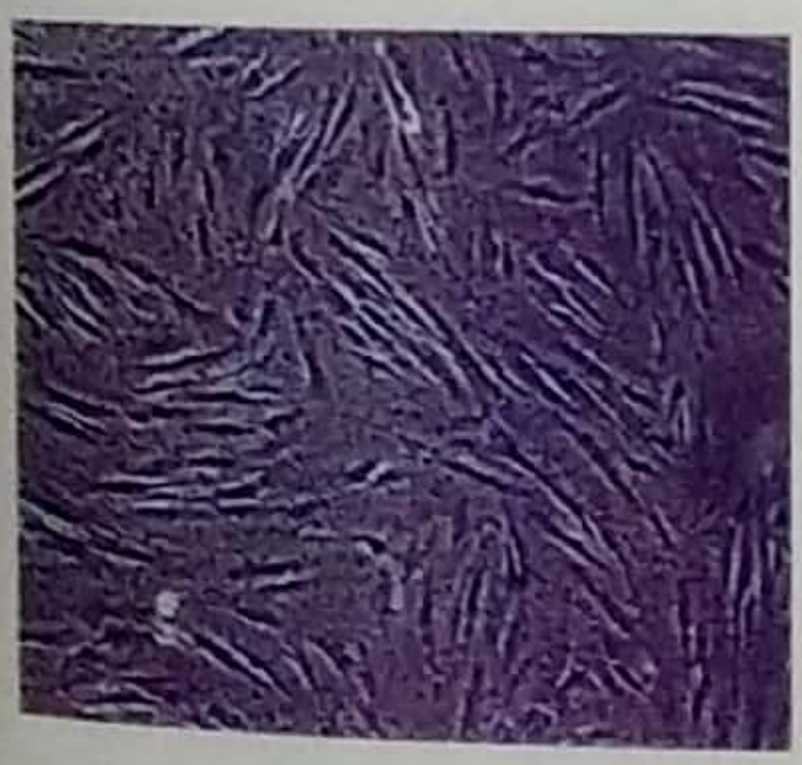


FIGURE: 7 CHX 100%

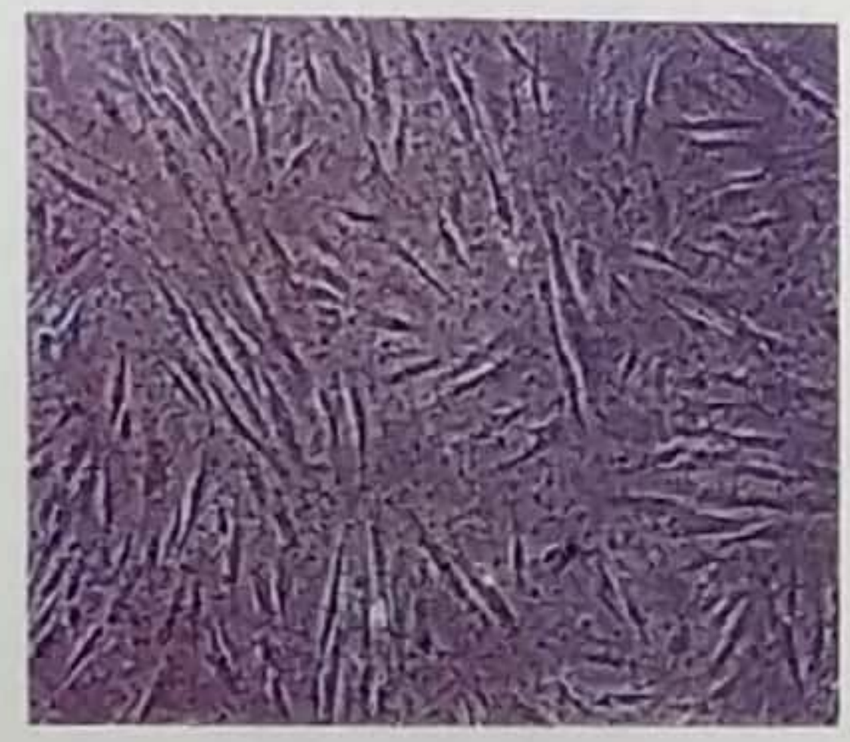


PLATE NO. XV

EXPOSURE OF *ALOE VERA* ON hGF (100X)

FIGURE:8 AVE 1%

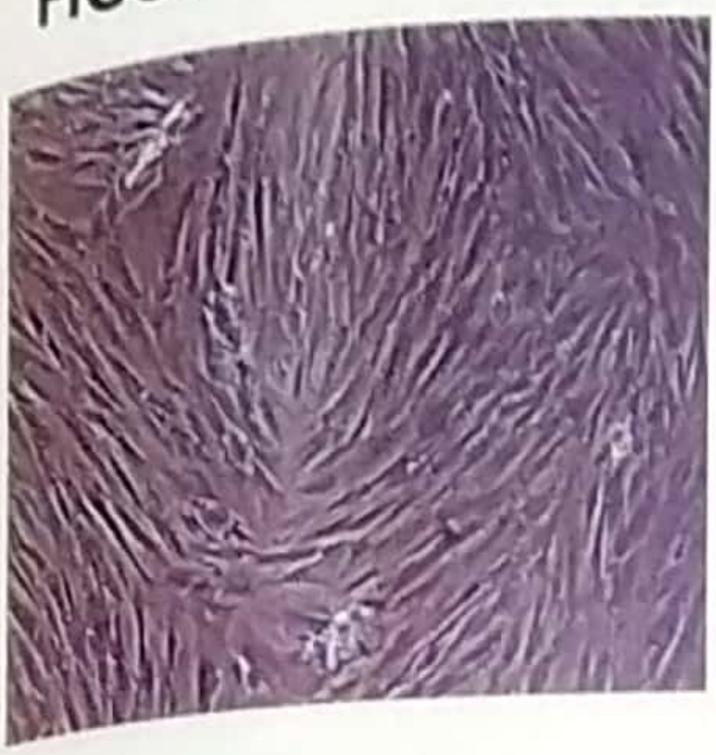


FIGURE:9 AVE 10%

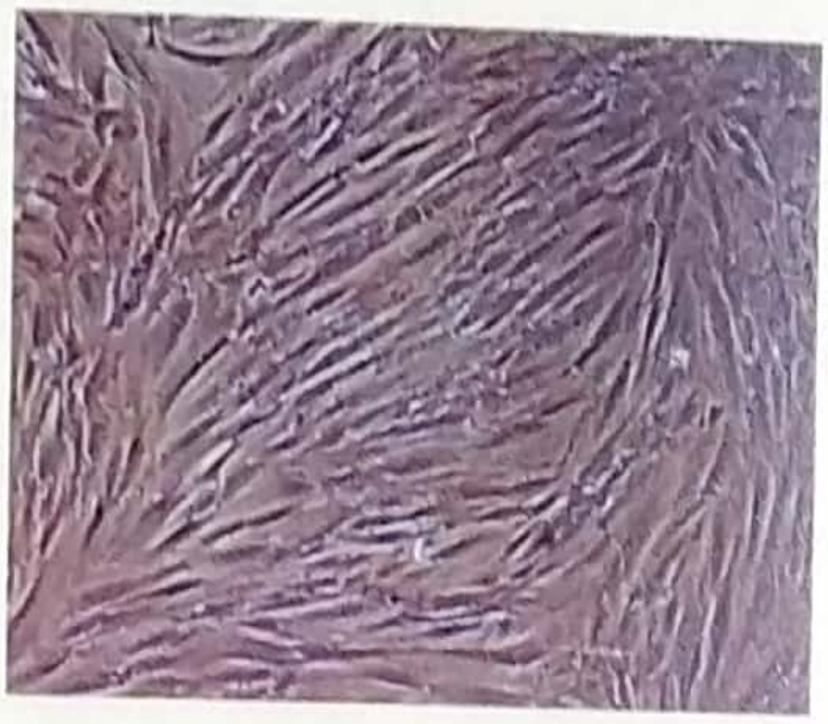


FIGURE:10 AVE 25%

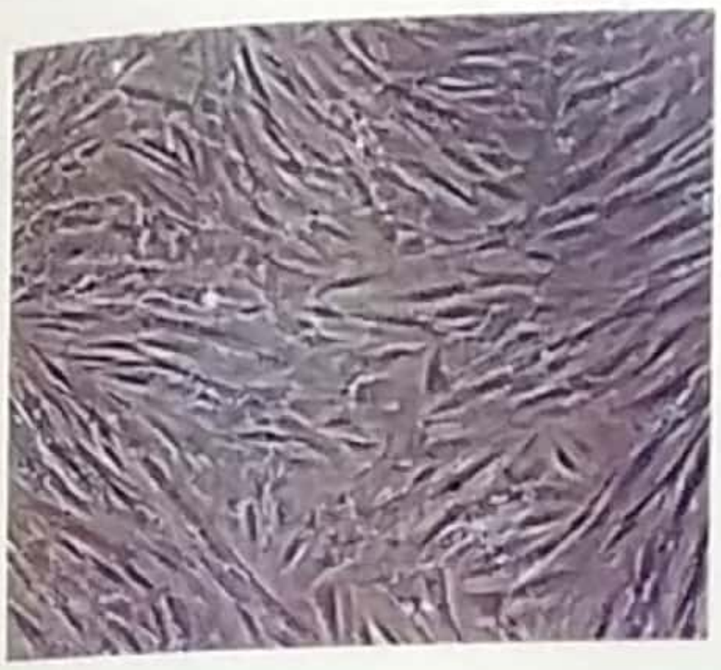


FIGURE:11 AVE 50%

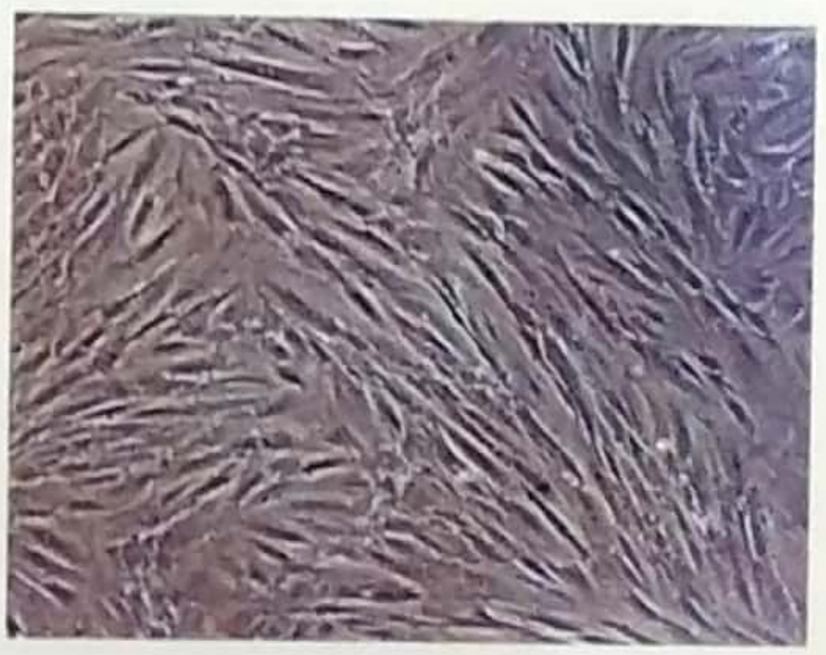


FIGURE:12 AVE 75%

FIGURE:13 AVE 100%

PLATE NO. XVI

B. SULFORHODAMINE B (SRB) DYE ASSAY

SRB assay has been performed to corroborate and confirm the results obtained by the microscopical evaluation of hGF. The effect of CHx and AVE on hGF exposure for 1 minute was undertaken and the results have been compared with the untreated control in different concentrations ranging from 1% – 100%.

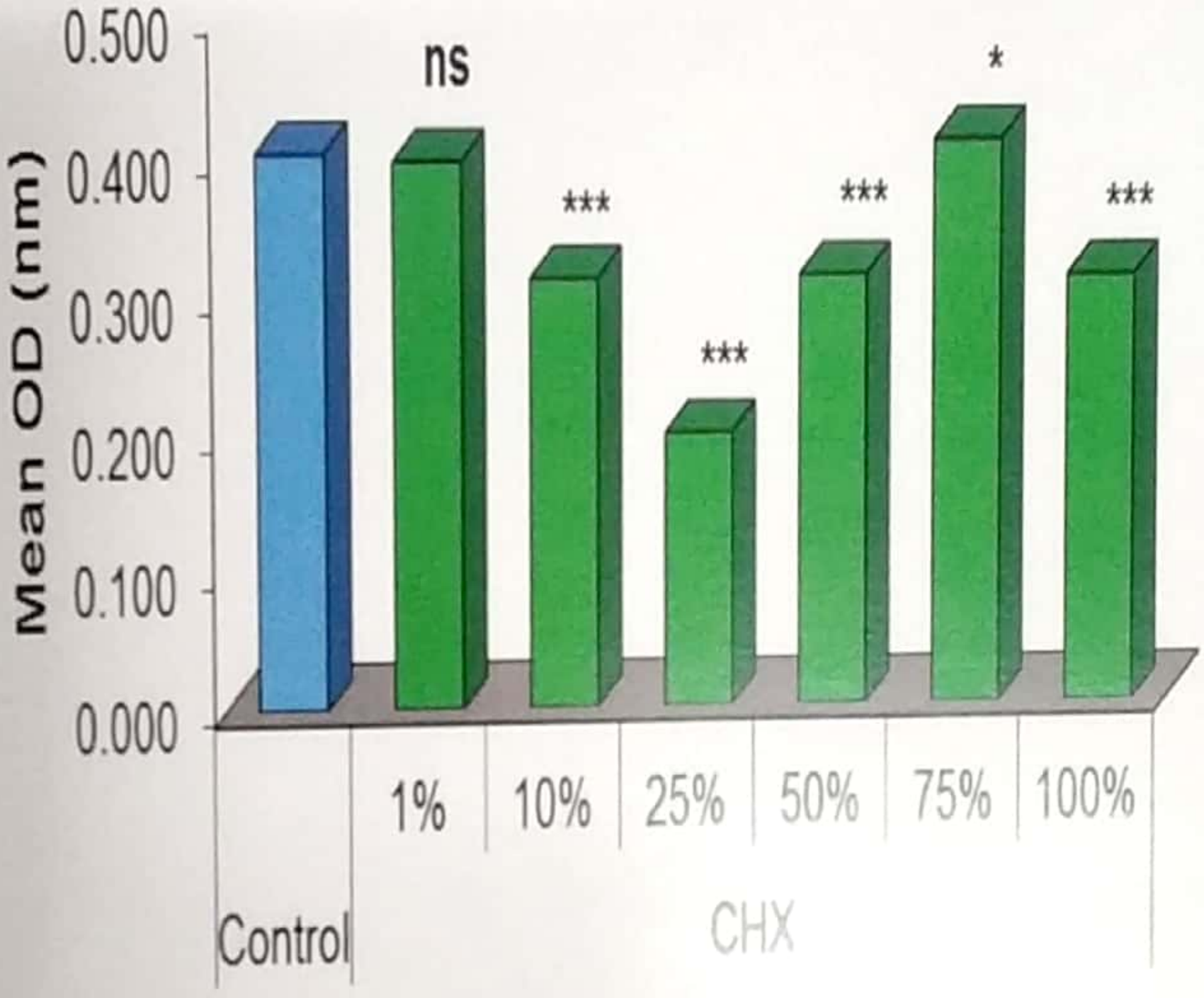
I. Cell survival/cytotoxicity at different concentrations of CHX

For analyzing the effect of CHX on cell survival (hGF), Sulforhodamine B assay was performed and the results shown in Table 1 graph 1. The results clearly indicate Cell survival rate and cytotoxic effects of CHx on hGF at 1%, 10 %, 25% concentrations, and viable cells have been reduced linearly at one minute exposure. At 50% and 75% concentrations and exposure of 1 minute increase in viable cells numbers have been seen, marked reduction at 100% concentration indicating no trend with different concentrations. Mean OD value i.e. cell survival rate decreased comparatively at all concentrations of CHX as compared to control except for 75%.

Evaluating the effect of chlorhexidine (concentrations or groups) on OD values, ANOVA showed significant effect of chlorhexidine on cell survival rate and cytotoxicity ($F=1966.26$, $p<0.001$)

Table 1: Cell survival rate and cytotoxicity (OD value in nm) at different concentrations of CHX

CHX (concentrations)	OD Mean ± SD (n=3)	F Value	p value
Control	0.403 ± 0.001	1966.26	<0.001
1%	0.397 ± 0.004		
10%	0.313 ± 0.003		
25%	0.200 ± 0.001		
50%	0.315 ± 0.005		
75%	0.412 ± 0.003		
100%	0.313 ± 0.003		



$^{ns}p>0.05$ or $^{*}p<0.05$ or $^{***}p<0.001$- as compared to control

Graph 1. Comparison of cell survival rate and cytotoxicity (mean OD value) between different concentrations of CHX.

II. Comparison of cell survival/cytotoxicity between different concentrations of CHX

Further, comparing the mean OD values between different groups, Tukey test showed significantly ($p<0.001$) different and lower cell survival rate and cytotoxicity in CHX at 10%, 25%, 50% and 100% while significantly ($p<0.05$) different and higher at 75% as compared to control. Further, cell survival rate and cytotoxicity also differed significantly ($p<0.01$ or $p<0.001$) between all concentrations of CHX except 10% and 50%, 10% and 100%, and 50% and 100% (Table 2).

Table 2: Comparison (p value) of cell survival/cytotoxicity (mean OD value) between different concentrations of CHX by Tukey test

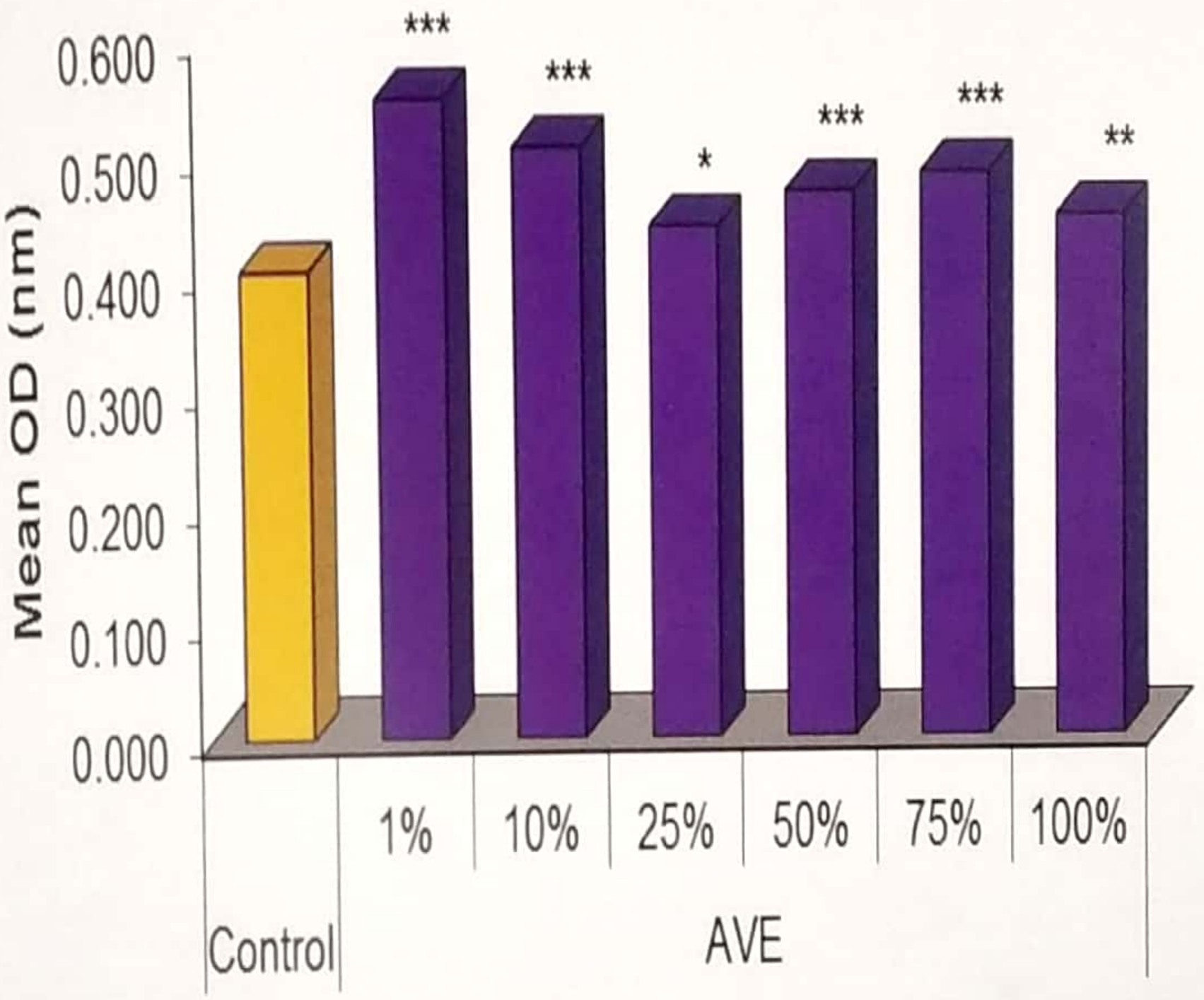
Comparisons- CHX concentrations	**p value**
Control vs. 1%	0.282
Control vs. 10%	<0.001
Control vs. 25%	<0.001
Control vs. 50%	<0.001
Control vs. 75%	0.035
Control vs. 100%	<0.001
1% vs. 10 %	<0.001
1% vs. 25%	<0.001
1% vs. 50%	<0.001
1% vs. 75%	0.001
1% vs. 100%	<0.001
10% vs. 25%	<0.001
10% vs. 50%	0.991
10% vs. 75%	<0.001
10% vs. 100%	1.000
25% vs. 50%	<0.001
25% vs. 75%	<0.001
25% vs. 100%	<0.001
50% vs. 75%	<0.001
50% vs. 100%	0.991
75% vs. 100%	<0.001

III. Cell survival rate and cytotoxicity of AVE

Results in (Table 3 & Graph 2) indicate the effects of AVE on hGF at 1% to 25% concentrations for 1 min exposure, maximum numbers of viable cells are seen at 1% concentration and reduction at 10% and 25% concentration respectively. At 50% and 75% concentrations and exposure of 1 min increase in the number of viable cells was observed, a reduction at 100% concentration is observed. Cell viability and number of viable cells at each concentration were observed to be comparatively higher as compared to control. Evaluating the effect of AVE (concentrations or groups) on OD values, ANOVA showed significant effect of AVE on cell survival rate and cytotoxicity ($F=51.48$, $p<0.001$).

Table 3: Cell survival rate and cytotoxicity (OD value in nm) at different concentrations of AVE

AVE (concentrations)	OD Mean ± SD (n=3)	F Value	P value
Control	0.403 ± 0.001	51.48	<0.001
1%	0.549 ± 0.007		
10%	0.508 ± 0.005		
25%	0.440 ± 0.010		
50%	0.470 ± 0.027		
75%	0.486 ± 0.002		
100%	0.450 ± 0.006		



*p<0.05 or ***p<0.001- as compared to control

Graph 2. Comparison of cell survival rate and cytotoxicity (mean OD value) between different concentrations of AVE.

IV. Comparison of mean cell survival/cytotoxicity between different concentrations of AVE

Comparison of the mean OD values between different groups, showed significant difference ($p<0.05$ or $p<0.01$ or $p<0.001$) when Tukey's test was performed. Higher cell survival rate and cytotoxicity at all concentrations of AVE was observed as compared to those in control (Table 4). Further, cell survival rate and cytotoxicity also differed significantly ($p<0.05$ or $p<0.01$ or $p<0.001$) between all concentrations of AVE except 10% and 75%, 25% and 50%, 25% and 100%, 50% and 75%, and 50% and 100% .

Table 4: Comparison (p value) of mean cell survival rate and cytotoxicity (mean OD value) between different concentrations of AVE by Tukey test

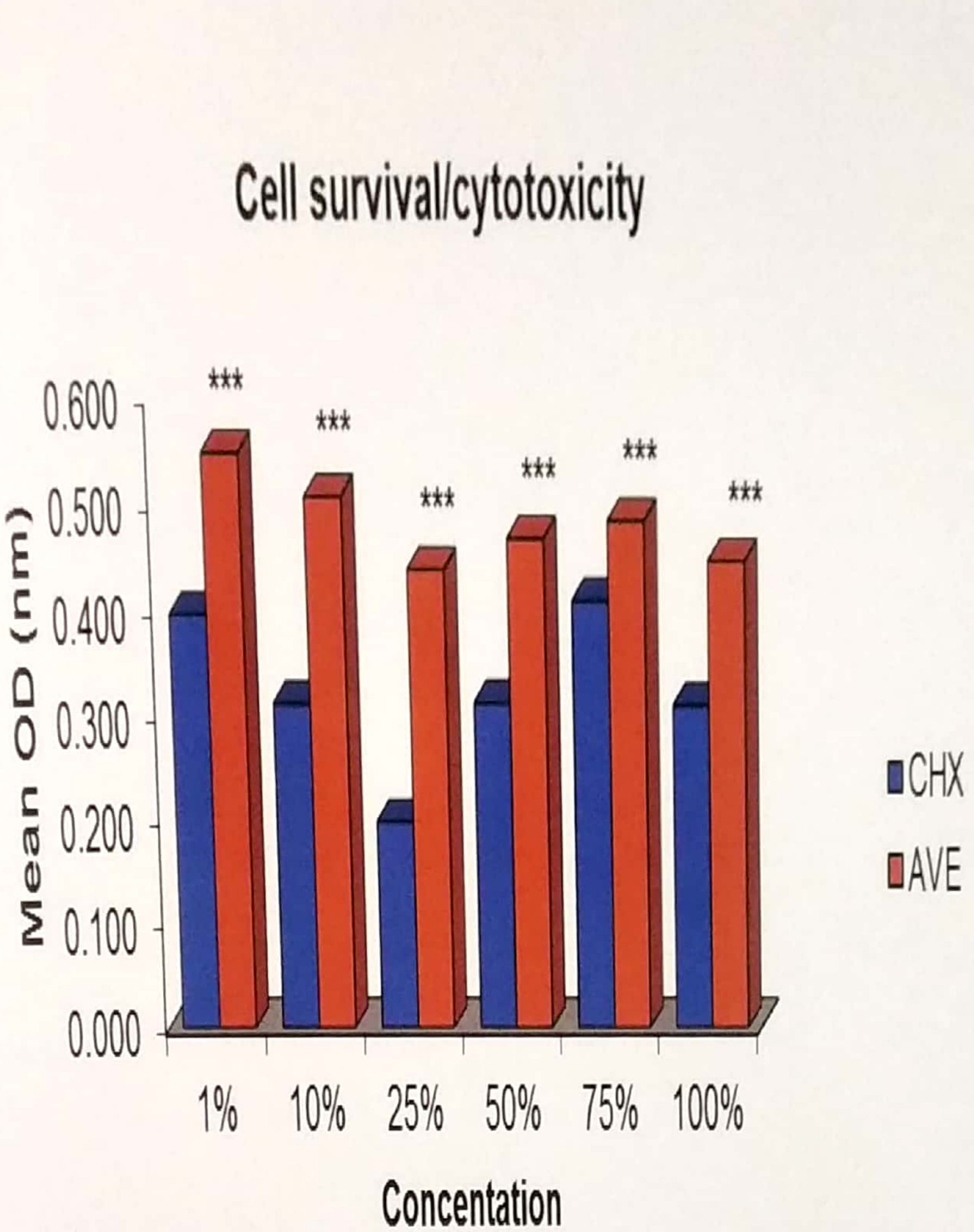
Comparisons- *Aloe vera* concentrations	**p value**
Control vs. 1%	<0.001
Control vs. 10%	<0.001
Control vs. 25%	0.020
Control vs. 50%	<0.001
Control vs. 75%	<0.001
Control vs. 100%	0.003
1% vs. 10 %	0.009
1% vs. 25%	<0.001
1% vs. 50%	<0.001
1% vs. 75%	<0.001
1% vs. 100%	<0.001
10% vs. 25%	<0.001
10% vs. 50%	0.016
10% vs. 75%	0.322
10% vs. 100%	0.001
25% vs. 50%	0.079
25% vs. 75%	0.003
25% vs. 100%	0.939
50% vs. 75%	0.584
50% vs. 100%	0.389
75% vs. 100%	0.021

IV. Comparison of cell survival and cytotoxicity between CHX and AVE at different concentrations

The comparison of cell survival rate and cytotoxicity was also done between CHX and AVE. On comparing, Student's t test showed significantly ($p<0.001$) different and higher cell survival rate and cytotoxicity (mean OD) in AVE as compared to CHX at all concentrations (Table 5 and Graph. 3).

Table 5: Comparison of cell survival rate and cytotoxicity (OD value in nm) between CHX and AVE at different concentrations

Concentration	Chlorhexidine (Mean ± SD, n=3)	AVE (Mean ± SD, n=3)	t value	p value
1%	0.397 ± 0.004	0.549 ± 0.007	33.84	<0.001
10%	0.313 ± 0.003	0.508 ± 0.005	64.49	<0.001
25%	0.200 ± 0.001	0.440 ± 0.010	40.64	<0.001
50%	0.315 ± 0.005	0.470 ± 0.027	9.90	<0.001
75%	0.412 ± 0.003	0.486 ± 0.002	36.34	<0.001
100%	0.313 ± 0.003	0.450 ± 0.006	35.55	<0.001



***p<0.001- as compared to chlorhexidine

Graph 3. Comparison of cell survival rate and cytotoxicity (mean OD value) between chlorhexidine and *Aloe vera* at different concentrations.

C. FACS ANALYSIS (Fluorescent activated cell sorter analysis)

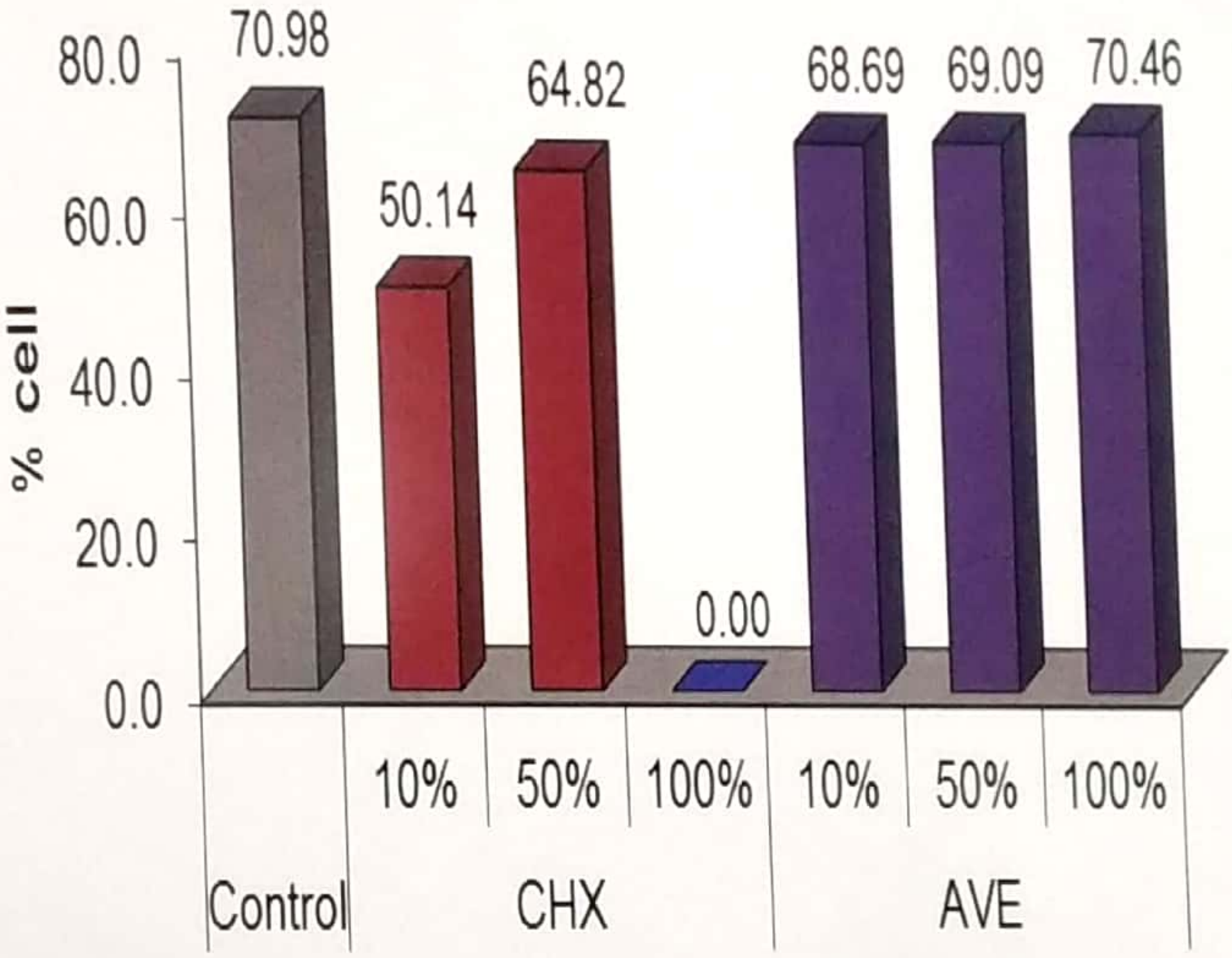
Cell cycle analysis was done by using flow cytometer. The cytometer is capable of analyzing cells treated with a fluorescent stain. The fluorescence intensity is directly proportional to the DNA content of each cell. The control cells have resolved into maximal G_0/G_1 (resting phase), minimal S (synthetic phase) and residual G_2/M (mitotic phase). The distribution of percent cell cycle in G0/G1 phase, S phase and G2/M phase of two treatment groups (chlorhexidine and *Aloe vera*) at three different concentrations (10%, 50% and 100%) is summarized in Table 1 and also depicted in Fig. 14 to 20, respectively**(PLATE – XVII-XXIII)**.

The percent cell cycle in G0/G1 phase was observed to be lowered in both the treatment group at all three concentrations as compared to control. Further, in *Aloe vera*, percent cell cycle increases with increase in concentrations but in chlorhexidine it did not show any trend, increase at 50% and decrease at 100% (Table 6 and Graph 4,5,6). In contrast, the percent cell cycle in S phase was found to be higher in both chlorhexidine (10% and 50%) and *Aloe vera* (10%, 50% and 100%) as compared to control. Further, percent cell cycle showed linear decrease with concentrations in chlorhexidine but no trend in *Aloe vera*, increase at 50% and decrease at 100% was observed (Table 6 and Graph 4,5,6).

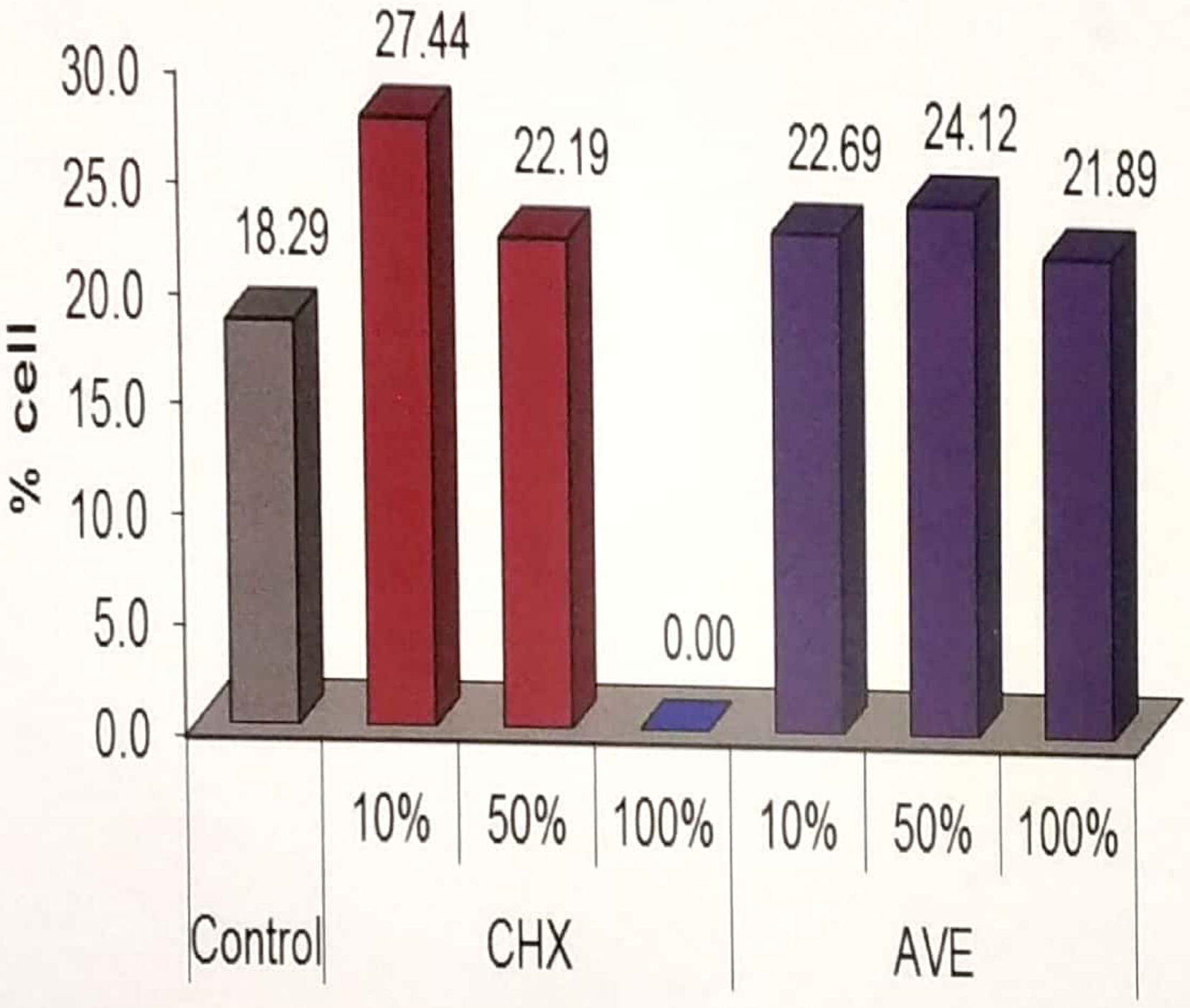
Conversely, the percent cell cycle in G2/M phase was found to be higher in all concentrations of *Aloe vera* while only 50% in chlorhexidine as compared to control but no trend with concentrations in both groups, increase at 50% and decrease at 100% was observed (Table 6 and Graph 4,5,6).

Table 6: % cell cycle distribution of two treatment groups at different concentrations at G0/G1, S and G2/M phases

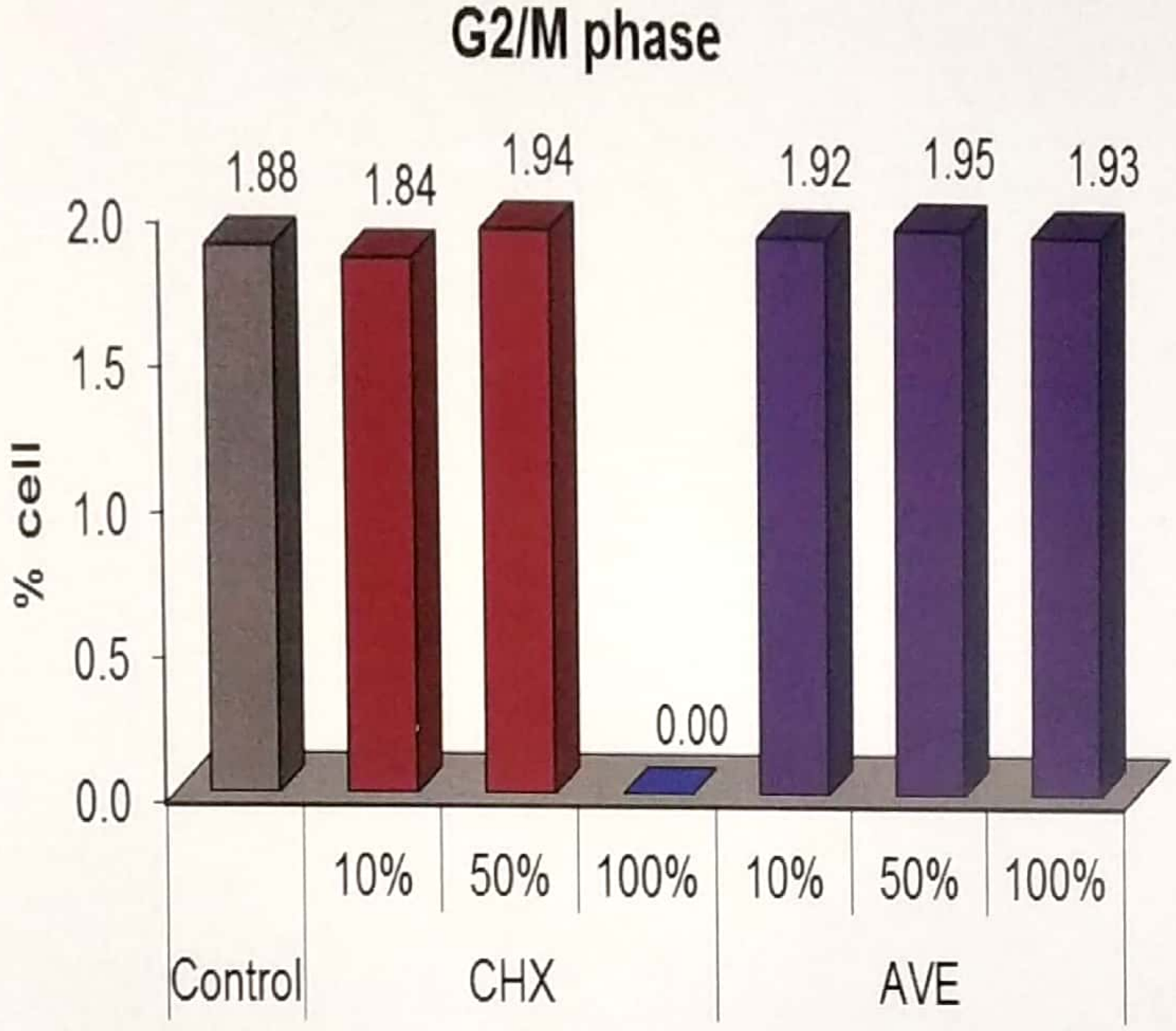
Groups	Apoptotic cells	G0/G1 phase	S phase	G2/M phase
Control	0%	70.98	18.29	1.88
CHX:				
10%	1.63%	50.14	27.44	1.84
50%	80.87%	64.82	22.19	1.94
100%	100.00%	0.00	0.00	0.00
AVE:				
10%	0.00%	68.69	22.69	1.92
50%	0.00%	69.09	24.12	1.95
100%	0.04%	70.46	21.89	1.93



Graph 4. Percent cell cycle distribution of two treatment groups at three different concentrations at G0/G1 phase.

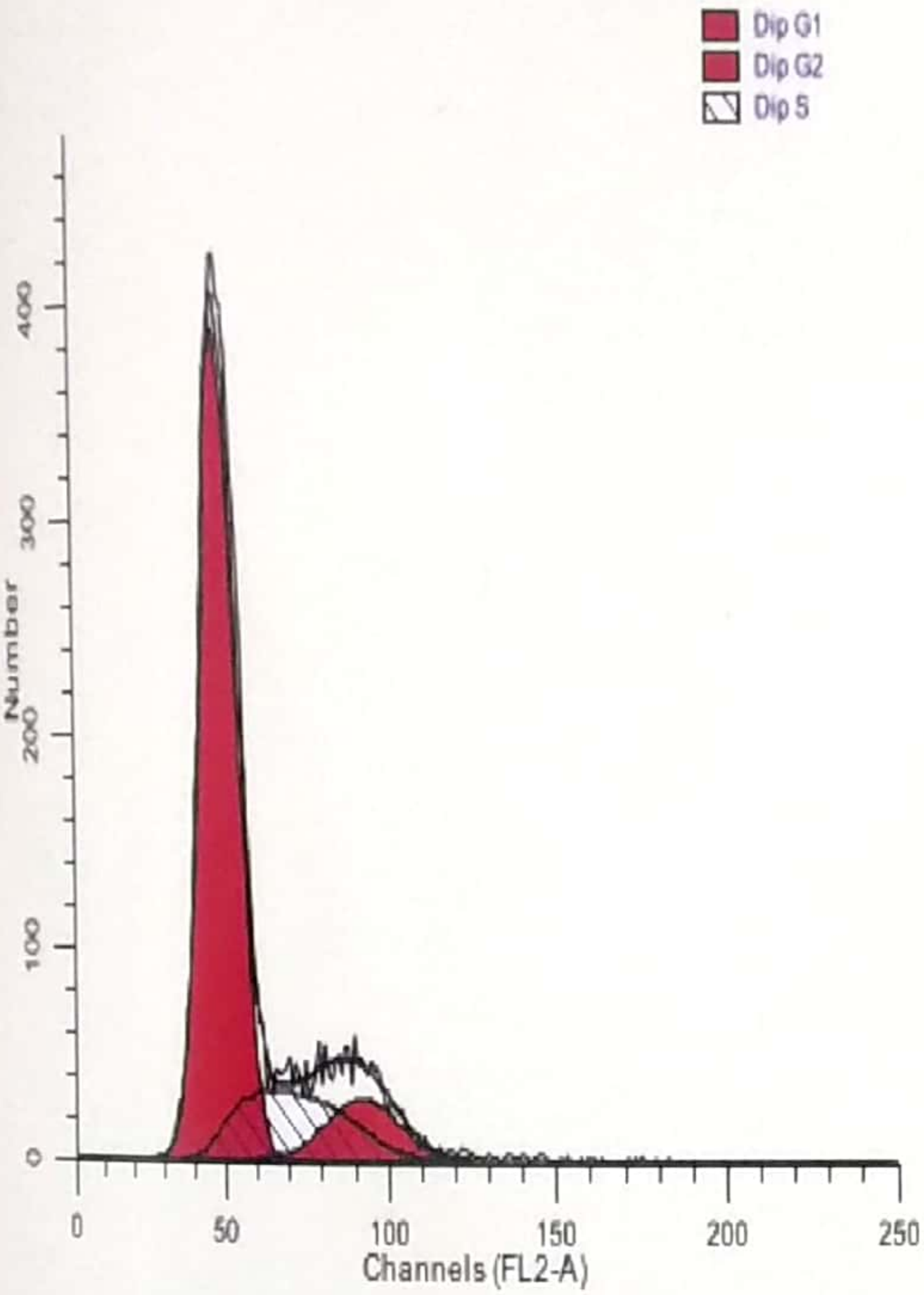


Graph 5. Percent cell cycle distribution of two treatment groups at three different concentrations at S phase.



Graph 6. Percent cell cycle distribution of two treatment groups at three different concentrations at G2/M phase.

FIG 14: FACS ANALYSIS OF hGF (control)



File analyzed: 06:05:16.001
Date analyzed: 9-May-2016
Model: 1nn0n_DSF
Analysis type: Manual analysis

Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 70.98 % at 49.22
Dip G2: 10.74 % at 92.54
Dip S: 18.29 % G2/G1: 1.88
%CV: 11.39

Total S-Phase: 18.29 %
Total B.A.D.: 0.00 % no debris no aggs

Debris: %
Aggregates: 0.00 %
Modeled events: 7682
All cycle events: 7682
Cycle events per channel: 173
RCS: 1.136

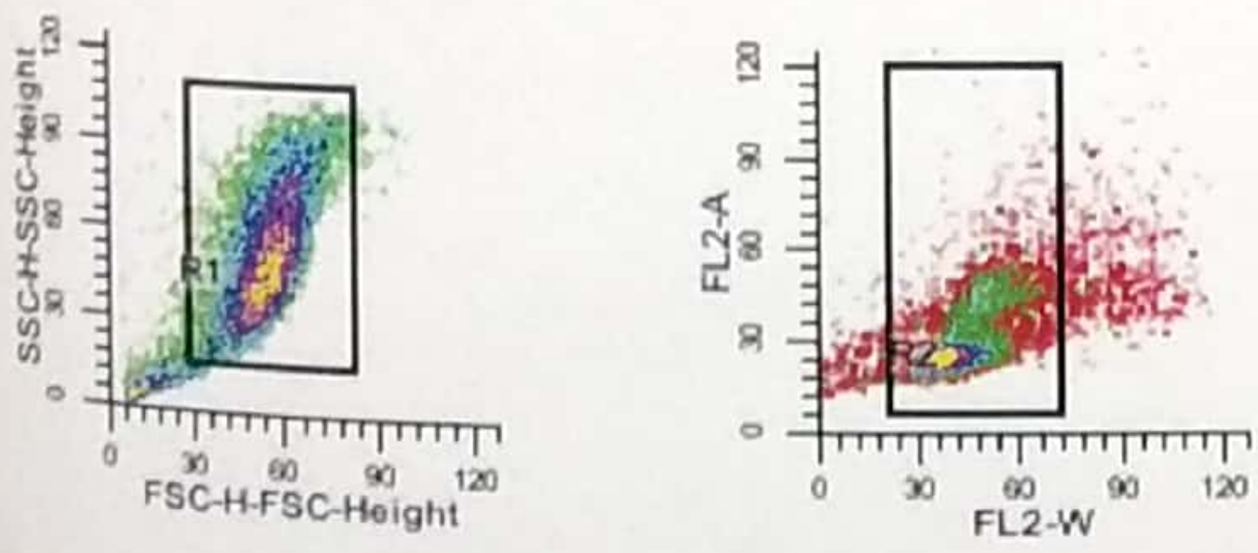
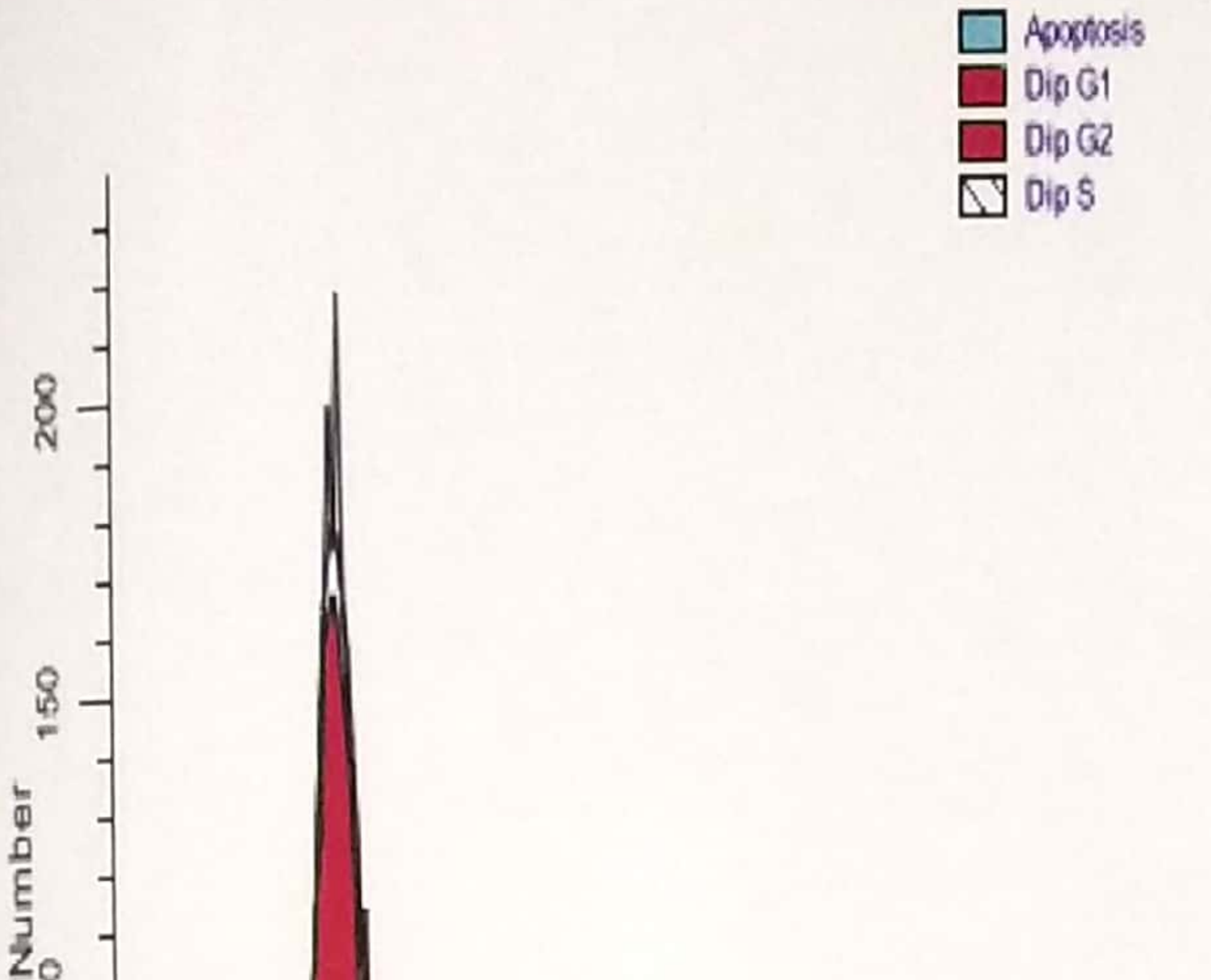


PLATE NO. XVII

FIG 15: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH CHX 10%



File analyzed: 06:05:16.002
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

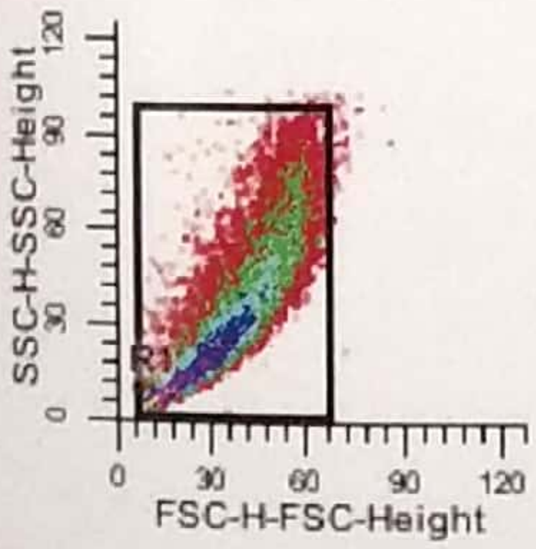
Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 58.14 % at 50.94
Dip G2: 14.43 % at 93.53
Dip S: 27.44 % G2/G1: 1.84
%CV: 10.18

Total S-Phase: 27.44 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: 1.63 % Mean: 19.27

Debris: %
Aggregates: 0.00 %
Modeled events: 3798
All cycle events: 3737
Cycle events per channel: 86
RCS: 0.806



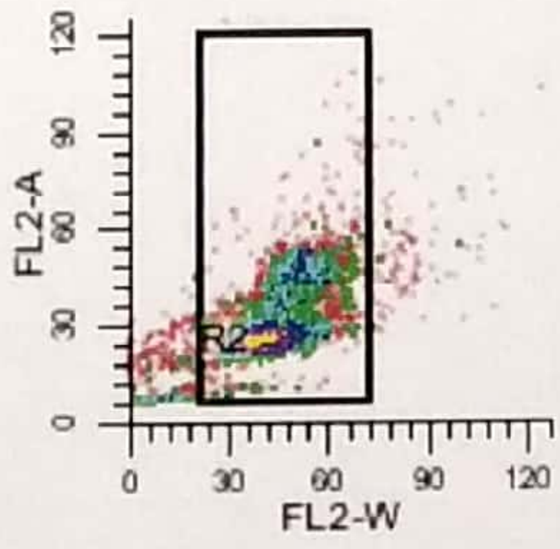


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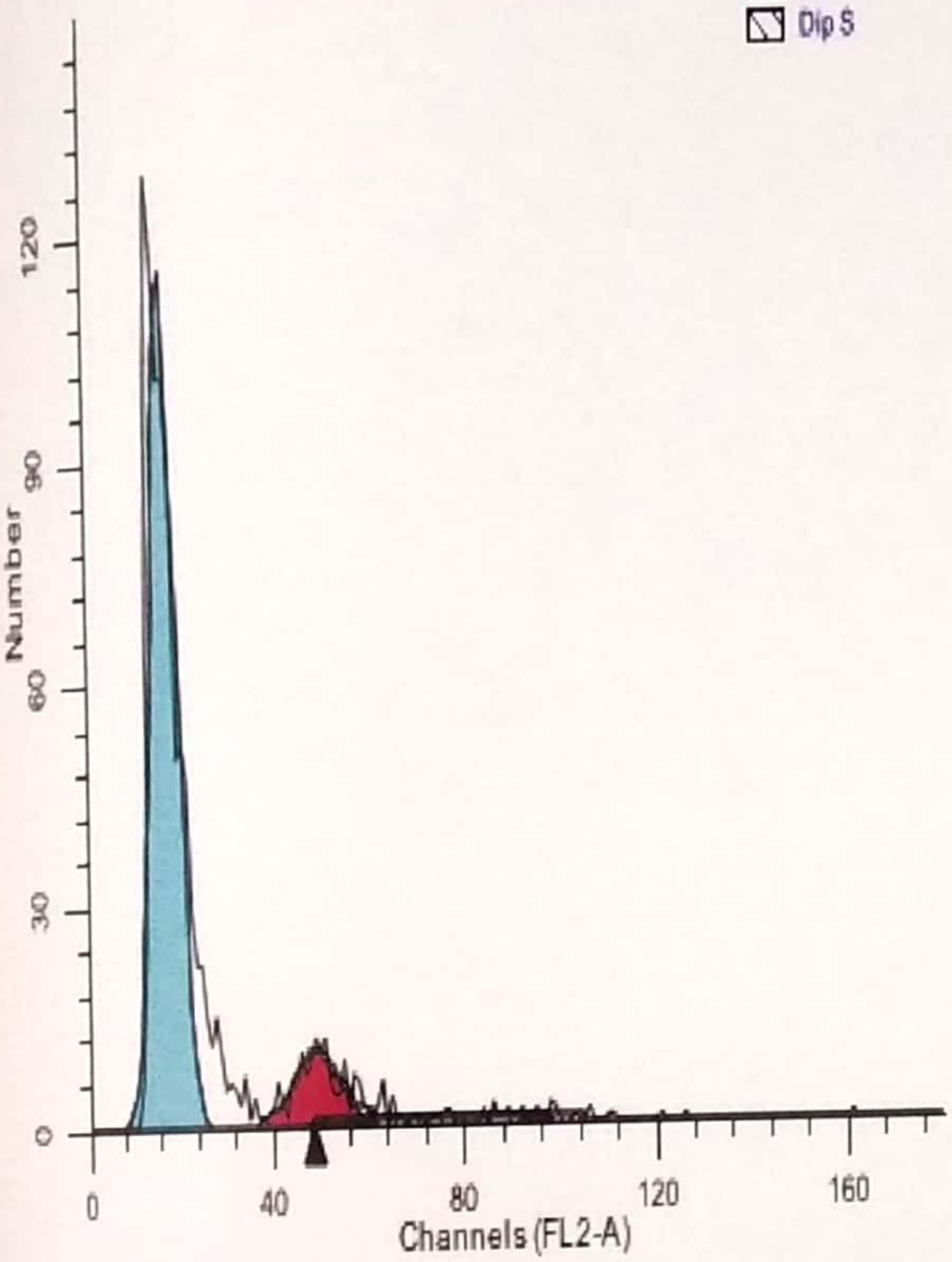
PLATE NO.XVIII

FIG 16: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH CHX 50%



File analyzed: 06:05:16.003
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

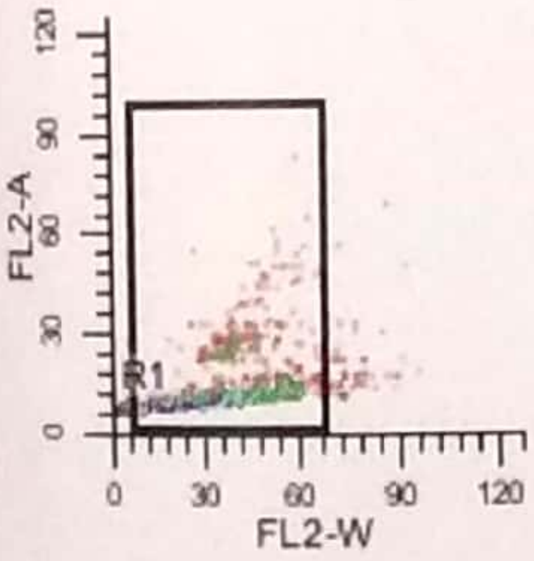
Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 64.82 % at 49.29
Dip G2: 6.99 % at 95.66
Dip S: 28.19 % G2/G1: 1.94
%CV: 10.04

Total S-Phase: 28.19 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: 80.87 % Mean: 17.73

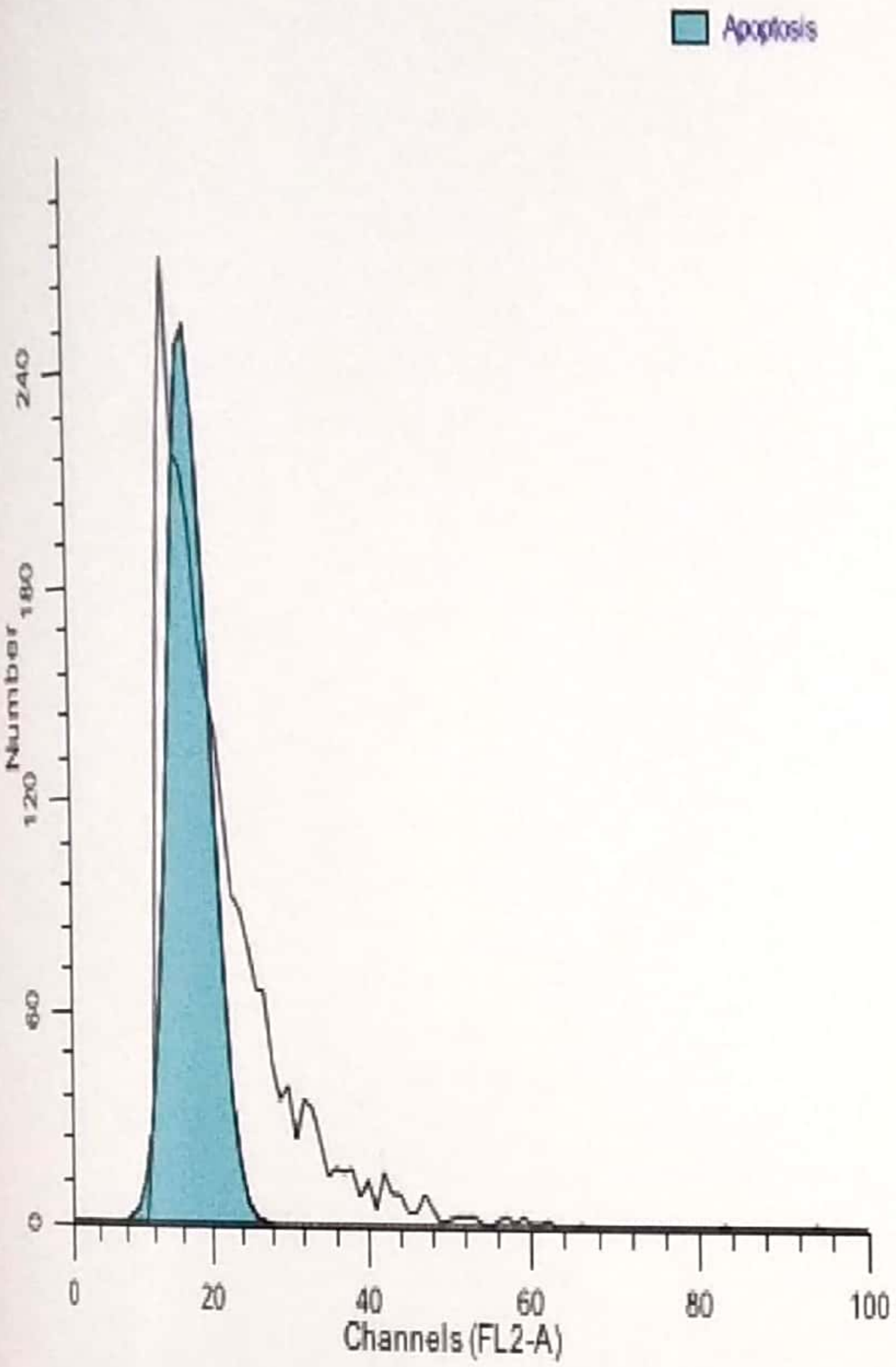
Debris: %
Aggregates: 0.00 %
Modeled events: 970
All cycle events: 186
Cycle events per channel: 4
RCS: 1.873



ModFitLT V3.2.1(Mac)

PLATE NO. XIX

FIG 17: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH CHX 100%



File analyzed: 06:05:16.004
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

Ploidy Mode: First cycle is diploid

Diploid: 0.00 %
Dip G1: % at
Dip G2: % at
Dip S: 0.00 % G2/G1:
%CV:

Total S-Phase: 0.00 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: 100.00 % Mean: 17.67

Debris: %
Aggregates: 0.00 %
Modeled events: 1721
All cycle events: 0
Cycle events per channel: 0
RCS: 12.974

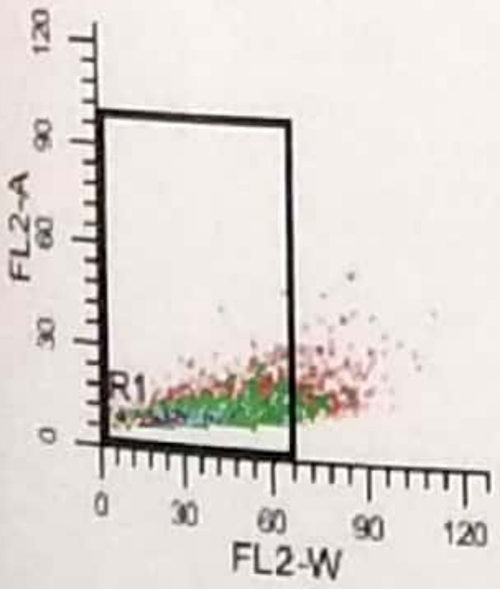
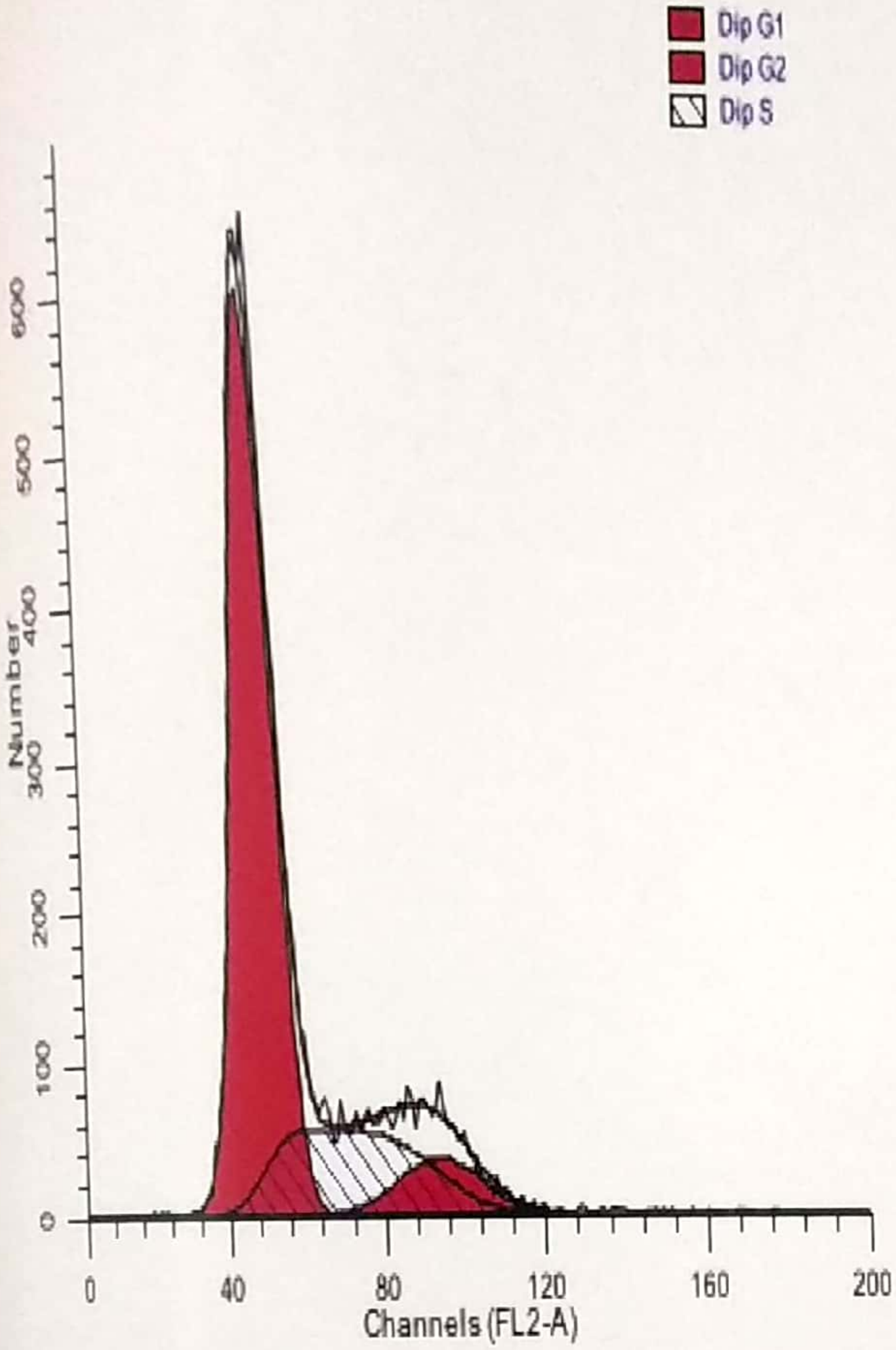


PLATE NO. XX

FIG 18: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH AVE 10%



File analyzed: 06:05:16.005
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 68.69 % at 48.84
Dip G2: 8.62 % at 94.01
Dip S: 22.69 % G2/G1: 1.92
%CV: 10.58

Total S-Phase: 22.69 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

Debris: %
Aggregates: 0.00 %
Modeled events: 11307
All cycle events: 11307
Cycle events per channel: 245
RCS: 1.107

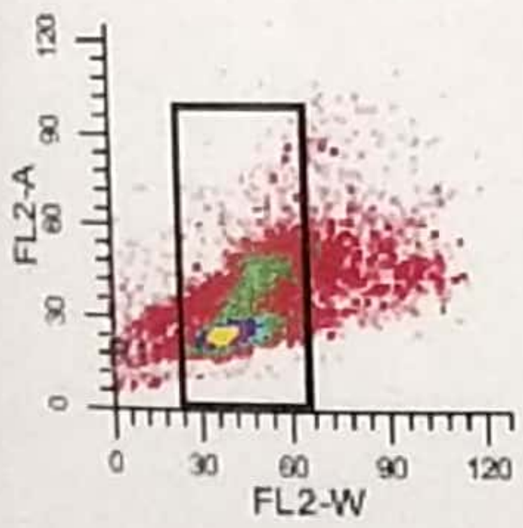


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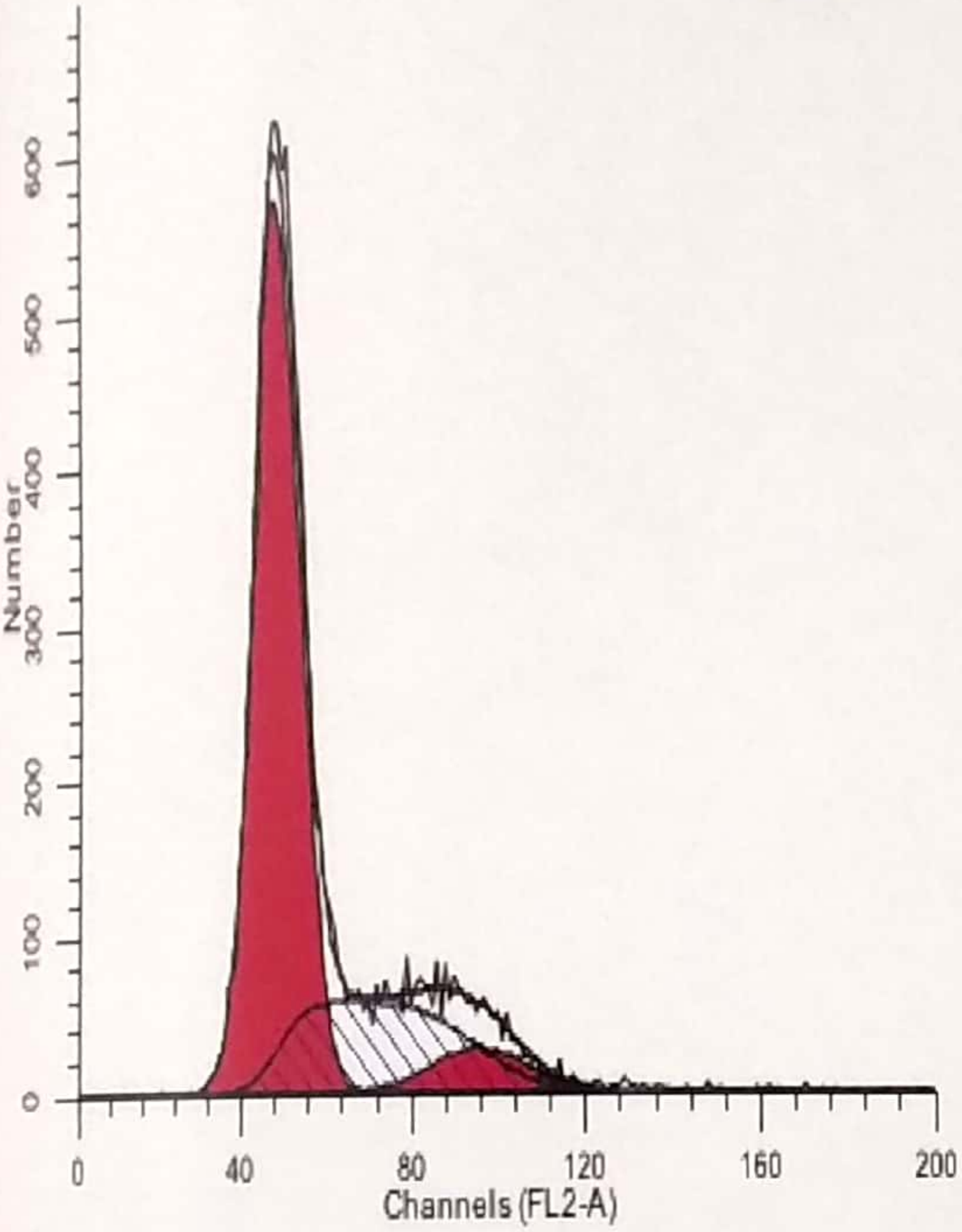
PLATE NO. XXI

FIG 19: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH AVE 50%



File analyzed: 06:05:16.006
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

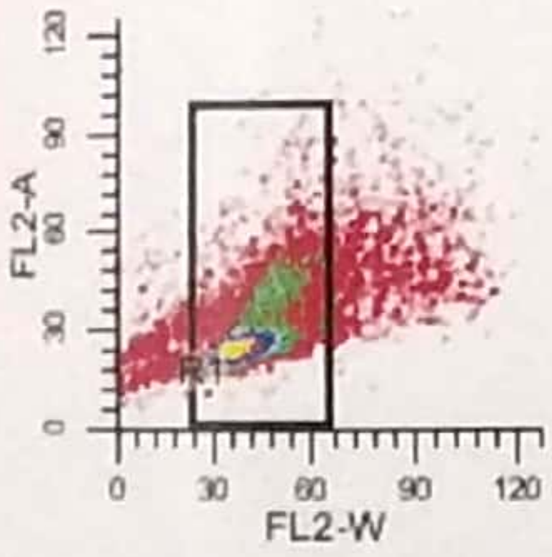
Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 69.09 % at 49.22
Dip G2: 6.80 % at 95.91
Dip S: 24.12 % G2/G1: 1.95
%CV: 11.17

Total S-Phase: 24.12 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

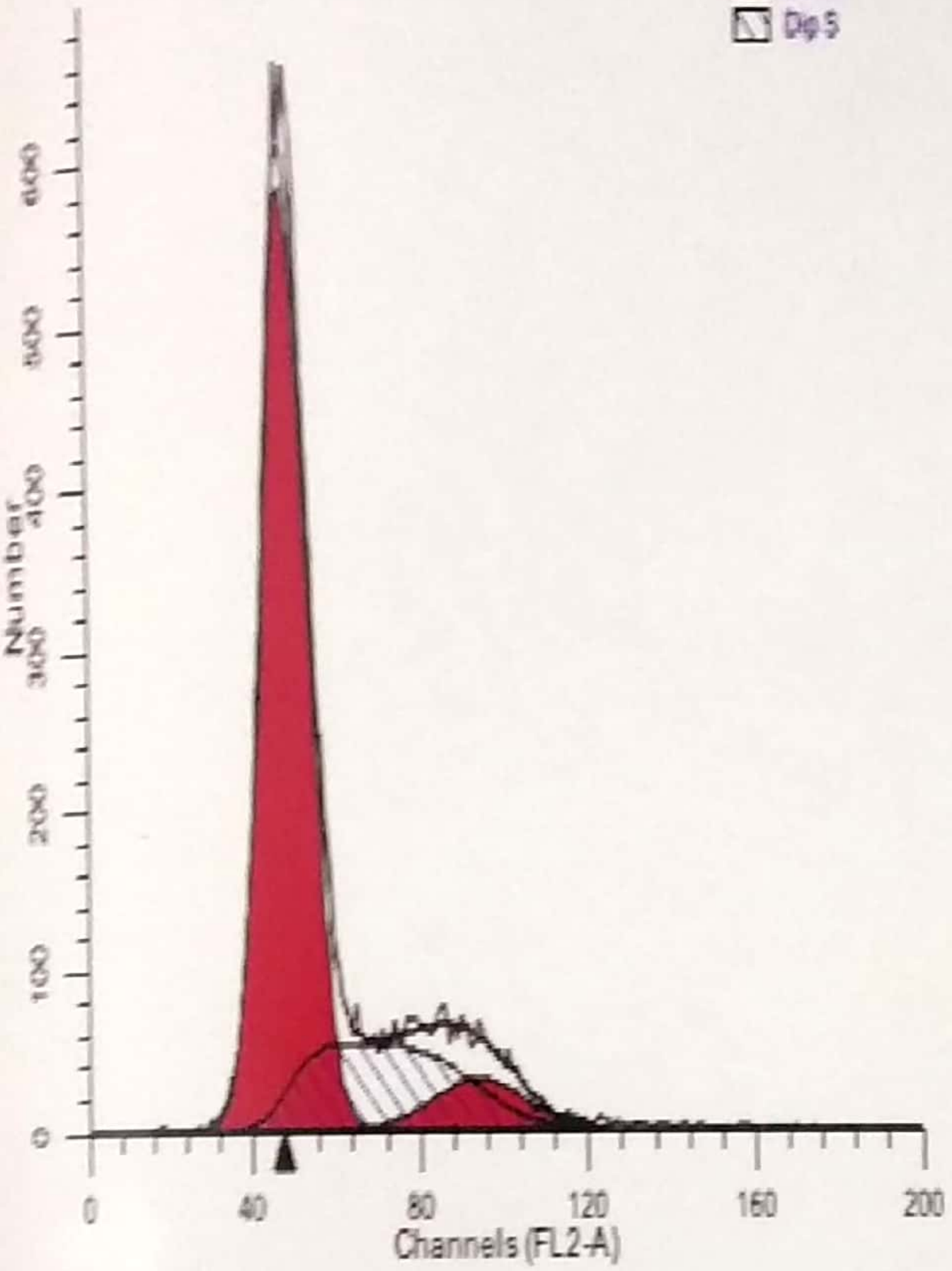
Debris: %
Aggregates: 0.00 %
Modeled events: 11318
All cycle events: 11318
Cycle events per channel: 237
RCS: 1.365



ModFit V3.2.1(Mac)

PLATE NO. XXII

FIG 20: FACS ANALYSIS OF hGF SHOWING TREATMENT WITH AVE 100%



File analyzed: 06:05:16.007
Date analyzed: 9-May-2016
Model: 1nn0A_DSF
Analysis type: Manual analysis

Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 70.46 % at 48.86
Dip G2: 7.64 % at 94.14
Dip S: 21.89 % G2/G1: 1.93
%CV: 11.15

Total S-Phase: 21.89 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: 0.04 % Mean: 18.48

Debris: %
Aggregates: 0.00 %
Modeled events: 11289
All cycle events: 11284
Cycle events per channel: 244
RCS: 1.393

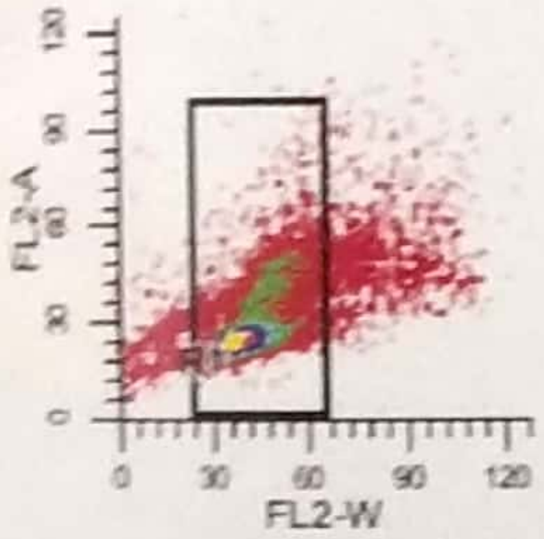


PLATE NO. XXIII

D. WOUND HEALING ASSAY

Wound healing assay was performed to study the effect on proliferation/migration of Human Gingival Fibroblasts by CHX and AVE at various concentrations after 1 minute of their exposure. After 48 hours wound gap showed poor mitotic index in the control cells. (Figure 22)

The cytotoxicity was seen with rounded shrunken cells in the wound area at 10% CHX exposure (Figure 23). Subsequently, widening of wound at concentration of 50% to 100% CHX (Figure 24-25) exposure showed cytotoxicity. Marked reduction in the size of the gap width was observed at 10 % (Figure 26) AVE. At 100 % AVE (Figure 28) exposure, improved closure of wound area with aggregated fibroblasts was observed. **(PLATE XXIV-XXVI)**

EFFECT OF CONTROL ON WOUND CLOSURE IN hGF MONOLAYER (NIKON ECLIPSE TI E 200 FLUORESCENCE MICROSCOPE)

FIGURE: 21
CONTROL(DAY ZERO)

FIGURE:22
CONTROL(POST 48 h)

PLATE NO. XXIV

EFFECT OF CHX ON WOUND CLOSURE IN hGF MONOLAYER (NIKON ECLIPSE TI E 200 FLUORESCENCE MICROSCOPE)

FIGURE: 23 CHX 10%

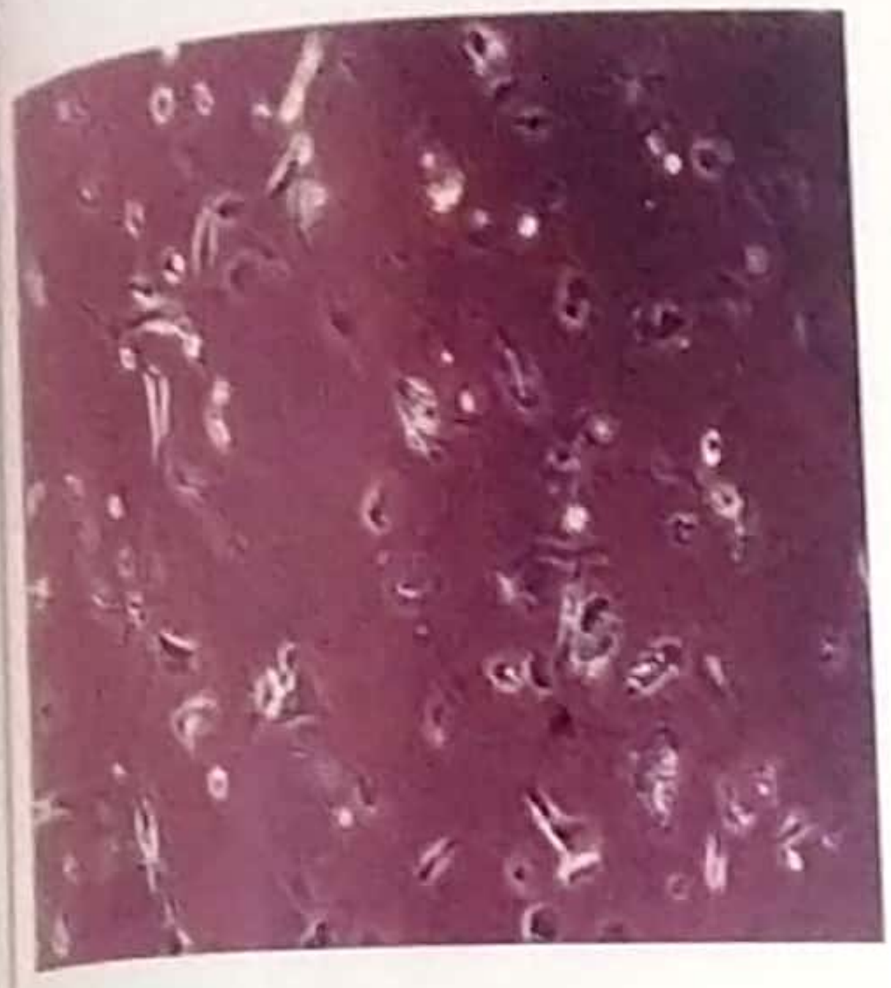


FIGURE: 24 CHX 50%

FIGURE: 25 CHX 100%

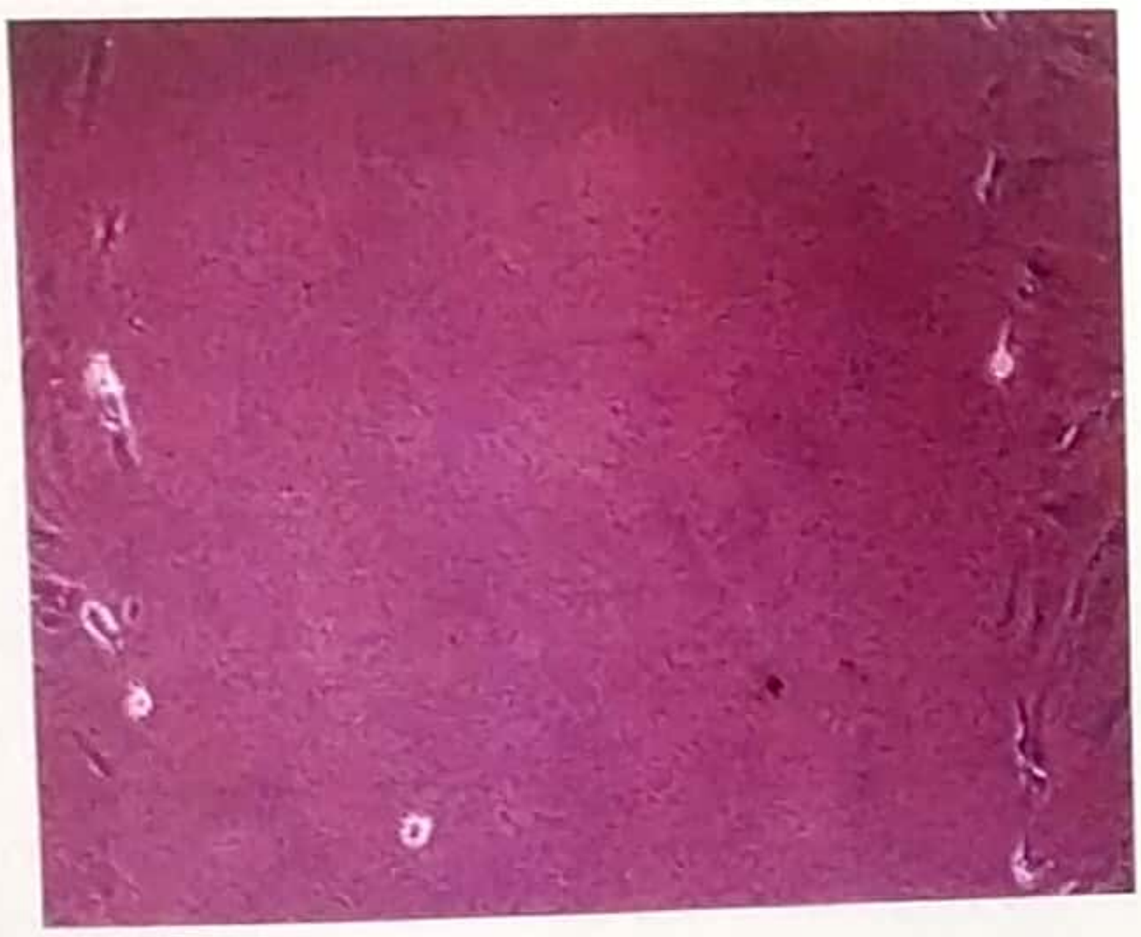


PLATE NO. XXV

EFFECT OF AVE ON WOUND CLOSURE IN hGF MONOLAYER (NIKON ECLIPSE TI E 200 FLUORESCENCE MICROSCOPE)

FIGURE: 26 AVE 10%

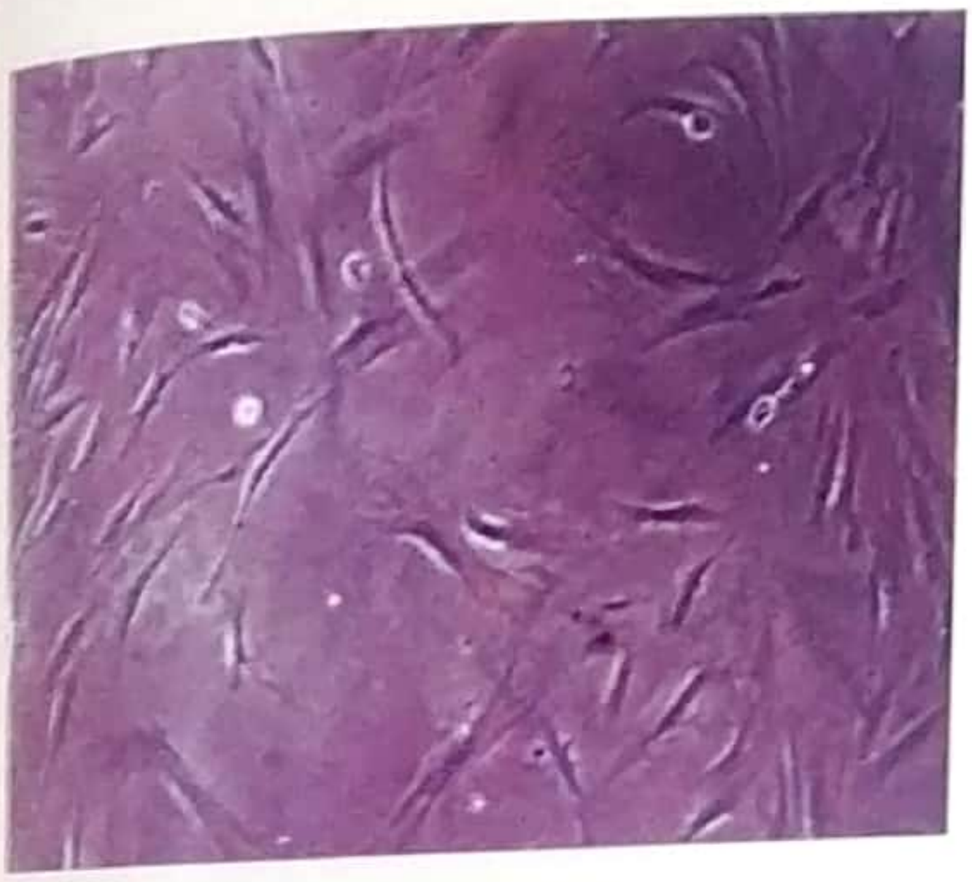


FIGURE: 27 AVE 50%

FIGURE: 28 AVE 100%

PLATE NO. XXVI

Discussion

The doses of mouthwashes employed have been 1-100%, 0.2% CHX which is commercially available in the market is regarded as 100%. AVE is considered as 100%.

Cytomorphology:

The cultured hGF fibroblast cells were exposed to the above mouthwashes range from concentration of 1-100% of 1 minute. Generally, gargling for 1 min is recommended so we decided 1 min as time exposure for our study to titrate the exposure at various concentrations similar to Mariotti et al[22]. The untreated control (figure 1) clearly shows that all cells are in healthy condition. Morphological studies also indicate that hGF gingival fibroblast upon exposure to CHX and AVE at different doses for 1 minute display specific effects. In 1% concentration of CHX and AVE even in concentrations going up to 1 % to 100% minimal number of cells with distorted morphology are seen (figure 2). This possibly indicates no cytotoxicity. Immediately upon contact with 10% concentrations of CHX, most of the cell died and escape into the supernatant as has been indicated in fig. 3 .From 25%-75%remaining cells get rounded up and fixed to substratum as seen in fig 4 & 6. However, CHX at 100% concentration showed cell lysis depicting high toxicity over AVE (100%) as depicted in fig. 7.Similar cellular response has been observed with CHX by Goldschmidt et al[65]. They observed that high concentration of toxic substances may somehow fix non vital cells to the surface of petridish. In another study reported that when cultured gingival fibroblasts were exposed to 0.12 % CHX, they rounded up and detached from the substratum within a few hours. Pucher et al[14] observed CHX caused the loss of fibroblast from the dish at higher concentration and rounding of these cells in monolayer at lower concentrations. These above studies are in agreement with our findings.

SRB (SULFORHODAMINE B ASSAY):

The Sulforhodamine B (SRB) assay was developed by Skehan et al (1990)[58]. SRB dye is a protein stain for use of quantification of cellular proteins in cultured cells, hence measures the cell viability in term of optical density. Optical density is measured in an ELISA plate reader. The optical density is directly proportional to the number of viable cells present in the sample. The optical density is then plotted versus the concentration of mouthwash to which the cells have been exposed for a given time period to provide a measure of cell viability and corresponding toxicity.

The cytotoxicity data compared with untreated control obtained from SRB assay clearly indicates that CHX dose-dependently induces cytotoxicity initiating at 1% (43.10%). However, NE did not show any dose-dependent cytotoxicity and maintained the level of cell death to about 31% even at 100% concentration.

Bamour et al in similar studies have observed the cytotoxic effect of 6 antibiotics and 6 antiseptics used on cultured human gingival fibroblast and keratinocytes and viability was determined using SRB test. At therapeutic concentrations, all the antiseptics are cytotoxic for fibroblasts and keratinocytes whereas antibiotics were not cytotoxic to the tested cell.

In the present study the cytotoxicity assay shows that, the cell survival decreases at all concentration of CHX except 75% as indicated in table1 and graph 1, whereas AVE shows maximum cell survival even upto concentration of 100 % except 10% and 75%, the cell survival on an average increases with the increase in concentration shows cell viability dependence on concentration as can be observed from table 3 and graph 2. All the above data has been compared with untreated control group. Overall, the data of present cytotoxicity

assay clearly indicate that CHX show significant increase in cytotoxicity from 10%-100% (table1 and graph1) concentration for 1 minute of treatment suggesting cell death. But in AVE no significant cytotoxicity was observed with the treatment up to 100% (table 3 and graph 2). From the above data it is clearly evident that the CHX is more cytotoxic as compared to AVE, which is further evaluated in next parameter.

Chang et al studied the effects of sodium hypochlorite (NaOCl) and CHX on cultured human periodontal ligament (PDL) in vitro. The effect of irrigation solution on human PDL cells was evaluated by propidium iodide fluorescence, cytotoxicity assay, protein synthesis assay and mitochondrial activity. Both NaOCl and CHX were cytotoxic to human PDL cells in a concentration and contact time dependent manner. And Verma UP et al[39] studied the influence of Chlorhexidine (CHX) and Neem Extract (NE) on Cultured Human Gingival Fibroblasts (hGF). The effects of CHX and NE were evaluated on cultured hGF through morphological and biochemical assays. Morphological studies with hGF indicate altered morphology beyond 1% CHX. However, NE shows similar results at higher concentrations. Cytotoxicity analysis reveals CHX beyond 1% concentration exhibits toxic effect on hGF at 1 minute time exposure. However, NE does not adversely affect the fibroblasts even up to 50% concentration showing less toxic effect in comparison with CHX on these cells. The cytoprotective, oral friendly quality of NE emphasize the superiority of NE over CHX.The observations of these studies are in agreement with the findings of the present study.

Comparison of the cell survival/cytotoxicity b/w different concentration of CHX and different concentration of AVE, response of fibroblast to mouthwash exposure within the same table were the p values (significance) reveal critical information as in table for CHX,

table for AVE.

For CHX (table 2), a careful perusal reveals the cytotoxic effect significance at 10%-100% concentration exposure except at 75% of concentration as compared to control since there is enormous difference between the optical density, i.e. the number of live cells, however at the time comparison b/w different concentration of CHX reveals highly significant values throughout from 1 % vs. 10%-100%, 10% vs. 25 % & 75%, 25% vs. 50% to 100% and 75% vs. 50% &100%. Similarly, for 10% vs. 50% , 10% vs. 100% and 50% vs. 100% exposure reveal insignificant values which can be confirmed through a table, graph. No significance between p values is observed because of non-prominent alteration in cell number. Thus, it can be concluded that cells can safely survive only at 1 % concentration of CHX exposure but none beyond.

In the case of AVE (table 4), reveals highly significant cell survival at all concentration of AVE as compared to control values decreased the significance at 1% vs.10% , a fact that can be confirmed from table. The reason again as before is that the viability is comparable at 1% and 10% but is significantly different at 10% than 1%.However, until 100% concentration of the mouthwash the optical density is not affected drastically meaning hereby that it can be safely tolerated until 1 minute without compromising on viability. On an overall basis, a comparison of the t values for the 2 mouth rinses tested that is CHX and AVE, it can be concluded that cultured fibroblast can safely survive between 1 %-100 % mouthwash exposures respectively.

Comparison of cell survival/cytotoxicity between CHX and AVE at different concentrations reveals significantly increased cell survival in AVE at all concentrations to CHX as can be seen in table 5 and graph 3.

FACS (FLUORESCENCE ACTIVATED CELL SORTER) ANALYSIS:

Apoptosis induced by CHX on hGF cells at different concentrations (10%, 50,100%) was studied by flow cytometry. Effects were observed, with Control and different concentrations of AVE (10%, 50%, 100%).

0% apoptosis of hGF cells was in control, and with CHX it increased to 1.63%. With subsequent increase in concentrations of CHX percentage of apoptotic cells increase reaching 80.87% % with 50% of CHX, these findings also co relate with cellular morphology. Maximum apoptosis were seen with 100% concentration of CHX i.e. 100%.

The effects of AVE on hGF cells at different concentrations were observed with 10%, 50% and 100% concentrations of AVE showing 0.00%, 0.00% and 0.04% respectively which denotes equal to negligible and non-significant apoptosis. Flowcytometric analysis was also used to study cell cycle kinetics and it was also observed that with control viable cells in G0/G1 phase were 70.98 %, in S phase they were 18.29% and in G2M phase they were 1.88%. It has been observed that with CHX viable cells in G0/G1 phase were 50.14, in S phase they were 27.44 % and in G2M phase 1.84% at 10% concentration, it was also observed that as the CHX concentration increased the percentage of apoptotic cells were increased as with 50% concentration viable cells in G0/G1 phase were 64.82, in S phase they were 22.19

% and in G2M phase 1.94%, also in 100% concentration CHX viable cells in G0/G1 phase were 0.00, in S phase they were 0.00 % and in G2M phase 0.00% indicating cell death.

Similarly using different concentrations of AVE viable cells in G0/G1 phase were 68.69%, in S phase 22.69% and in G2M phase they were 1.92 % at 10% concentration. It was observed that with 50% AVE the viable cells in G0/G1 phase reduced to 69.09%, in S phase 24.12% and in G2M phase they were 1.95%. With 100% AVE the viable cells in G0/G1 phase reduced to 70.46%, in S phase 21.89% and in G2M phase they were 1.93%.

Changes in stages of cell cycle i.e. distribution of cell-population in G0/G1, S and G2/M phases are explained herewith. Figure clearly shows the distribution cell in various phases of cell cycle in normal control cells which have not undergone any treatment. The majority of cells are in G0/G1 phase, less in S phase to decrease again in G2M phase after which they again enter into G0/G1 stage and beyond.

The results of fibroblasts membranal damage upon exposure to CHX as previously discussed are carried downstream to the adverse changes in the cell cycle where 1 % exposure (figure) shows adverse effects in all the phases as compared to normal control (fig). Phases G0/G1, S, G2/M shows the toxic effects with reduced distribution cells in each. The cell cycles are progressively affected until 100 % CHX concentration (fig) taken together with the fluorescence data, the results of cell cycle showed complete agreement with each other , the cytotoxicity assay(graph) for CHX vs. fibroblasts also do not support the above conclusion .

The regulation of fibroblasts cellular characteristics exposed to different concentrations of NVC yielded results dissimilar to CHX and is indicative of better tolerance of former mouth

rinse. The improvement of NVC over CHX exposure was more evident in the changes in the cell cycle despite the membranal alterations. The PI uptake at 1 %, 25 % NVC exposure of fibroblast is similar to that observed in necrotic control (fig) rather than normal control. At 50%NVC, the pi uptake declined drastically to rise again at 100 % concentration to minimal level indicating maximum membrane changes. The results had 100 % NVC exposure are akin to those witnessed with necrotic control. It can be summarized that when compares with the cytotoxicity data using MTT and SRB assays, that cultured fibroblast can suitably handle 75%NVC exposure without compromising much in the cell cycle but with some adverse changes in plasma membrane characteristics.

Despite the fact that the plasma membrane architecture of fibroblast undergoes changes with NVC, it seems though that cell remain evenly distributed in the different phases of cell cycle evenly from 1%-50% concentration of NVC(fig). It is interesting to note the uncoupling between the fluorescence vs. cell cycle changes. At 100% NVC concentration the phases i.e. G0/G1-G2M including the S phase become indistinguishable as in figure .No resemblance with the profile observed at 100% NVC with either the normal control or the necrotic control could be seen. It can be therefore concluded that NVC up to 75% where the cytotoxicity is minimal with MTT and SRB assays, the survival of cell is optimal as far as cell cycle maintenance is concerned. Comparison with CHX clearly indicated that even 1 % exposure causes deviation from normal control making them look more like necrotic control, the exposure up to 75%NVC is very well tolerated by cultured fibroblast i.e. NVC is better than CHX as a rinse.

The third and most important mouth rinse evaluated through FACS in the present study i.e. Neem extract revealed most interesting of the data when compared to the results obtained with CHX and NVC. At 1% Neem exposure, a careful comparison with normal control suggest that the Neem extract although the pi uptake is slightly of a higher order but the cell cycle data reveals that cells are more sharply in all the three phases, more distinct than even the normal control this clearly suggest some unknown factor that may reside in Neem extract causing the cells to withstand the slight protruberations in the fluorescence but yet providing more crisper and distinct phases over the normal control. The results on NE are in complete disagreement over the necrotic control suggesting its utility. As the concentration of Neem extract is raised from 25%-100% the pi uptake does not alter much to adverse status at 50% and 100% concentrations respectively. This data when evaluated in terms of the cytotoxicity assays clearly indicate good tolerance of Neem extract by fibroblasts up to only 25% concentration beyond which the toxic effects become evident. Apoptosis induced by CHX on hGF cells at different concentrations (10%, 50, 100%) was studied by flowcytometry. Effects were observed, with Control and different concentrations of AVE (10%, 50, 100%). 0% apoptosis of hGF cells was in control, and with CHX it increased to 1.63%. With subsequent increase in concentrations of CHX percentage of apoptotic cells increase reaching 80.87% % with 50% of CHX, these findings also co relate with cellular morphology. Maximum apoptosis were seen with 100% concentration of CHX i.e. 100%. The effects of AVE on hGF cells at different concentrations were also studied using 10%-100% concentration.

Finding was observed with 10%, 50% and 100% concentrations of AVE showing 0.00%, 0.00% and 0.04% respectively which denotes equal to negligible and non-significant

apoptosis. Flow cytometric analysis was also used to study cell cycle kinetics and it was also observed that with control viable cells in G0/G1 phase were 70.98 %, in S phase they were 18.29% and in G2M phase they were 1.88%. It has been observed that with CHX viable cells in G0/G1 phase were 50.14, in S phase they were 27.44 % and in G2M phase 1.84% at 10% concentration, it was also observed that as the CHX concentration increased the percentage of apoptotic cells were increased as with 50% concentration viable cells in G0/G1 phase were 64.82, in S phase they were 22.19 % and in G2M phase 1.94%, also in 100% concentration CHX viable cells in G0/G1 phase were 0.00, in S phase they were 0.00 % and in G2M phase 0.00% indicating cell death.

Similarly using different concentrations of AVE viable cells in G0/G1 phase were 68.69%, in S phase 22.69% and in G2M phase they were 1.92 % at 10% concentration. It was observed that with 50% AVE the viable cells in G0/G1 phase reduced to 69.09.%, in S phase 24.12% and in G2M phase they were 1.95% . With 100% AVE the viable cells in G0/G1 phase reduced to 70.46 %, in S phase 21.89% and in G2M phase they were 1.93%.

WOUND HEALING ASSAY:

A novel, in vitro hGF cell based wounding assay was optimized, calibrated, and validated to observe qualitative, directional cell migration and proliferation employing hGF, CHX and *Aloe vera*. CHX exposure showed cytotoxicity as at 10% CHX exposure, the cytotoxic potential could be visualized with rounded shrunken cells in the wound area(figures [23,24,25]). Subsequently, widening of wound at 50% to 100%. However, our results differed

from those of Giannelli et al[66] demonstrated that the number of cells of the human osteoblastic cell line Saos-2 undergoing apoptotic nuclear fragmentation increased upon exposure to higher concentration of CHX, reaching almost 80% of the total cells. The above result is in harmony with the study of Bassetti and Kallenberger described that the use of CHX delays the process of wound healing[67]. The reason for contrast results could be use of different test applied for the study of cytotoxic effects in comparison to our work. Marked reduction in the size of the gap width was seen at 10 % *Aloe vera* exposure, at 50 % *Aloe vera* exposure, improved closure of wound area with aggregated fibroblasts was observed. Rehabilitation of the damage to the cells owing to 100 % *Aloe vera* exposure was done (figures 26,27,28). Hence, this may serve as an example for understanding in vivo gingival wound healing and its regulation with various ligands, its clinical implication in the management of periodontal diseases needs investigation under in vivo environment.

On the basis of our in-vitro study it was observed that the individual effect of CHX on Hgf was not as significant as that of *Aloe vera* .Also, *Aloe vera* has shown to have no cytotoxicity[68], as compared to CHX assessed in few studies[69,70]. Thus, it can prove to be a beneficial and more cost effective alternative to CHX. However, further longitudinal studies are required to assess the efficacy of the above mentioned plants as an adjunct to periodontal therapy.

Conclusion

CONCLUSION

Within limitations, the present study concluded that:

- **Cytomorphological study**: Exposure of CHx on hGF showed adverse effects on Cytomorphological feature of fibroblasts dose dependently. On exposure of control on hGF the cells appeared healthy. On the contrary, the fibroblasts growth and proliferation was unaffected up to 100% concentration of AVE. At 1%-50% concentration AVE displays no alteration in cellular morphology. Even at 75% and 100% concentration, AVE did not display adverse effects on fibroblasts. Cytoarchitecture was found to be normal and mitotic cells were also clearly observed.

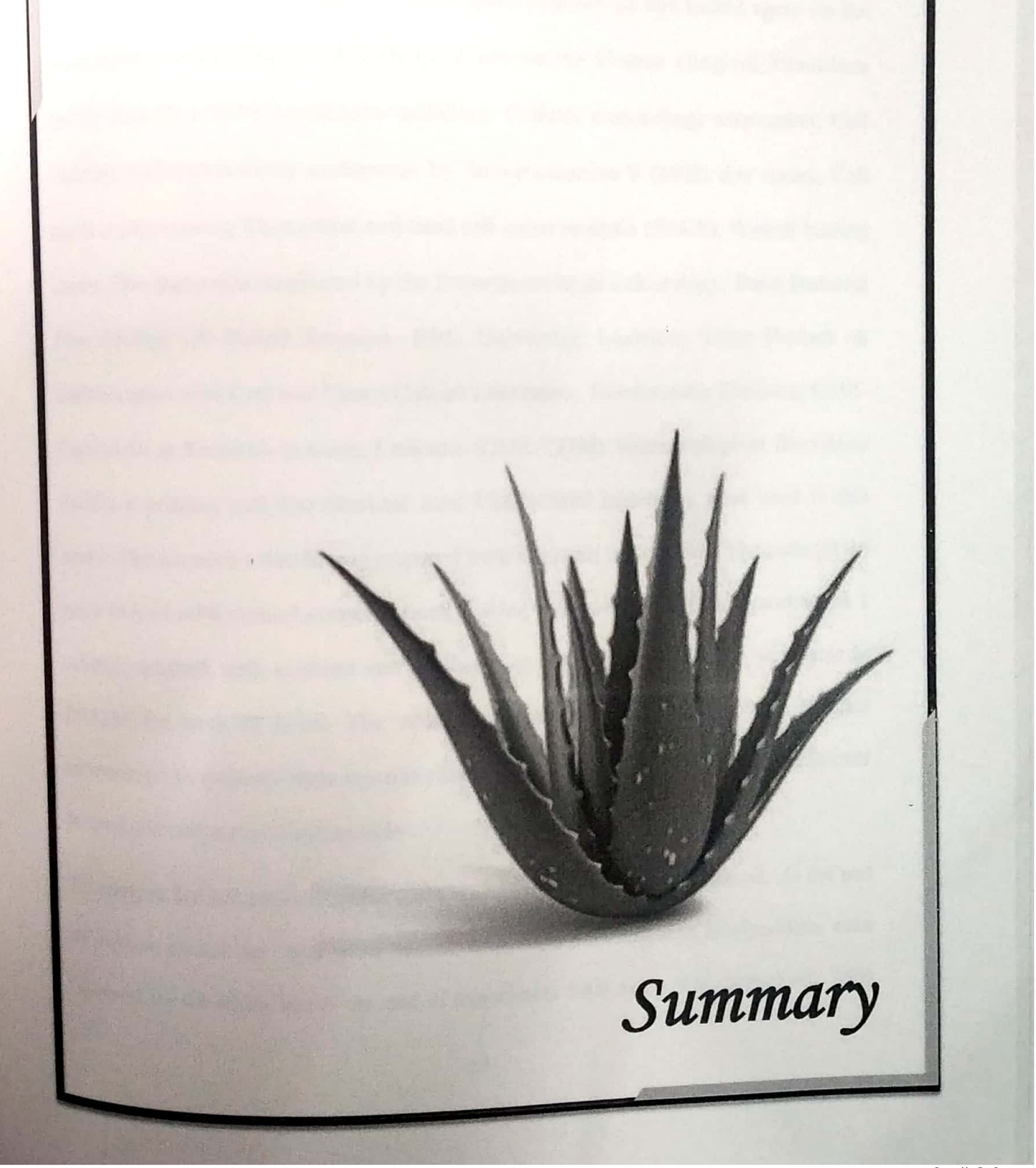
- **SRB Assay:** The cytotoxicity data compared with untreated control obtained from SRB assay clearly indicates that CHX dose-dependently induces cytotoxicity. However, AVE did not show any dose-dependent cytotoxicity and maintained the level of cell survival even at 100% concentration.

- **FACS Analysis** : Apoptosis induced by CHx on hGF cells at different concentrations showed subsequent increase in concentrations of CHx percentage of apoptotic cells increase. Concentrations of AVE showed equal to negligible and non-significant apoptosis.

- **Wound healing Assay:** Wound healing assay performed on hGF clearly demonstrated that upon treatment with various concentration of CHX the cell proliferation and migration was considerably inhibited showing cytotoxic potential of CHX on hGF. However, treatment with AVE displayed improved reduction of wound size with growth and migration of cells.

Hence, evaluating the results it can be concluded that Aloe vera is better when compare with Chlorhexidine in terms of cytotoxicity, percent cell survival with wound healing characteristics.

Further in vivo studies are required for the incorporation of Aloe vera derived mouthwashes for various parameters of gingivitis and periodontitis.



Summary

SUMMARY

Aloe vera plant has been used since the 4th century BC to treat minor burns, intestinal problems like stomach ulcers, and to help speed wound healing. Scientific studies have shown that a complex carbohydrate in aloe vera, acemannan, combines antiviral properties, antibacterial action. We investigated efficacy of this herbal agent on the proliferation, morphology and toxicity, if any on the Human Gingival Fibroblasts (hGF) through various techniques including: Cellular morphology assessment, Cell viability and Cytotoxicity assessment by Sulforhodamine B (SRB) dye Assay, Cell cycle analysis using Fluorescent activated cell sorter analysis (FACS), Wound healing assay. The study was conducted by the Department of periodontology, Babu Banarasi Das College of Dental Sciences, BBD University, Lucknow, Uttar Pradesh in collaboration with Cell and Tissue Culture Laboratory, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI). Human gingival fibroblasts (hGF), a primary cell line obtained from CSIR-CDRI laboratory were used in this study. The aqueous solution was prepared from the fresh leaf of Aloe. The cells (hGF) were treated with various concentrations of *Aloe vera* extract and Chlorhexidine for 1 minute, washed with medium and incubated at 37°C humidified CO_2 incubator in DMEM for next 48 hours. The cells were examined daily under phase contrast microscope to evaluate their cytomorphology. In addition SRB assay was performed to evaluate cell survival/cytotoxicity.

To analyse the cell cycle Kinetics (G0/G1) FACS analysis was performed. At the end of culture period the cells were treated with Aloe vera extract/Chlorhexidine were cultured for 48 hours and at the end of experiment SRB assay was carried out. SRB

bound to the cellular protein was removed with 10 mM tris solution (pH 10.5) and optical density was determined at 560 nm spectrometer (SpectraMax M2: Molecular devices). To analyse cell cycle kinetics/apoptosis, human gingival fibroblasts (0.2 × 10^6) were plated in a 6-welled plate and cultured for 24 hours in DMEM following treatment with *Aloe vera*/CHX and further cultured for next 48 hours. Later cells were analysed by Flow cytometry for cell cycle studies on a Beckton-Dickinson Fluorescence-Activated Cell Sorter (FACS) employing the Cell Quest Software. All the data was summarised as Mean ± SD (standard deviation). Groups were compared by Student's t test. Groups were also compared by one way analysis of variance (ANOVA) and the significance of mean difference between the groups was done by Tukey's post hoc test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variance between groups by Levene's test. A two-tailed $p<0.05$ was considered statistically significant. Analyses were performed on STATISTICA software (Windows version 7.1, StatSoft, Inc., USA). Results of cytomorphology assay depicted typically fibroblastic morphology of control, exposure of CHX showed more number of cell death with the increase in its concentration. Cell deaths were observed when it was exposed to >10% concentration. At 25%-75% concentration, cells were found rounded up and fixed to the substratum. On exposure with Aloe vera extract, fibroblasts were observed to be unaffected up to 100% concentration. At 1%-50% concentration AVE displays no cells alteration in their cellular morphology. Even up to 75 % to 100% concentration AVE does not seem to be adversely affecting the fibroblasts which is conclusive of the fact that AVE has no altered (cytotoxic) effects on morphology of hGF, whereas CHX exhibited toxic effects on hGF dose

dependently. Results obtained from SRB (statistical analysis) when compared with untreated control obtained from cytotoxicity data clearly indicates that CHX dose-dependently induces cytotoxicity whereas AVE did not induce cytotoxicity whatsoever and maintained the level of cell survival even at 100% concentration which is suggestive of the safety of AVE over CHX. In FACS analysis apoptosis induced by CHX on hGF cells at different concentrations showed subsequent increase in concentrations of CHX percentage of apoptotic cells increase. Concentrations of AVE showed equal to negligible and non-significant apoptosis. Wound healing assay performed on hGF demonstrated that upon treatment with various concentration of CHX the cell proliferation and migration was considerably inhibited showing cytotoxic potential of CHX on hGF and treatment with AVE displayed improved reduction of wound size with growth and migration of cells showing its wound healing properties. Evaluating the results of these tests it can be concluded that Aloe vera is better when compare with Chlorhexidine in terms of cytotoxicity, percent cell survival and wound healing characteristics.

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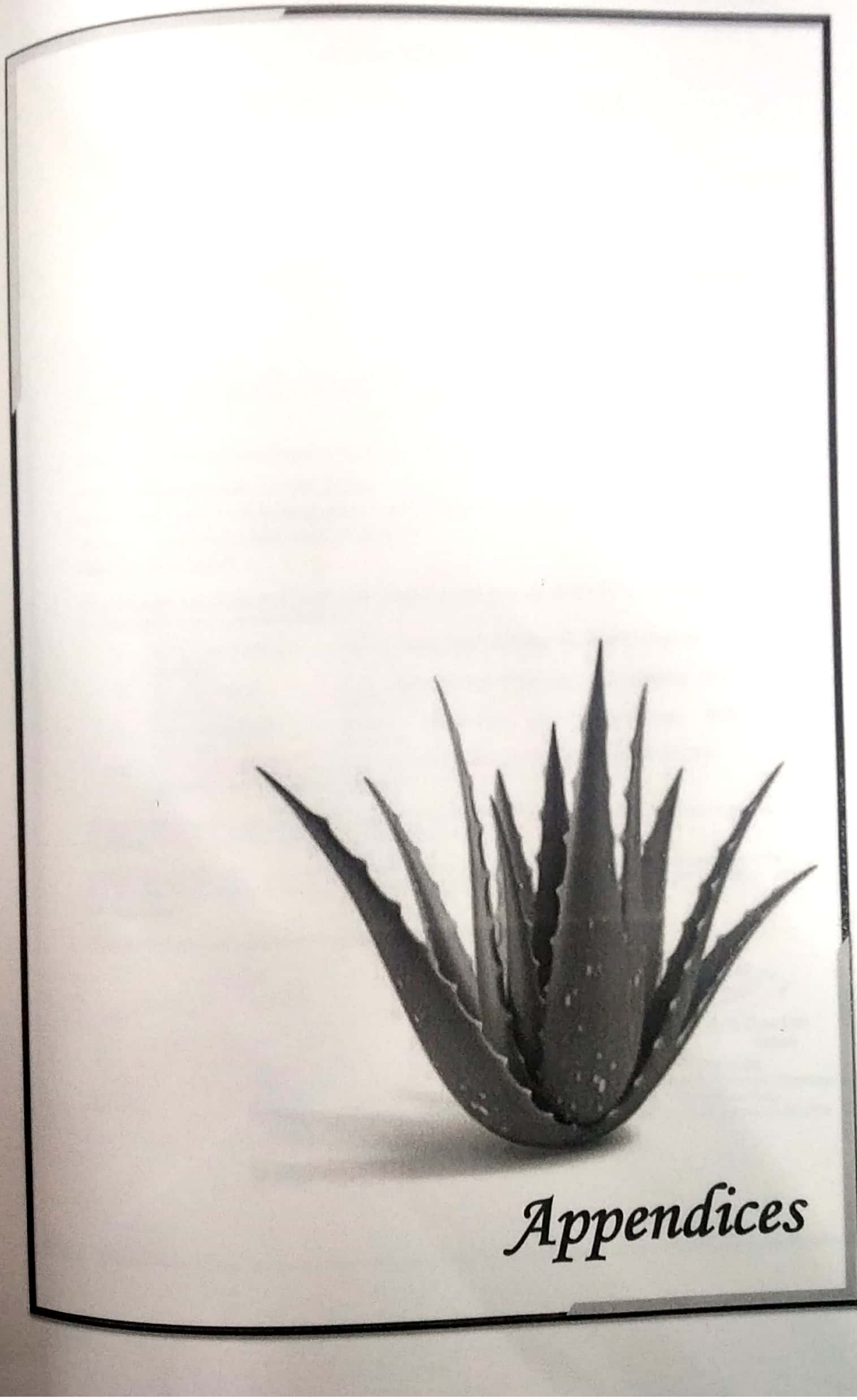

Appendices

APPENDIX – I

ETHICAL COMMITTEE APPROVAL FORM

Babu Banarasi Das College of Dental Sciences
(A Faculty of Babu Banarasi Das University)
BBD City, Faizabad Road, Lucknow – 227105 (INDIA)

Dr. Lakshmi Bala
Professor and Head Biochemistry and
Member-Secretary, Institutional Ethics Committee

Communication of the Decision of the 3rd Institutional Ethics Sub Committee Meeting.

BBDCODS/ 24 /2015

IEC Code: 24

Title of the Project: The Effect Of Aloe Vera On Human Gingival Fibroblast –An In vitro Study.

Principal Investigator: Dr. Nida Ansari **Department:** Periodontology

Name and Address of the Institution: BBD College of Dental Sciences Lucknow

Type of Submission: New, MDS Protocol

Dear Dr. Nida Ansari

The Institutional Ethics Sub Committee meeting was held on 09-01-2015. The sub committee comprises following four members :

1. Dr. Amrit Tandon — Prof. & Head, Deptt. of Prosthodontics BBDCODS, Lucknow. — Member
2. Dr. Jiji George — Prof., Deptt. of Oral Pathology & Microbiology, BBDCODS, Lucknow. — Member
3. Dr. Ashish Saini — Reader, Department of Periodontology, BBDCODS, Lucknow. — Member
4. Dr. Lakshmi Bala — Prof. and Head, Deptt. of Biochemistry, BBDCODS, Lucknow. — Member Secretary

The committee reviewed and discussed your submitted documents of the research study in the meeting. The proposal was reviewed and thoroughly revised.

Decisions of the IEC : As per the recommendations I.E.C. has taken following decisions for the current protocol of study "The Effect Of Aloe Vera On Human Gingival Fibroblast –An In vitro Study."

The committee approved the above proposal from ethics point of view.

Lakshmi Bala 14/2/15

(Dr. Lakshmi Bala)
Member-Secretary IEC

Member-Secretary
Institutional Ethics Committee
BBD College of Dental Sciences
BBD University
Faizabad Road, Lucknow-226028

Forwarded by:

(Dr. Vivek Govila)
Dean

DEAN
BBD College of Dental Sciences
BBD University
Faizabad Road Lucknow-226028

APPENDIX – II

FORMULA USED FOR THE ANALYSIS

The Arithmetic Mean

The most widely used measure of central tendency is arithmetic mean, usually referred to simply as the mean, calculated as

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

The Standard Deviation

The standard deviation (SD) is the positive square root of the variance, and calculated as

$$SD = \sqrt{\frac{\sum X_i^2 - \frac{(\sum X_i)^2}{n}}{n-1}}$$

where, n= no. of observations

Student's t-test

Student's t-test was used to calculate the differences between the means of two groups

$$t = \frac{\bar{X}_1 - \bar{X}_2}{SE}$$

where, $$SE = \sqrt{S^2 X \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}$$

S^2 is the pooled variance and n_1 and n_2 are number of observations in group 1 and 2 respectively. The degrees of freedom (DF) is calculated as

$$DF = n1 + n2 - 2$$

Analysis of Variance

Analysis of variance (ANOVA) is used when we compare more than two groups simultaneously. The purpose of one-way ANOVA is to find out whether data from several groups have a common mean. That is, to determine whether the groups are actually different in the measured characteristic. One way ANOVA is a simple special case of the linear model. For more than two independent groups, simple parametric ANOVA is used when variables under consideration follows Continuous exercise group distribution and groups variances are homogeneous otherwise non parametric alternative Kruskal-Wallis (H) ANOVA by ranks is used. The one way ANOVA form of the model is

$$Y_{ij} = \alpha_{.j} + \varepsilon_{ij}$$

Where;

- Y_{ij} is a matrix of observations in which each column represents a different group.
- $\alpha_{.j}$ is a matrix whose columns are the group means (the "dot j" notation means that α applies to all rows of the j^{th} column i.e. the value α_{ij} is the same for all i).
- ε_{ij} is a matrix of random disturbances.

The model posits that the columns of Y are a constant plus a random disturbance. We want to know if the constants are all the same.

Tukey Multiple Comparison Test

After performing ANOVA, Tukey HSD (honestly significant difference) post hoc test is generally used to calculate differences between group means as

where,
$$q = \frac{\bar{X}_1 - \bar{X}_2}{SE}$$

$$SE = \sqrt{\frac{S^2}{2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

S^2 is the error mean square from the analysis of variance and n_1 and n_2 are number of data in group 1 and 2 respectively.

Statistical significance

Level of significance "p" is the probability signifies level of significance. The mentioned p in the text indicates the following:

$p > 0.05$	Not significant (ns)
$p < 0.05$	Just significant (*)
$p < 0.01$	Moderate significant (**)
$p < 0.001$	Highly significant (***)