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MY PARENTS

EVALUATION OF MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN HUMAN GINGIVAL FIBROBLASTS (hGF) TREATED WITH *ACACIA NILOTICA*

Dissertation

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By

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"Keep on going, and the chances are that you will stumble on something, perhaps when you are least expecting it. I never heard of anyone ever stumbling on something sitting down."

-- Charles F. Kettering

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Dr. Himangi Dubey

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"EVALUATION OF MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN HUMAN GINGIVAL FIBROBLASTS (hGF) TREATED WITH *ACACIA NILOTICA*"** is a bonafied and genuine research work carried out by me under the guidance of ***Dr. Ashish Saini***, Reader, Department of Periodontics, Babu Banarasi Das College Of Dental Sciences, Babu Banarasi Das University, Lucknow, Uttar Pradesh.

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Place:

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ABBREVIATIONS

A. nilotica	*Acacia nilotica*
AE	*Acacia* Extract
ANBE	*Acacia nilotica* Bark Extract
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CDRI	Central Drug Research Institute
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EO	Essential Oils
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
HCV	Hepatitis C Virus
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
Hgf	Human Gingival Fibroblasts
H_2O_2	Hydrogen Peroxide
Kcl	Potassium Chloride
L. acidophilus	*Lactobacillus acidophilus*
LPO	Lipid Peroxidation
LPS	Lipopolysaccharide
MS	*Mutans streptococci*
MTAD	Mixture of Tetracycline, Citric Acid and Detergent

NDEA	N-Nitrosodiethylamine
NaOCL	Sodium Hypochlorite
OD	Optical Density
PBS	Phosphate Buffered Saline
PDL	Periodontal Ligament
PI	Propidium iodide
PMA	Phorbol 12-Myristate 13-Acetate
RNase	Ribonuclease
S-G	Smulow-Glickman
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
S. mutans	*Streptococcus mutans*
SRB assay	Sulforhodamine-B assay
TCA	Tetrachloroacetic Acid
TFC	Total Flavonoid Contents
TPC	Total Phenolic Contents
Viz.	Namely

ABSTRACT

Prevention of periodontal diseases can be achieved by both mechanical and chemical means of plaque control. In chemical plaque control, mouthwashes are a commonly used method to deliver the anti-microbial agent and can be used by the patient as an oral hygiene aid. Chemical plaque inhibitors are toxic if used for longer duration. However, herbal preparations are less toxic as compared to chemical mouthwashes and show better results. Owing to these factors, *Acacia nilotica* (*A. nilotica)* has been used for the inhibition of gingivitis and factors that lead to gingival diseases such as plaque. In this study attempts to evaluate the influence of Chlorhexidine (CHx) and *Acacia* extract (AE) on cultured Human Gingival Fibroblasts (hGF) is made. Experiments were conducted with the routinely maintained hGF cell line. The effects of CHx and AE were evaluated on cultured hGF through cellular morphological study, cell growth/cytotoxicity assessment, cell-cycle analysis and wound healing assay. Morphological studies with hGF indicate altered cellular morphology beyond 1% CHx. However, AE shows similar results at higher concentrations. Cytotoxicity analysis of AE displayed safety of AE as compared with CHx. Beyond 1% concentration CHx exhibits toxicity on hGF at 1 minute time exposure. However, AE does not adversely affect the fibroblasts even up to 50% concentration showing less toxic effect in comparison with CHx on these cells. No noticeable apoptotic changes were evident in hGF cells with the distribution of % cell cycle in G0/G1 phase diminished, S phase was found enhanced in both the treatment group, G2/M phase was found enhanced in all concentrations of AE while only 50% in CHx as compared to control. The *in vitro* model for wound healing indicates that AE significantly

enhanced wound healing faster than CHx treated hGF cells over 48 hours. The cytoprotective, oral friendly quality of AE emphasize the superiority of AE over CHx.

INTRODUCTION

Mouthwashes are a commonly used means to deliver the anti-microbial agent (after toothpastes), which can be used by the patient as an oral hygiene aid[1]. Schroeder reported the antiplaque activity of Chlorhexidine (CHx) in 1969[2]. It is a cationic bis-biguanide with broad-spectrum activity effective against an array of microorganisms. It exhibits both anti-plaque and anti-bacterial properties[3]. It's highly effective anti-plaque activity is the result of its substantivity[4, 5]. However, its prolonged use may cause local side effects like, altered taste sensation, brown discoloration of the teeth and tongue, oral mucosal ulcerations which reduce its acceptability in patients[6]. CHx has shown toxic effects on neutrophils, human epithelial cells, gingival fibroblasts, new born fibroblasts[7] and also cause delay in wound healing.

Owing to these limitations, there has been an increasing trend for development of natural formulations using medicinal plants as an adjunct. A number of herbs have been recommended and used in dentistry.

Acacia nilotica (A. nilotica) is known for its high medicinal properties. It is also known as Babul, Gum Arabic, and Egyptian thorn. It is widely spread in subtropical and tropical Africa from Egypt to Mauritania Southwards to South Africa, and in Asia eastwards to Pakistan and India[8, 9]. It serves as the source of polyphenols. Pods, leaves and bark of the plant possess cytotoxic, anti-pyretic, anti-fungal, anti-spasmodic, anti-bacterial properties[10]. Anticancer, antioxidant and anti-malarial properties of *A.*

nilotica extracts have been confirmed in recent studies[11]. Branches of the plant are used as tooth sticks for strengthening of the gingiva[12].

Bark of *A. nilotica* is also used commonly as tooth brush. It removes the plaque adhering on tooth surfaces and all together massages an individual's gingiva. Repeated chewing releases valuable chemical constituents: Tannin acts as astringent on mucous membrane, as a result, reducing gingivitis and bactericidal action of Alkaloids in oral cavity have been evaluated[13].

The fibroblast is responsible for the production of structural proteins and extracellular matrix and is the predominant cellular element in the gingival & periodontal connective tissue, thus any toxic effect on this cell has a major role in the maintenance and repair of gingival connective tissue[14].

Although *A. nilotica* had been used in the oral cavity in the past, but its effects, if any, on Human Gingival Fibroblasts (hGF) remained unexplored till date. Thus, the present study was undertaken to analyze the cytomorphological characterization and biochemical effects of *A .nilotica* on hGF and to compare these effects with CHx.

AIM AND OBJECTIVES

The Aim of the present study was:

To evaluate the effects of *A. nilotica* on hGF.

The Objectives of the present study were:

1. To evaluate the effects of the aqueous extract of *A. nilotica* on hGF, their morphology, proliferation and cell cycle upon exposure to the aqueous extract.
2. To compare the changes of A. nilotica vis-à-vis CHx on hGF.

CHLORHEXIDINE

CHx is the most commonly used among the various mouthwashes in the treatment of periodontal disease. It was developed in 1940's by Imperial Chemical Industries (ICI, Macclesfeild, England), marketed in 1954 as a general disinfectant[15].In 1970, Loe and Schiott reported that 5, 2 or even 1 daily rinse of 0.2% chlorhexidine gluconate virtually prevented plaque accumulation[16, 17]. Like other chemical agents, it is also toxic as suggested by various evidences showing deleterious effects on gingival fibroblast proliferation as well as collagen and non collagen protein production in cell culture.

Greenstein G, Berman C, Jaffin R (1986)[18] reviewed the use of CHx as an adjunct to periodontal therapy. Approximately 30% of the drug is retained in oral cavity following a rinse with 10ml of 0.2% solution for a minute. The drug is released over next 8 to 12 hours, thereby prolonging the bactericidal effect. Systemic absorption of the drug was poor and the side effects were minimal.

Brecx M, Netusehil L, Reiehert B, Schreil G (1990)[19] compared the anti-plaque, anti-gingivitis and antimicrobial efficacies of a phenolic compound (Listerine) and a stannous fluoride mouthwash (Meridol), using a placebo preparation as a negative control and a CHx solution as a positive control. The results demonstrated that CHx was superior to others in its ability to maintain low plaque scores and gingival health during this 3-week period of no mechanical oral hygiene.

Burchard WB, Cobb CM, Drisko DL, Killoy WJ (1991)[20] evaluated the *in vitro* attachment of gingival fibroblasts to dental implants of differing surface character treated with 0.12% CHx, 1.64% stannous fluoride, or sterile saline. Analysis of results showed a significantly greater number of fibroblasts attachment to specimens treated with saline or CHx than to those treated with stannous fluoride.

Pucher JJ, Daniel JC (1992)[21] concluded that effect of 0.002% CHx on human fibroblasts derived from skin and oral tissues, shows minimal cytotoxicity, but is able to suppress cell division almost completely. Collagen gel contraction, as a model of wound contraction, was also severely affected by all the concentrations of CHx used. Total protein synthesis was suppressed by CHx in collagen gel culture.

Babich H, Wurzburger BJ, Rubin YL, Sinensky MC, Blau L (1995)[22] evaluated the *in vitro* cytotoxicity of CHx with the Smulow-Glickman (S-G) gingival epithelial cell line. The toxicity of a 24-h exposure to CHx to the S-G cells was progressively lessened as the content of Fetal bovine serum (FBS) in the exposure medium was increased from 2% to 8%. The potency of a 1-h exposure to CHx was reduced in medium amended with albumin, lecithin, and heat-killed Escherichia coli.

Ernst CP, Prockl K, Willershausen B (1998)[23] concluded that increase in concentration of CHx provided no clinical advantage or disadvantage, on evaluation of two commercial CHx mouthwashes (0.1% and 0.2%) for its efficacy on dental plaque and gingival inflammation, their undesirable effects. Results demonstrated sulcular bleeding index, approximal plaque index, gingival index decreased significantly except discoloration index which was increased in both groups.

Mariotti AJ, Rumpf DA (1999)[24] concluded that CHx induces a dose dependent reduction in cellular proliferation and that concentrations of CHx that have little effect on cellular proliferation can significantly reduce both collagen and non collagen protein production of hGF *in vitro*.

Chang YC, Huang FM, Tai KW, Chou MY (2001)[25] examined the in vitro effects of sodium hypochlorite (NaOCl) and CHx on cultured human periodontal ligament (PDL) cells, suggesting that these irrigation fluids may cause detrimental effects on vital tissues. The concentration, exposure time of the agent, and exposure surface area are important factors affecting the resulting effect.

Wilken R, Botha SJ, Grobler A, Germishuys (2001)[26] observed that cells were immediately fixated by 10% CHx in water, 20% povidone iodine and 70% benzydamine-HCl. Fibroblasts survived and recovered from the exposure to 8.5% and 10% ethanol, which indicated that the fixation effect is not the result of the ethanol, but should be ascribed to the activity of the active ingredients in the mouthrinses.

Dogan S, Gunay H, Leyhausen G, Geurtsen W (2003)[27] investigated the in vitro effects of low concentrations of CHx on the proliferation of Streptococcus sobrinus (ATCC 33478) and primary hGF. The results revealed that chlorhexidine inhibits proliferation of Streptococcus sobrinus even at very low concentrations while concentrations of CHx≤5µM are not cytotoxic to hGF.

Bonacorsi1 C, Raddi MSG, Carlos IZ (2004)[28] evaluated in vitro CHx induced cytotoxicity and its effects on reactive oxygen/nitrogen intermediate induction by murine peritoneal macrophages. The results demonstrated that CHx has no

immunostimulating activity and sub-toxic concentrations did not affect the response of macrophages to the phorbol 12-myristate 13-acetate (PMA) but can interfere with the receptor-dependent stimulus lipopolysaccharide (LPS).

Flemingson, Emmadi P, Ambalavanan N, Ramakrishnan T, Vijayalakshmi R (2008)[29] examined the effect of three commercial mouth rinses (Hexidine 0.2%, Listerine Cool Mint, Betadine 1%) upon cultured hGF proliferation. The results suggested that CHx, Listerine and Povidone-Iodine are capable of inducing a dose-dependent reduction in cellular proliferation of fibroblasts.

Rajabalian S, Mohammadi M, Mozaffari B (2009)[30] showed the cytotoxic effects of Persica and CHx mouthwashes on cultured human and mouse cell lines, indicating that both Persica and CHx mouthwashes are toxic to macrophage, epithelial, fibroblast, and osteoblast cells in a concentration-dependent manner.

Lee TH, Hu CC, Lee SS, Chou MY, Chang YC (2010)[31] exhibited the in vitro mechanisms of cytotoxicity of CHx in human osteoblastic cells, demonstrating a cytotoxic effect to U2OS cells in a dose-dependent manner ($P < 0.05$), inhibiting cell proliferation and collagen synthesis ($P < 0.05$).

Lessa FCR, Aranha AMF, Nogueira I, Giro EMA, Hebling J, Costa CAS (2010)[32] examined toxicity of CHx on odontoblast-like cells. The result suggested that CHx concentrations had a high direct cytotoxic effect to cultured MDPC-23 cells.

Verma UP, Dixit J (2011)[33] assessed the influence of CHx and Neem Extract on Cultured hGF, indicating hGF with altered morphology beyond 1% CHx and Neem Extract showing similar results at higher concentrations. Cytotoxicity and Antioxidant

analysis of Neem Extract displayed remarkable safety as compared with CHx with less than 32% cytotoxicity even at 100% conc. The cytoprotective, oral friendly quality of Neem Extract emphasized the superiority of Neem Extract over CHx.

Tsourounakis I, Palaiologou-Gallis AA, Stoute D, Maney P, Lallier TE (2013)[34] investigated the in vitro effect of commercially available mouthwashes on the survival and migratory capacity of human fibroblasts. The results indicated that diluted essential oils (EO) displayed no detectable detrimental effects on human gingival and PDL fibroblasts, whereas diluted CHx reduced both cell migration and long-term survival. Both solutions retained their antimicrobial activity in lower concentrations.

Ghabanchi JA, Moattari AB, Darafshi RC, Andisheh AD, Khorshidi HE, Shakib MF (2013)[35] determined the cytotoxic effect of three commercial mouthwashes (CHx, Persica and Irsha) on the cultured fibroblasts. The results showed that, three mouth washes show cytotoxic effect on the cultured cells, at commercially available concentration and even diluted and Irsha was found to be the most toxic one. Cytotoxicity of three mouthwashes was reduced with decreasing concentration.

Mirhadi H, Reza Azar M, Abbaszadegan A, Geramizadeh B, Torabi S (2014)[36] concluded that 2% CHx alone and in combination with either 1 or 3% Hydrogen Peroxide (H_2O_2) are significantly more toxic than 0.2% CHx alone and in combination with 1 and 3% H_2O_2, on cultured human PDL fibroblasts. Therefore, to benefit from the synergistic antimicrobial effect, it is recommended to use 0.2% concentration of CHx combined with 3% H_2O_2.

Li YC , Kuan YH, Hsin T L, Huang FM, Chang YC (2014)[37] evaluated the potential toxicological implications of CHx employing an *in vitro* mammalian test system, stating that CHx demonstrated to exhibit cytotoxicity that could disrupt the stable cellular redox balance, resulting in increasing levels of free radical generation and subsequent cell death.

Shetty KP, Venkata S, Kilaru K, Ponangi KC, Venumuddala VR, Ratnakar P (2014)[38] analyzed the cytotoxicity of various volumes of 5.25% of sodium hypochlorite, 2% of CHx and mixture of a tetracycline isomer, an acid and a mixture of Tetracycline, Citric Acid and Detergent (MTAD) by checking for hemolysis of human red blood corpuscles. The study suggested that the three irrigating solutions do cause detrimental effects on the diluted red blood corpuscles.

Tu YY, Yang CY, Chen RS, Chen MH (2015)[39] investigated the effects of CHx on Stem cells from human exfoliated deciduous teeth (SHED). The results demonstrated that different concentrations of CHx had cytotoxic effects on SHED cells in a dose- and time-dependent manner. The proliferation of SHED cells was inhibited by approximately 50% by the use of 0.01% CHx.

Bowen J, Cole C, Glennen R (2015)[40] compared commercial available cleansing rinses (CHx, carbamide peroxide, aloe vera, essential oils with and without alcohol and a combination product with essential oils and carbamide peroxide), measuring possible toxic effects on gingival fibroblasts invitro and their bactericidal effects in-vivo. The results suggested that antimicrobial rinses can also be harmful to gingival fibroblasts.

Dr. Vanaki SS, Dr. Rudrayya S (2016)[41] conducted a study to evaluate frequency of micronuclei in buccal epithelial cells of patients using CHx containing mouthrinses, suggesting that CHx has cytotoxic effects.

Acacia nilotica

Acacia is the most significant genus of family: Leguminosae, first of all described by Linnaeus in 1773; with an estimation of roughly 1380 species present worldwide[42]. Acacia species—commonly known as Babool (or babul), Egyptian mimosa, Egyptian thorn, kikar, Indian gum, and red thorn—have long been used for the treatment of various ailments. It has been recognized worldwide as a multipurpose tree (National Academy of Sciences 1980). O. Solomon-Wisdom has extensively studied their antimicrobial activities are reviewed elsewhere (Farzana M et al.)[43].

Singh BN, Singh BR, Singh BK, Singh HB (2009)[44] studied Chemo preventive potential of *Acacia nilotica* bark extract (ANBE) against single intraperitoneal injection of N-nitrosodiethylamine (NDEA, 200mg/kg). The results strongly supported that *A. nilotica* bark prevents lipid peroxidation (LPO) and promotes the enzymatic and non-enzymatic antioxidant defense system during NDEA-induced hepatocarcinogenesis which might be due to activities like scavenging of oxy radicals by the phytomolecules in ANBE.

Pai MBH, Prashant GM, Murlikrishna KS, Shivakumar KM, Chandu GN (2010)[45] evaluated the *in vitro* antifungal efficacy of *Punica granatum, Acacia nilotica, Cuminum cyminum* and *Foeniculum vulgare* on *Candida albicans.* All the above-mentioned ingredients showed antifungal property, with *Punica granatum* showing the highest inhibition of *Candida albicans* with a mean zone of inhibition of 22 mm.

Rehman S, Ashfaq UA, Riaz S, Javed T, Riazuddin S (2011)[46] screened out thirteen medicinal plants against Hepatitis C Virus (HCV) by infecting HCV inoculums of 3a genotype in liver cells. The results demonstrated that acetonic and methanolic extract of *Acacia nilotica* showed more than 50% reduction at non toxic concentration, concluded that by selecting different molecular targets, specific structure-activity relationship can be achieved by doing mechanistic analysis. So, additional studies are required for the isolation and recognition of antiviral compound in *Acacia nilotica* to establish its importance as antiviral drug against HCV.

Khanam Z, Adam F, Singh O, and Ahmad J (2011)[47] studied medicinal importance of *Acacia nilotica* and the therapeutic utility of flavanoids. A new acylated flavanoidic glycoside tricin-4'-O-beta- (6"–hydroxycinnamic)–glycoside was isolated from the wood of *Acacia nilotica* together with two known compounds, gallic acid and apigenin. The yield of novel tricin glucoside showed that it makes up to 0.0786 % of mixed ethyl acetate and acetone extract.

Kalaivani T, Rajasekaran C, Suthindhiran K, Mathew L (2011)[48] conducted a study to determine the free radical scavenging, cytotoxic and hemolytic activities of leaves of *Acacia nilotica* by using various methods. The results revealed that ethanol extract was the most effective and IC_{50} value was found to be 53.6 $\mu g\ mL^{-1}$ for Vero cell lines and 28.9 $\mu g\ mL^{-1}$ for Hela cell lines in cytotoxicity assays. None of the tested extracts possessed any hemolytic activity against rat and human erythrocytes revealing their cytotoxic mechanism and non-toxicity.

Riaz S, Faisal M, Hasnain S, Khan NA (2011)[49] conducted a study to investigate lysates from *Acacia nilotica* pods for their antimicrobial and cytotoxic activities against a variety of extended spectrum beta-lactamase -producing Enterobacteriaceae as well as methicillin resistant Staphylococci aureus. The results revealed that the lysate of *Acacia nilotica* pods is a potentially good candidate for the therapy of antibacterial-resistant bacteria, and would therefore require further studies.

Rasool N, Tehseen H, Riaz M, Rizwan K , Zubair M, Mahmood Y et al (2012)[50] evaluated the antioxidant activity by the measurement of total phenolic contents (TPC), total flavonoid contents (TFC), reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and antioxidant activity in linolieic acid oxidation. The results concluded that plant roots may be used as a potential source of antioxidant agents.

Ali A,* Akhtar N, Khan BA, Khan MS, Rasul A, Shahiq-UZ-Zaman *et al (2012)[51] reviewed on the detailed phytochemical composition, medicinal uses, along with pharmacological properties of different parts of multipurpose plant *Acacia nilotica*, acknowledged to be rich in phenolics, consisting of condensed tannin and phlobatannin, gallic acid, protocatechuic acid, pyrocatechol, (+) -catechin, (-) epi-gallocatechin-7-gallate and (-) epigallocatechin-5, 7-digallate etc.

Gupta RK, Gupta D (2013)[52] evaluated the clinical effects of 3 mouthrinses (50% *Acacia nilotica*, 0.2% CHx, saline water) against salivary mutans streptococci (MS). The results suggested that there were significant decreases in the MS colony count in the *Acacia nilotica* and CHx groups at 30 days (85% and 83%, respectively) and at 60

days (65% and 63%, respectively) (P < 0.0001). The antibacterial action of *Acacia nilotica* against MS was similar to that of CHx.

Pote M, Hirapure P (2014)[53] evaluated the highest anti dental infection property of different extract of *Acacia nilotica*, crude extracts of bark, leaves and pod of *Acacia nilotica* against five dental pathogens (Lactobacillus acidophilus, Streptococcus sanguinis, Streptococcus salivarius etc.) using agar diffusion technique and determine the minimum inhibitory concentration of each extract against dental pathogen. The results demonstrated that the pod extract showed highest antibacterial potential followed by the bark extract and leaves extract.

Kumar S, Mohan K, Bhagavan R (2014)[54] carried out a study to evaluate the efficacy of methanol, chloroform, hexane and petroleum ether extracts of *Acacia nilotica* against gram-negative bacteria: *Pseudomonas aeruginosa*, using disc diffusion method. The result demonstrated that the methanol extract is more efficient than the other solvent extracts in suppressing the bacterial growth

Chandrashekar BR, Nagarajappa R, Singh R, Thakur R (2014)[55] compared the anti-microbial efficacy of herbal extracts against primary plaque colonizers. 0.2% CHx and Dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively. Agar well diffusion method was used for anti-microbial efficacy testing. The results suggested that P. *guajava, Eucalyptus, A. nilotica, M. koenigii L.S* and *H. sabdariffa L.* have the potential to inhibit primary plaque colonizers, and they could be used as anti-plaque agents.

Mohan S, Thiagarajan K, Chandrasekaran R, Arul J (2014)[56] investigated the antioxidant capacity and acute toxicity of ethyl gallate, a phenolic antioxidant present in the *A. nilotica* (L.) leaf extract. Results revealed no mortality or abnormal biochemical changes *in vivo* and the protective effect of *A. nilotica* (L.) leaf extract and ethyl gallate on DNA and protein against oxidative stress *in vitro*. Hence, *A. nilotica* (L.) leaf extract or ethyl gallate could be used as potential antioxidants with safe therapeutic application in cancer chemotherapy.

Ker-Woon C, Ghafar NA, Hui CK, Yusof YA (2014)[57] explored the effects of *Acacia honey* on corneal keratocytes morphology, proliferative capacity, cell cycle, gene and protein analyses. The results of the present study show promising role of *Acacia honey* in accelerating the initial stage of corneal wound healing.

Chandrashekar BR, Nagarajappa R, Singh R, Thakur R (2015)[58] assessed and compared antimicrobial efficacy of four plant extracts (*A.nilotica*, *P.guajava*, *E. hybrid, Murraya koenigii L.*) derived using hot and cold extraction methods against *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius*. The results suggested that all the four plant extracts derived using either hot or cold extraction were effective against these bacteria and have the potential to be used as antiplaque agents.

Angelo RU (2015)[59] conducted a study to provide the health application of *Acacia nilotica*, based on its secondary metabolites. Phytochemical analysis of *Acacia nilotica* plant confirmed the presence of various phytochemicals saponins, terpenoids, steroids, anthocyanins, coumarins and tannins. The results suggested that the

phytochemical properties for curing various ailments and possess potential antimicrobial, antioxidant and leads to the isolation of new and novel compounds.

Arbab AH, Parvez MK, Al-Dosari MS, Al-Rehaily AJ, Al-Sohaibani M et al (2015)[60] investigated the hepatoprotective and anti-HBV efficacy of *Acacia mellifera* leaves extracts. The crude ethanolic-extract, including organic and aqueous fractions, were tested for cytotoxicity on HepG2 and HepG2.2.15 cells (IC_{50} = 684 μg/mL). In CCl_4-injured rats, oral administration of *Acacia mellifera* ethanol extract (250 and 500 mg/kg bw) normalized the sera aminotransferases, alkaline phosphatase, bilirubin, cholesterol, triglycerides, and lipoprotein levels and elevated tissue nonprotein sulphydryl and total protein.

Chandra Shekar BR, Nagarajappa R, Jain R, Singh R, Thakur R, Shekar S (2016)[61] conducted an *in-vitro* study to assess antimicrobial efficacy of *Acacia nilotica, Murraya koenigii* (L.)*Sprengel, Eucalyptus, Psidium guajava* extracts, and their combination on *Streptococcus mutans* and *Lactobacillus acidophilus*. The results showed that all the individual plant extracts and their combinations were effective against *S. mutans* and *L. acidophilus*. These could be tried as herbal alternates to chlorhexidine.

Kabbashi AS, Almagboul AZ, Garbi MI, Osman EB, Koko WS et al (2016)[62] investigated the *in-vitro* antigiardial activity and cytotoxicity of ethanol extract of *Acacia nilotica* (bark). *Acacia nilotica* bark ethanolic extract exhibited 100% mortality within 96 h, at a concentration of 500 ppm; this was compared with Metronidazole which gave 96% inhibition at the concentration of 312.μg/ml at the

same time. In addition cytotoxicity verified the safety of the examined extract with an IC_{50}less than 100µg/ml.

***Emad MA (2016)*[63]** conducted a test on the methanol extract of the pods of *Acacia nilotica* against four human bacterial pathogens using disc diffusion method, minimal inhibition concentration and minimal bactericidal concentrations tests. The results revealed that the extract exhibited a broader spectrum of antibacterial activity and revealed potent bactericidal effect at high concentrations (up to 100 mg/ml) and bacteriostatic at lower concentrations (as low as 12.5 mg/ml). In general, all tested bacteria were susceptible, however the tested gram positives (Bacillus cereus ATCC 11778 and Staphylococcus aureus) were much more susceptible than the gram negatives (Escherichia coli and Acinetobacter baumannii), supporting the traditional medical application and suggest it as a source for new antibacterial drugs.

An *in vitro* study evaluating the effects of *A. nilotica* on hGF was conducted by the Department of Periodontics, Babu Banarasi Das College of Dental Sciences, Lucknow, Uttar Pradesh in collaboration with Tissue & Cell Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI). Ethical clearance was obtained from the institutional ethical committee. **(Appendix-I)**

MATERIALS:

The hGF were cultured in the Tissue & Cell Culture Lab, Biochemistry Division, (CSIR-CDRI). The effect of the *Acacia* extract (AE) and CHx were evaluated on cultured hGF cells. Prepared the extract of *A .nilotica* from the fresh soft twigs of the plant at CSIR-CDRI, as explained underneath. Commercially available CHx 0.2 % w/v (Lot no-ND 15-11) manufactured by Kayvee Aeropharm Private Limited (Kadi, Gujarat) was procured. All the reagents, Sterile Plastic were, flasks, multiwelled tissue culture plates, filterware etc were mainly procured from Sigma Chemicals Co. St. Louis, MO (USA). Fetal Bovine Serum (FBS) was purchased from GIBCO BRL Laboratories, New York, USA.

STUDY DESIGN:

The study was divided into four parts:

1. Cellular morphological study - The cultured cells examined under Nikon Phase Contrast Microscope and photographed at the end of the experiment. The details of the morphological characteristics of treated cells studied and compared with Control.

2. Cell growth/Cytotoxicity assessment- Sulforhodamine-B (SRB) assay was conducted to analyse the effects of CHx and AE on cell survival, based on measurement of cellular protein content.

3. Cell-cycle analysis- Cell-cycle analysis was carried out by using flow cytometer. The cytometer is capable of analyzing cells treated with a fluorescent stain Propidium Iodide (PI).

4. Wound healing assay- *In vitro* cellular response on proliferation /migration of hGF was assessed.

A. nilotica **(Plate -I)**

The present study was undertaken to evaluate the effect of *A. nilotica* plant extract on hGF:

Scientific Name: *A. nilotica* (L.) Delile

Family: Leguminosae/Fabaceae-Mimosoideae

Common Name: Acacia, Egyptian mimosa, Egyptian thorn, Red thorn, Babul (in India).

Part Used: Soft twig **(Plate -I)**

Chemical Constituents: The bark and pods contains 12-20% of tannin. Several polyphenolic compounds have been reported from the bark and pods of the plant Acacia nilotica. The gum resin of the plant contains galactose, aldobio uronic acid and arabinobioses. It also contains about 52% of calcium and 20% of magnesium. The flowers contain flavonoids- kaempferol-3-glucoside, iso-quercitrin and leucocyanidin[64].

Toxicology: It has no toxic effect on human body if taken in limited amount[65].

Ayurvedic medicines containing Babul tree:

Dasanakanti churnam- It is an herbal toothpowder used for strengthening of the gums and tooth.

Trayodashang guggulu- It is an ayurvedic tablet used for low back ache, sciatica, arthritic pain, locked jaw.

Pepcer capsule- It is a capsule used to treat peptic ulcer, gastritis, heart burn condition.

Khadiradi gutika- The decoction of babul plant bark is used in binding the powders used to prepare khadiradi tablet, which is used to treat cough[66].

COLLECTION AND VALIDATION OF PLANT MATERIAL:

Collection and validation of *A. nilotica* (soft twig) was done by Tissue Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI).

PREPARATION OF EXTRACT:

Aqueous solution of *A. nilotica* was prepared from the fresh soft twigs 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 at 1000 × g for 5 minutes at 4° C to rid of debris and finally sterilized it by filtering through 45µm syringe filter at Tissue & Cell

Culture Lab, Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow (CSIR-CDRI). **(Plate -II, III)**

ISOLATION AND CULTURE OF HUMAN GINGIVAL FIBROBLASTS:

Experiments was conducted with the hGF cell line routinely maintained at Tissue Culture Laboratory, Biochemistry Division, CSIR- Central Drug Research Institute For experiment the cells were cultured in Dulbecco's Modified Eagle Medium DMEM,PH 7.4) containing penicillin (100 U/ml), streptomycin (200 µg/ml), gentamycin (50 µg/ml) supplemented with 10% FBS and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in a humidified CO_2 incubator at 37°C[67] .

CELLULAR MORPHOLOGICAL STUDY:

Cellular morphological study was performed according to Nigam et al; 2008[68]. A confluent flask of hGF was trypsinized using 0.5% trypsin in Ethylenediaminetetraacetic acid (EDTA) to obtain single cell suspension and the cells counted by trypan blue dye exclusion test. $0.2x10^6$ cells were plated onto a 6 welled plate and cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (200 µg/ml), gentamycin (50 µg/ml) and sodium bicarbonate (2.2 g/l) and incubated at 37° C in a humidified 5% CO_2 incubator. The cells were treated with various doses of AE (1%, 10%, 25%, 50% and 100%) /CHx (1%, 10%, 25%, 50% and 100%) respectively. After 48 hours of culture, the cells were exposed to various concentrations of solution for 1 minute, they were washed thrice with DMEM, followed by the addition of fresh medium and cultured for the next 48 hours at 37° C

in a humidified 5% CO_2 incubator. At the end of experiment the cells were photographed by Nikon™ ECLIPSE Ti Phase Contrast Microscope. **(PLATE-IV)**

CELL GROWTH/CYTOTOXICITY ASSESSMENT:

Sulforhodamine-B (SRB) assay[69] is an accurate and reproducible assay based upon the quantitative staining of cellular proteins by sulforhodamine B (SRB)[70] was performed to evaluate cell survival/cytotoxicity according to Skehan P et al;1990 & Nigam et al; 2008[68,71]. Briefly, 10^4 cells/well plated in a 96 welled plate. After 24 hours of culture the cells were treated with various concentration of AE (1%, 10%, 25%, 50% and 100%) /CHx (1%, 10%, 25%, 50% and 100%) for 1 minute followed by washing (with DMEM) and again cultured for next 48 hours. At the end of the culture period the cells were fixed with chilled 10% Trichloroacetic acid (TCA) and incubated at 4°C for 1 hour followed by washing thrice with distilled water and air dried. 0.4% (w/v) SRB solution dissolved in 1% acetic acid was added (100 µl/well) and incubated for 30 minutes at room temperature. Lastly, the SRB bound to the cellular protein was solubilized with 10 mM Tris (pH 10.5) and determination of optical density was done at 560 nm by Spectrophotometer (Spectra Max M2: Molecular Devices). **(PLATE-V)**

CELL-CYCLE ANALYSIS:

Cell-cycle analysis[72] employs flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, hGF cells (0.2×10^6) were plated on a 6-welled plate and cultured for 24 hours in DMEM solution following treatment with AE(10%,50% and 100%) /CHx (1%,50% and 100%) and further cultured for next 48

hours. Lastly, the cells were collected by trypsinization and washing with chilled Phosphate Buffered Saline (PBS, PH 7.4) and the cells with chilled 70% ethanol for 30 minutes at 4°C[73]. After rewashing with chilled PBS, the cells centrifuged at 100g for 10 minutes at 4° C, and cells was resuspended in 40 µg/ml of Propidium Iodide (PI) containing PBS (500 µl) and Ribonuclease (RNase) (100 µg/ml) and analyzed by Flow cytometry for cell-cycle studies on a Beckton-Dickinson Fluorescence-Activated Cell Sorter (FACS) employing the Cell Quest Software according to Perry et al; 1995 & Nigam et at 2008)[68, 74]. **(PLATE-VI, VII)**

WOUND HEALING ASSAY:

Wound healing assay[75] was performed to study hGF cell migration and interaction according to Rodriguez et al;2005[76]. 0.2×10^6 cells plated in a 6 welled plate and cultured in DMEM containing penicillin (100 U/ml), streptomycin (200 µg/ml), gentamycin (50 µg/ml), 10% FBS and cultured at 37° in a humidified CO2 incubator. One wound/well was created with a sterile 200 µl pipette tip after monolayer formation. The monolayer with the wound was washed with growth medium and treated with to AE (10%, 50% and 100%) / CHx (1%, 50% and 100%) for one minute and further cultured for next 48 hours. At the end of experiment, cells were observed for cell growth/migration under Nikon Eclipse Ti E200 fluorescence microscope. **(Plate-VIII)**

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)**

The present *in vitro* study was undertaken to evaluate the morphological and biochemical changes in hGF treated with *AE*. The effect at different concentrations of *Acacia* was observed on Cellular morphology, Cell growth/Cytotoxicity, cell cycle analysis by FACS (G0/G1, S and G2/M), and in cellular response concerning growth and migration of hGF Using Wound healing assay and compared with CHx.

CELLULAR MORPHOLOGICAL STUDY:

The cellular morphology of cultured hGF was observed after being treated with sequential concentrations of AE, CHx and untreated Control. After treatment the cells were further cultured for next 48 hours and at the end of experiment the cellular morphological features of the cells were examined under Phase Contrast Microscope and photographed.

I. Control (Untreated)

Figure 1 represents untreated Control. It could be observed that cells appear healthy, in abundance, depicting typical large flat, spindle-shaped (elongated) structure possessing extending processes out from the body of the cells with distinct flat and oval cell nucleus. **(PLATE-IX)**

II. Exposure of CHx on hGF

Figure 2, 3, 4, 5, 6, 7 shows the effect of CHx 1%, 10%, 25%, 50%, 75%, 100% on hGF. It could be observed that there is a dose dependent decline in the number of fibroblast.1% CHx displayed minimal affect on cells without much alteration on cellular morphology; this probably indicates nominal cytotoxic effects. However, at 10% concentration cell death was observed enormously. From concentration of 25%-75% cells were found rounded up and fixed to the substratum, floating cells were present along with cell shrinkage. With increasing concentration, more number of cell

deaths is observed. However, at 100% of CHx concentration it exhibited maximal cytotoxic effects on hGF. **(PLATE-X)**

III. Exposure of AE on hGF

Figure 8, 9, 10, 11, 12, 13 signify exposure of AE on hGF at concentration of 1%, 10%, 25%, 50%, 75%, 100%. Upto 100% concentration the fibroblasts are seen undeterred. From 1%-50% concentration of AE no morphological alteration in cells is displayed. AE does not adversely affect the fibroblast cells, even in concentrations going upto 75 % to 100%. Normal cell shape of the cells is clearly maintained. **(PLATE-XI)**

CELL GROWTH/CYTOTOXICITY ASSESSMENT:

In order to confirm and validate the results obtained from microscopical examination and evaluation of hGF, SRB assay was performed. These assays are the benchmarks for ascertaining the cytostatic/proliferative/toxic effect of any drug or ligand such as CHx and AE. The respective effects of CHx and AE on hGF exposure were undertaken (for 1 minute) and the results of different concentrations (ranging from 1 – 100%) are compared with the untreated Control.

I. Cell survival /cytotoxicity at different concentrations of CHx on hGF

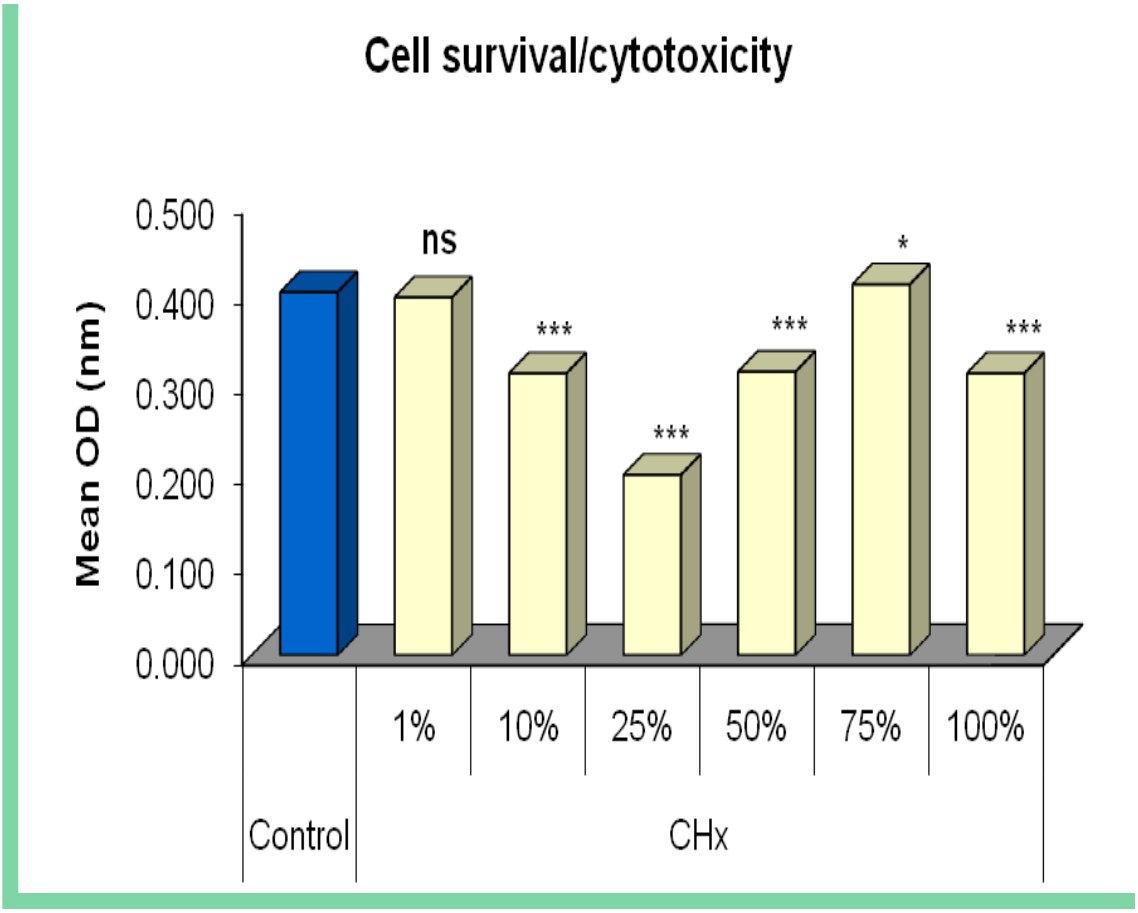
To analyse the effects of CHx on hGF cell survival/cytotoxicity, SRB assay was performed and results are summarized in Table 1 and Graph 1. CHx treatment at concentrations of 10%, 25%, 50% and 100% displayed highly significant cytotoxic effects on cells ($p<0.001$) in respect of control. However, at 50% to 100% concentration the OD was found to be increased as comparison with 10% and 50% treated cells. The mean OD value i.e. cell survival lowered comparatively at all concentrations of CHx as compared to control.

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

Table 1: Cell survival/cytotoxicity (OD value in nm) at different concentrations of CHx on hGF by ANOVA

CHx (concentrations)	**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		

Graph 1: Comparison of cell survival/cytotoxicity (mean OD value) between different concentrations of CHx



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

II. Comparison of cell survival/cytotoxicity between different concentrations of CHx on hGF

On comparing the mean OD values between different groups, significant ($p<0.001$) lowering of cell viability in CHx at 10%, 25%, 50% and 100% concentrations was shown by Tukey test, while cell viability is significantly ($p<0.05$) higher at 75% as compared to control. Further, cell survival 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 rate and 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/cytotoxicity at different concentrations of AE on hGF

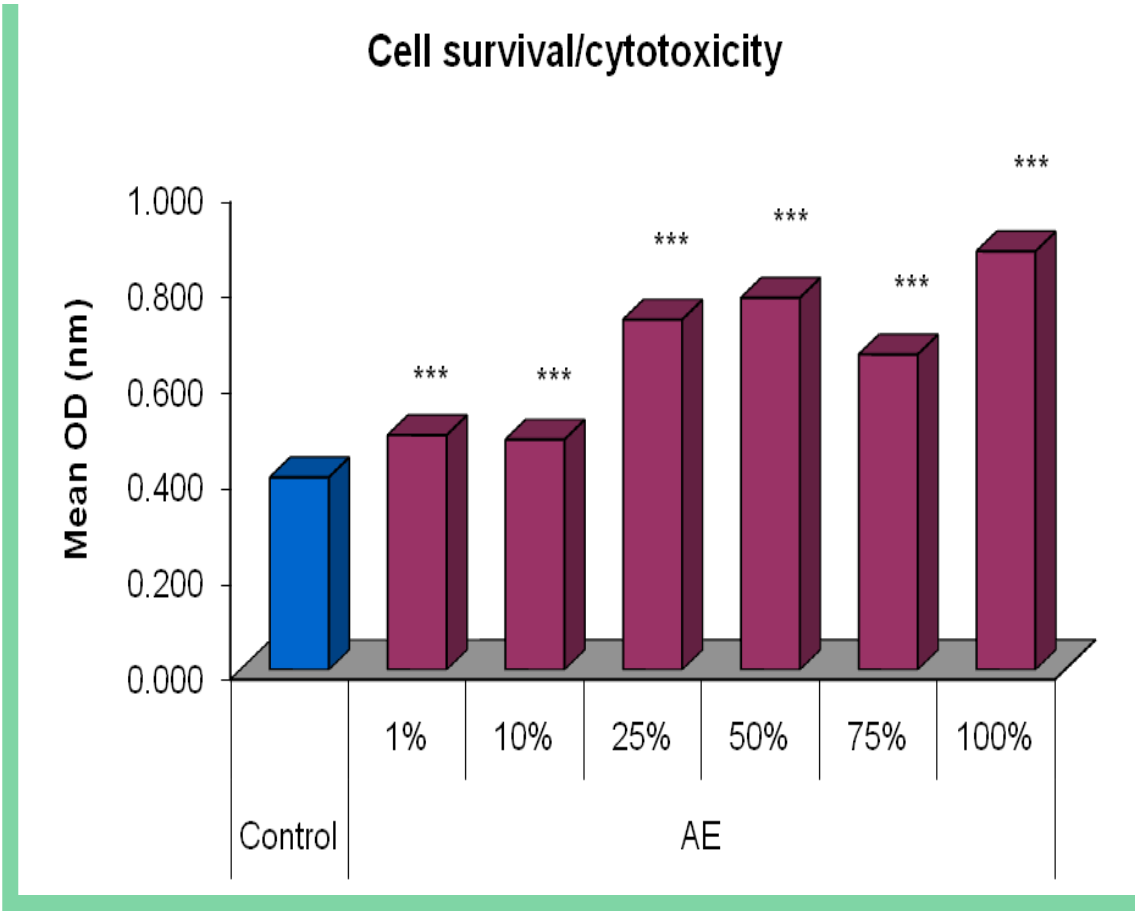
Cell viability assay was performed by SRB after hGF cells treated with AE for 1 minute exposure at various concentrations. The results of cell viability performed as mean OD in nm are summarized in Table 3 and Graph 2.It has been observed that OD significantly increased ($p<0.001$) at every dose of treatment from 1% to 100% dose dependently except at 10% of AE treatment. Mean OD value on an average increases with the increase in concentration shows cell viability dependence on concentration.

Evaluating the effect of AE (concentrations or groups) on OD Values, ANOVA showed significant effect of AE on cell survival rate and cytotoxicity ($F=2880.68$, $p<0.001$).

Table 3: Cell survival/cytotoxicity (OD value in nm) at different concentrations of AE by ANOVA

AE (concentrations)	**Mean ± SD (n=3)**	**F Value**	**P Value**
Control	0.403 ± 0.001	2880.68	<0.001
1%	0.491 ± 0.001		
10%	0.482 ± 0.004		
25%	0.733 ± 0.010		
50%	0.779 ± 0.002		
75%	0.660 ± 0.005		
100%	0.876 ± 0.009		

Graph 2: Comparison of cell survival/cytotoxicity (mean OD value) between different concentrations of AE



***p<0.001- as compared to control

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

On comparing the mean OD values between different groups, Tukey test showed significantly ($p<0.001$) higher cell viability at all concentrations of AE as compared to control. Cell viability also differed significantly ($p<0.001$) between all concentrations of AE except 1% and 10 %. (Table-4)

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

Comparisons- AE Concentrations	**p value**
Control vs. 1%	<0.001
Control vs. 10%	<0.001
Control vs. 25%	<0.001
Control vs. 50%	<0.001
Control vs. 75%	<0.001
Control vs. 100%	<0.001
1% vs. 10 %	0.452
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%	<0.001
10% vs. 75%	<0.001
10% vs. 100%	<0.001
25% vs. 50%	<0.001
25% vs. 75%	<0.001
25% vs. 100%	<0.001
50% vs. 75%	<0.001
50% vs. 100%	<0.001
75% vs. 100%	<0.001

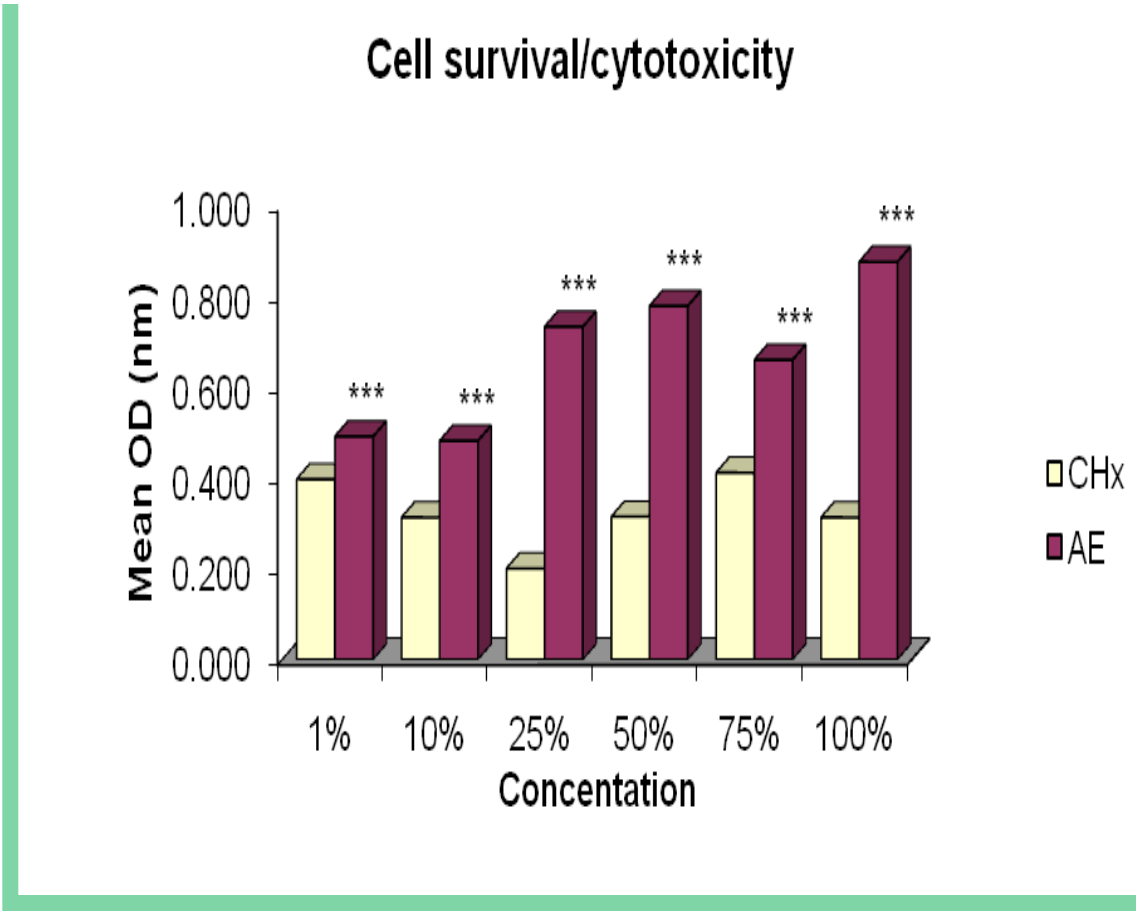
V. Comparison of cell survival/cytotoxicity between CHx and AE at different concentrations

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

Table 5: Comparison of cell survival/cytotoxicity (OD value in nm) between CHx and AE at different concentrations by Student's t-test

Concentration	**CHx (Mean ± SD, n=3)**	**AE (Mean ± SD, n=3)**	**T Value**	**P Value**
1%	0.397 ± 0.004	0.491 ± 0.001	45.75	<0.001
10%	0.313 ± 0.003	0.482 ± 0.004	61.04	<0.001
25%	0.200 ± 0.001	0.733 ± 0.010	91.56	<0.001
50%	0.315 ± 0.005	0.779 ± 0.002	163.00	<0.001
75%	0.412 ± 0.003	0.660 ± 0.005	73.15	<0.001
100%	0.313 ± 0.003	0.876 ± 0.009	103.20	<0.001

Graph 3: Comparison of cell survival/cytotoxicity (mean OD value) between CHx and AE at different concentrations



***p<0.001- as compared to CHx

CELL-CYCLE ANALYSIS:

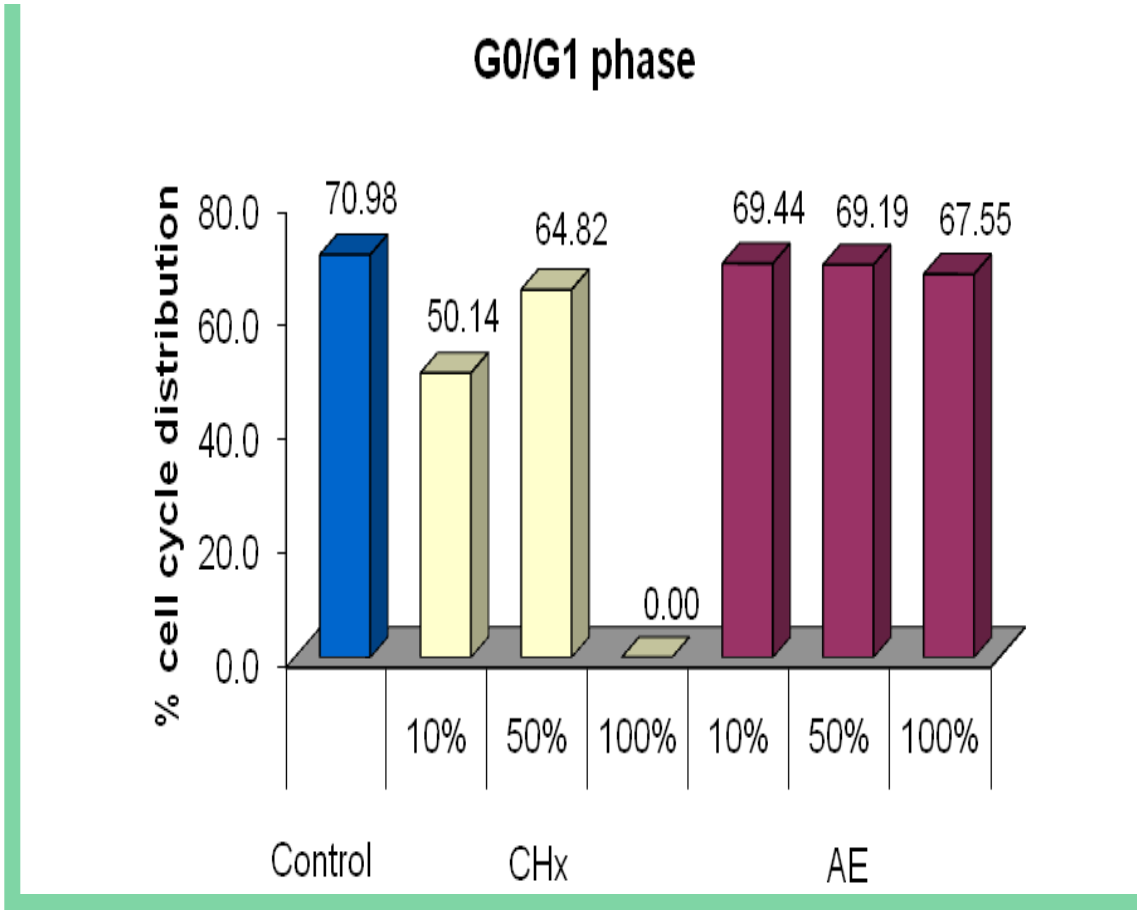
Flow cytometry was done to reveal the stages of the cell-cycle. The control cells resolve into maximal G_0/G_1 (resting phase), minimal S (synthetic phase) and residual G_2/M (mitotic phase).The distribution of % cell cycle in G0/G1 phase, S phase and G2/M phase of two treatment groups (CHx and AE) at three different concentrations (10%, 50% and 100%) is summarized in Table-6 and Graph-4,5,6 and also depicted in Fig. 14 to 20 **(PLATE XII-XVIII)**, respectively.

The % cell cycle in G0/G1 phase diminished in both the treatment group at all three concentrations as compared to control. Further, in AE, % cell cycle diminished with increase in concentrations but in CHx it did not display any trend, increase at 50% and decrease at 100% (Table-6, Graph-4).

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

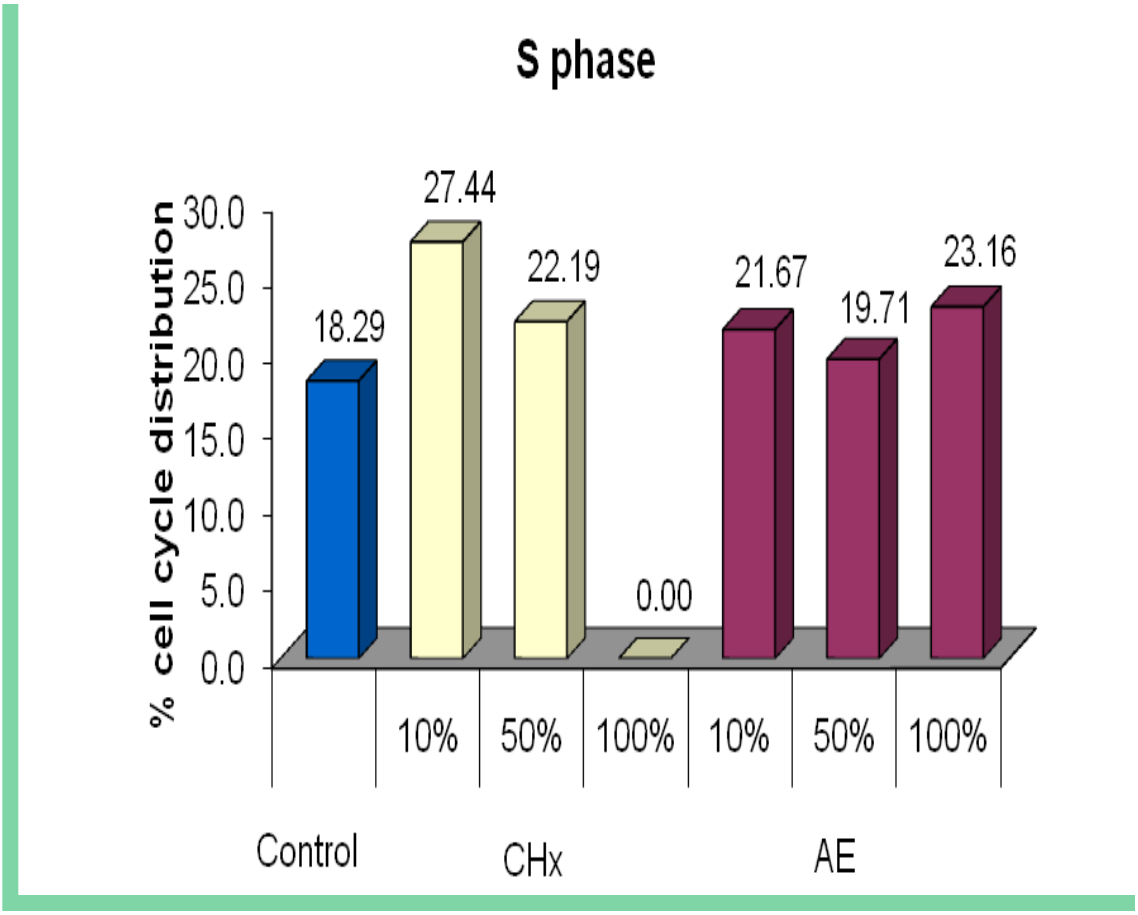
Groups	**Apoptotic cells**	**G0/G1 phase**	**S phase**	**G2/M phase**
Control	0%	70.98	18.29	1.88
CHx:				
10%	1.63%	50.14	27.44	1.84
50%	80.87%	64.82	22.19	1.94
100%	100.00%	0.00	0.00	0.00
AE:				
10%	0.05%	69.44	21.67	1.90
50%	0.02%	69.19	19.71	1.91
100%	0.10%	67.55	23.16	1.92

Graph 4: % cell cycle distribution of CHx and AE at different concentrations at G0/G1 phase



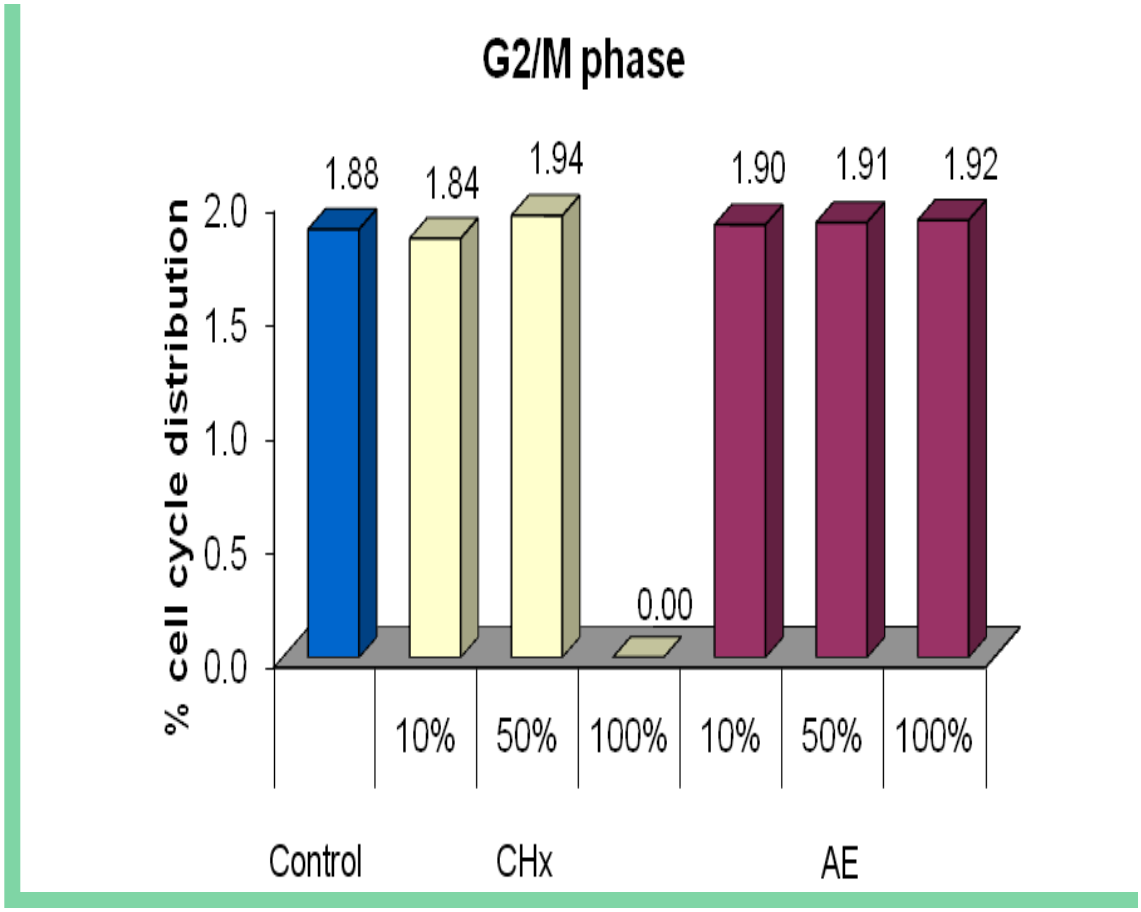
In contrast, the % cell cycle in S phase was found enhanced in both CHx(10% and 50%) and AE (10%, 50% and 100%) as compared to control but showed linear decrease with concentrations in CHx while no trend in AE, decrease at 50% and increase at 100% (Table-6, Graph-5).

Graph 5: % cell cycle distribution of CHx and AE at different concentrations at S phase



Conversely, the % cell cycle in G2/M phase was found enhanced in all concentrations of AE while only 50% in CHx as compared to control and showed a slight increase with increase in concentration in AE but no trend with concentrations in CHx, increase at 50% and decrease at 100% (Table-6 ,Graph-6).

Graph 6: % cell cycle distribution of CHx and AE at different concentrations at G2/M phase



WOUND HEALING ASSAY

Different concentrations of CHx and AE were studied by Wound healing assay to see their role and effectiveness on proliferation /migration of hGF which is essential for rapid and effective repair of necrosed or diseased tissue. Post-48 hours wound gap devoid of fibroblasts indicated poor mitotic index amongst the control cells (Figure 22). At 10% CHx exposure, the cytotoxic potential could be visualized with rounded shrunken cells in the wound area (Figure 23). Subsequently, widening of wound at 50% to 100% CHx exposure showed cytotoxicity (Figure 24 and 25). Marked reduction in the size of the gap width was seen at 10 % AE exposure (Figure 26), the gap obliterated with healthy looking cells, unlike the control. At 50 % AE exposure (Figure 27), improved closure of wound area with aggregated fibroblasts was observed. Rehabilitation of the damage to the cells owing to 100 % AE exposure was done (Figure 28). **(PLATE-XIX, XX)**

Within limitations, the present study concluded that:

- Cellular morphological study show dose-dependent cytotoxicity of CHx on hGF at concentration ranging from 10%-100%.However, AE displayed no distorted morphology even at higher concentration.
- Cell viability assessment conducted on hGF using SRB assay clearly indicates that CHx dose-dependently induces cytotoxicity initiating beyond 1 % concentration for 1 minute of treatment suggesting cell death. However, AE did not show any dose-dependent cytotoxicity and maintained the level of cell survival and doesn't adversely affect the cultured fibroblasts even upto concentration of 100 %. This therefore suggests that CHX at 1% only and AE until 100% was well tolerated by hGF hinting at their relative safety.
- Cell-cycle analysis of the cells exposed to CHx at various concentrations as low as 1% for 1 minute indicates adverse effects on cell cycle when compared to untreated control cells. Percentage of apoptotic cells showed that CHx concentration increased percent apoptotic cells increased reaching maximum at100% concentration. Whereas AE interestingly shows no adverse effect on cell cycle phases and also very few apoptosis was found.
- Wound healing assay conducted on hGF exposed to CHx showed cytotoxicity at 10%, subsequently, widening of wound at 50% to 100%. Contrarily improved closure of wound area with aggregated fibroblasts was seen at sequential AE exposure.

Hence, evaluating the results it can be concluded that AE exhibits the best results as compared to CHx and Untreated Control. Moreover AE shows the cells undamaged in terms of their morphology, viability and cell cycle stages even over the Untreated Control. The Fact That AE enhance the wound closure, a crucial feature, makes us choose it as the best amongst compared groups.

The in vitro studies conducted here would greatly help to extrapolate the situation under intact in vivo conditions that exist in a human where fibroblasts interaction with other cellular components is crucial. Further clinical studies may play an imperative role to investigate the properties of *Acacia* derived mouthwashes.

Mouthwashes are a universally used method to deliver the anti-microbial agent (after toothpastes), which can be used by the patient as an oral hygiene aid. Chlorhexidine gluconate (CHX) with its broad-spectrum activity is effective against an array of micro-organisms and is considered a gold standard in chemical plaque control. Medicinal plant formulations can be considered a feasible alternative as these are widely available, cost-effective and have fewer side effects than the presently available chemical anti-plaque agents. Although *Acacia nilotica* had been used in the oral cavity in past but its effects, if any, on Human Gingival Fibroblasts (hGF) remain undetermined till date. Thus, the present study was undertaken to analyze the effects of *A. nilotica* on hGF on the basis cytomorphological characterization and biochemical of and to compare these effects with CHx.

Aqueous solution of *A. nilotica* was prepared from the fresh soft twigs of the plant, extracted with 10% w/v chilled 1.15% potassium chloride (KCl) by grinding with a pestle and mortar and then centrifuged at 1000 × g for 5 minutes at 4° C to remove the debris and finally sterilized it by filtering through 45µm syringe filter at Tissue & Cell Culture Lab, Biochemistry Division, CSIR-CDRI, Lucknow. The study was divided into two parts. The first part of the study dealt with evaluation of morphology of hGF, performed by Nikon Phase Contrast Microscope. The second part of the study dealt with analyzing the effects of CHx and AE on cell survival, based on measurement of cellular protein content by SRB assay. The third part of the study dealt with Cell cycle

analysis by using flow cytometry. The fourth part of the study dealt with in *vitro* cellular response on proliferation /migration of Hgfusing wound healing assay.

The observation of the Cellular morphological study shows that hGF upon exposure to CHx and AE at different concentrations for 1 minute display specific effects. It has been observed that CHx at 1% concentration and AE from 1 % to 100% concentrations displayed normal morphologicalfeatures possibly indicating no cytotoxicity. However CHx at 10% concentrations, exhibited cytotoxic effects and most of the cell deaths clearly observed and found floated in medium, From 25%-75% of CHx displayed rounded cells and some of them got fixed to substratum. Lastly, CHx at 100% concentration showed cell death indicating high toxicity over AE (100%).

Cell viability assessment conducted on hGF using SRB assay clearly indicates that CHx induces dose dependent cytotoxicity initiating beyond 1 % concentration for 1 minute of treatment suggesting cell death. Although, AE did not show any cytotoxicity as the optical density was above the control also, it doesn't adversely affect the cultured fibroblasts even upto concentration of 100 %.Hence suggestive of the fact that CHX at 1% only and AE until 100% is well tolerated by hGF hinting at their safety relatively.

Cell-cycle analysis showed adverse effects on cell cycle when compared to untreated control cells when exposed to CHx at various concentrations as low as 1% for 1 minute. Percentage of apoptotic cells showed that increase in CHx concentration increased percent apoptotic cells when reaches maximum as much as 100%

concentration. Whereas AE did not show adverse effect on cell cycle phases and very less apoptosis was observed.

Wound healing assay performed on hGF showed cytotoxicity at 10% upon treatment with to CHx, and widening of wound at 50% to 100% is clearly observed. On the contrary improved closure of wound area with aggregated fibroblasts was seen at exposure of AE.

It can be concluded that AE exhibits better results as compared to CHx. AE treatment shows that the cells are undamaged in terms of their morphology and cell cycle stages even over the Untreated Control, also promote cell growth and migration and augment growth as well as seen in cell growth/cytotoxicity assay. AE enhances wound closure, which is a crucial feature, making it the best amongst other compared groups.

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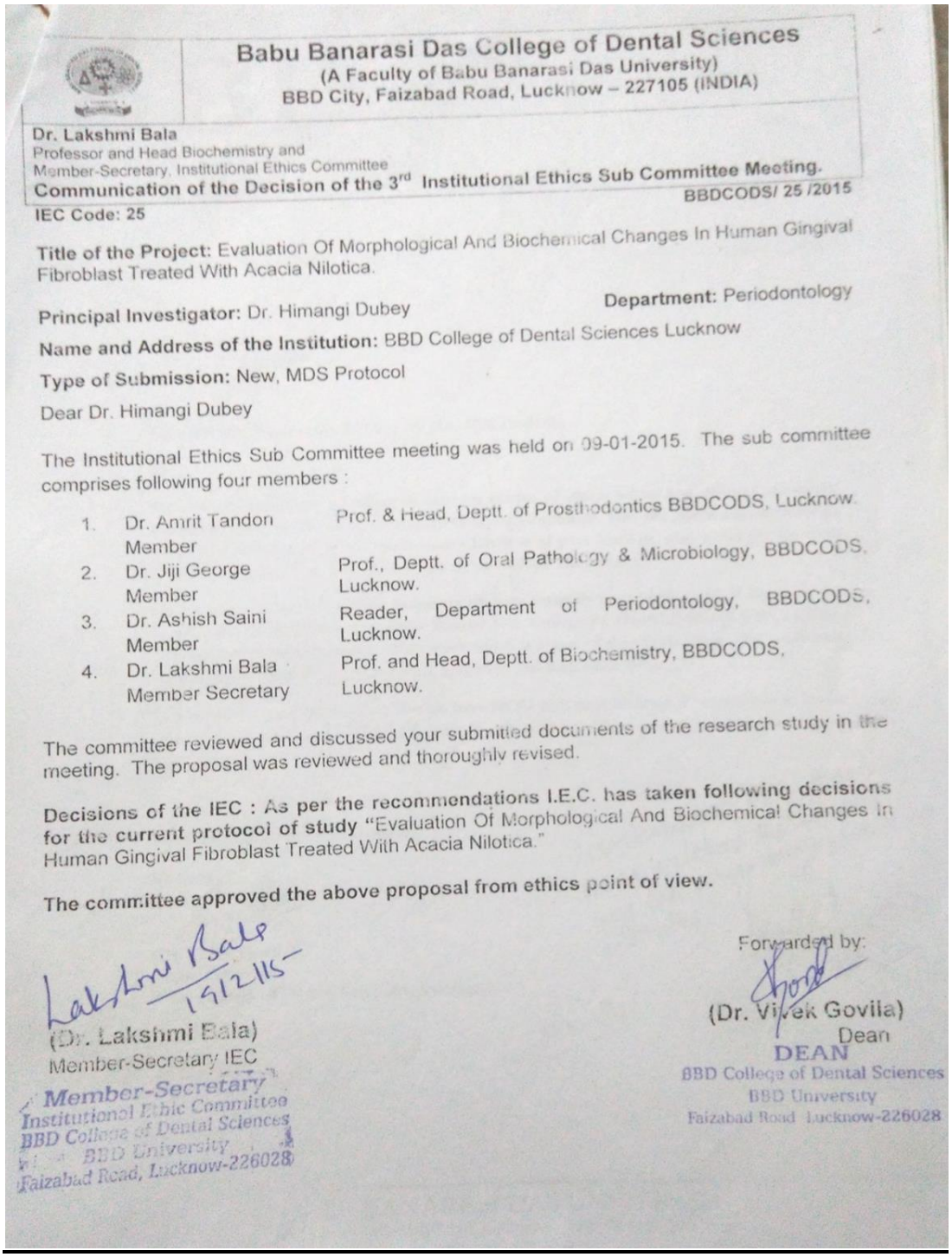
APPENDIX – I

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/ 25 /2015

IEC Code: 25

Title of the Project: Evaluation Of Morphological And Biochemical Changes In Human Gingival Fibroblast Treated With Acacia Nilotica.

Principal Investigator: Dr. Himangi Dubey **Department:** Periodontology

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

Type of Submission: New, MDS Protocol

Dear Dr. Himangi Dubey

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

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

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 "Evaluation Of Morphological And Biochemical Changes In Human Gingival Fibroblast Treated With Acacia Nilotica."

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

Lakshmi Bala 19/2/15

(Dr. Lakshmi Bala)
Member-Secretary IEC

Member-Secretary
Institutional Ethic 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

STATISTICAL TOOLS USED

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).
- $\boldsymbol{\varepsilon_{ij}}$ 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

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

where,

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

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 (***)

Antiseptic mouth rinses play an important role in daily dental care, and their use is strongly encouraged throughout the world, particularly for the prevention and treatment of periodontal diseases. Broad spectrum antimicrobial mouthwashes have gained great importance as adjuncts to conventional periodontal therapy. Loe and Schiott in 1970 established the potential for antimicrobial mouthrinses in clinical practice using an experimental gingivitis study model and found that 0.2% chlorhexidine mouthrinse can effectively prevent plaque and gingivitis in the absence of other chemical and mechanical oral hygiene procedures[77].

Chlorhexidine (CHx) was used in our study as it is recognized as the "gold-standard" or positive control against which the efficacy of alternative anti-plaque agents can be measured[78, 79]. It exhibits both anti-plaque and anti-bacterial properties and acts by altering integrity of cell membrane of bacteria[80].An important property of chlorhexidine is its substantivity referring to the oral retentiveness[81].However, its extended use may cause local side effects like, altered taste sensation, brown discoloration of the teeth and tongue, oral mucosal ulcerations which reduce its acceptability in patients[82].

Due to these limitations a continuous search for alternatives is being carried out. Nature has been a source of medicinal plants for many years. A number of studies have been done to explore the medicinal properties of plants in a quest to formulate new, economical, a widely available antibacterial agent with fewer side effects. Acacia has attracted the widest attention globally for a myriad of reasons and is used to maintain the oral hygiene[8, 9]. The bark contains a large quantity of tannin and is a powerful astringent; its decoction is largely used as a gargle and mouth wash in cancerous and syphilitic affections[83].The tender twigs are used as

toothbrushes[12]. Acacia gum consists primarily of Arabica, a complex mixture of calcium, magnesium and potassium salts of Arabic acid. It contains tannins which are reported to exhibit astringent, hemostatic and healing properties. It also contains cyanogenic glycosides in addition to several enzymes such as oxidases, peroxidases and pectinases, all of which have been shown to exhibit antimicrobial properties. The bark constituents of Acacia catechuit are used to treat stomatitis, gingival bleeding, improve appetite (Gazi, 1991). P, gingivalis and P. intermedia are strongly implicated in the pathogenesis of chronic periodontitis (Moore; 1987) and the proteolytic activity of P. gingivalis is recognised as a potential virulence factor (Slots and Genco 1984). The in vitro inhibitory action of acacia gum against these organisms and their enzymes is thus of possible clinical significance (Clark et al; 1993). Pradeep AR have reported that gumtone gel (containing *Acacia arabica*) was not associated with any discolouration of teeth or unpleasant taste and it may be a useful herbal formulation for chemical plaque control agent and in the improvement of plaque and gingival status. When evaluated the short term clinical effects of commercially available gel containing *Acacia arabica* in the reduction of plaque and gingival inflammation in subjects with gingivitis. Nagarajappa R et al (2015) has reported antimicrobial efficacy of acacia extracts derived using hot and cold extraction methods against *Streptococcus mutans, Streptococcus sanguis* and *Streptococcus salivarius a*nd found effective. Several studies have also proven the efficacy of *A. nilotica* as an aantioxidant, antibacterial, anti-microbial agent etc[10, 11].However; to best of our knowledge no systematic study is available regarding its cytotoxicity on human Gingival Fibroblast (hGF) and their impact on hGF in wound repair remains unknown.

The present study has been undertaken for this reason. Gingival fibroblast were selected for this study, as they are primarily responsible for synthesis of extracellular matrix and also involved in a number of regulatory processes necessary for the maintenance of tissue homeostatis (bartold et al; 2000).The efficacy of Acacia Extract (AE) has been compared with Chlorhexidine (CHx) on cultured hGF cells.Our study consists of: 1) Cellular morphological study 2) Cell growth/Cytotoxicity assessment 3) Cell-cycle analysis 4) Wound healing assay. The doses of mouthwashes employed have been 1-100%. 0.2% CHx which is commercially available in the market is regarded as 100%.AE is considered as 100%.

CELLULAR MORPHOLOGICAL STUDY:

The cultured hGF fibroblast cells were exposed to the above mouthwashes range from concentration of 1-100% for 1 minute. Generally, gargling is recommended for 1 min so we decided 1 min as time exposure for our study to titrate the exposure at various concentrations similar to Mariotti et al[24].The untreated control clearly exhibited all cells are in healthy conditions. Morphological studies also indicate that hGF upon exposure to CHx and AE at different doses for 1 minute display specific effects. It has been observed that CHx at 1% concentration and AE at various concentrations ranges from 1 % to 100% displayed minimal number of morphological alterations. This possibly indicates no cytotoxicity. However, CHx at 10% concentration exhibited cytotoxic effects and most of the cell died and floating in medium. From 25%-75% of CHx treatment the cells get rounded up and some of them fixed to substratum. However, CHX at 100% concentration showed cell death depicting high toxicity over AE (100%). Similar cellular response has been observed with CHx by Goldschmidt et al[84]. They observed that high concentration of toxic substances may somehow

fix non vital cells to the surface of petridish. In another study Prabhushankar et al[85] reported that when cultured gingival fibroblast were exposed to 0.12 % CHx, they rounded up and detached from the substratum within a few hours. Pucher et al[86] observed CHx caused the loss of fibroblast from the dish at higher concentrations and rounding of these cells in monolayer at lower concentrations. These above studies are in agreement with our findings.

CELL GROWTH/CYTOTOXICITY ASSESSMENT:

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.

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 assay. At therapeutic concentrations, all the antiseptics are cytotoxic for fibroblasts and keratinocytes whereas antibiotics were not cytotoxic to the tested cell.

The cytotoxicity data compared with untreated control obtained from SRB assay clearly indicates that the cell survival decreases at all concentration of CHx dose dependently except 75% as indicated in table1 and graph 1, whereas AE 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. All the above

data has been compared with an untreated control group. Overall, the data of present cytotoxicity assay clearly indicate that CHx dose-dependently induces cytotoxicity initiating beyond 1 % concentration for 1 minute of treatment suggesting cell death. However, AE did not show any dose-dependent cytotoxicity and maintained the level of cell viability to about 100% concentration.

From the above data it is clearly evident that the Chx induces more toxic effect on hGF at much lower concentrations as compared to AE. The cytotoxic effect of CHx has been well documented by several workers in detail. Our work is in accord with findings of Chang et al studied the effects of sodium hypochlorite (NaOCl) and Chx on cultured human periodontal ligament (PDL) in vitro. The effects of irrigation solution on human PDL cells were 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 dependant manner. In another study Verma et al 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 evaluations. 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. In addition, the cytopathic effect of CHx on human fibroblasts and HeLa cells has been

demonstrated by Goldschmidt et al[84]. The observations of these studies are in agreement with the findings of the present study.

The data of the in vitro study was analysed statistically and the observation was made comparing the cell survival/cytotoxicity between different concentration of CHx and different concentration of AE.

For CHx, 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 viable 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. No significance between p values was observed within various doses 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 above doses.

In the case of AE, reveals highly significant cell growth and development at all concentration of AE as compared to control. However, values decreased the significance at 1% vs.10%. 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 p values for the 2 mouth rinses tested that is Chx and AE, it can be concluded that cultured fibroblast can safely

survive between 1 % to 100 % by AE mouthwash exposure respectively. Comparison of cell survival/cytotoxicity between CHx and AE at different concentrations reveals significantly increased cell survival in AE at all concentrations to CHx.

These results corroborate well with the results of Cellular morphological study.The cytotoxic potential of CHx clearly observed even at low doses on hGF however, AE exhibited no cytotoxic effects on hGF even exposed at high concentration. This therefore suggests that CHx at 1% only and AE until 100% well tolerated by hGF hinting at their relative safety, which may be of relevance in the *in vivo* situations.

CELL CYCLE ANALYSIS:

Flow cytometry analysis was performed to analyze the cell cycle kinetics, It provides an idea of the percentage of apoptotic cells and viable cells in different phase of cell cycle (Nigam et al ; 2008). Distribution of cell cycle phases of hGF cells and Apoptosis induced by CHx at different concentrations (10%, 50%, and 100%) was studied by Flowctometry. Effects were observed, with Control and different concentrations of AE (10%, 50%, 100%). No apoptotic changes of hGF cells were noticed in the control group 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 may also co relate with cellular morphology. Maximum apoptosis were observed with 100% concentration of CHx. The effects of AE on hGF at different concentrations was observed (10%, 50% and 100%) which showed 0.05%, 0.02% and 0.10% of apoptotic cells respectively which denotes to normal in cell cycle.

Flowcytometric analysis was also used to study cell cycle kinetics and it was observed that in control group cells in G0/G1 phase were 70.98 %, in S phase 18.29% and in G2M phase 1.88%. Figure clearly shows the distribution cell in various phases of cell cycle in normal control cells, which have not undergone any treatment, 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.

It has been observed that with Chx cell population 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 further noticed that as the CHx at 50% concentration, cells in G0/G1 phase were 64.82, in S phase they were 22.19 % and in G2M phase 1.94%, In addition at 100% concentration of CHx the cell cycle phases were not detectable indicating cell death. Increasing CHx concentrations can affect the mode of cell death. When cytotoxicity was raised, CHx-induced cell death by necrosis rather than apoptosis. Our results were in agreement with Faria et al[87] who reported that CHx induced apoptosis at lower concentrations and caused necrosis at higher concentrations in L 929 fibroblasts. However, our results differed from those of Giannelli et al[88] 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 reason for this contrary result is not clear. It may be due to the different origins of the cells or different experimental protocols used in each laboratory. The cellular effects of CHx may not necessarily be comparable in all tissues.

Similarly using different concentrations of AE the cells observed in viable cells in G0/G1 phase was 69.44%, in S phase 21.67% and in G2M phase they were 1.90 % at 10%

concentration. Further, with 50% of AE exposure the cells in G0/G1 phase were found to be 69.19%, in S phase 19.71% and in G2M phase they were 1.91%. In addition, 100% AE exposure displayed cells in G0/G1 phase reduced to 67.55%, in S phase 23.16% and in G2M phase they were 1.92%. AE failed to show cytotoxicity or apoptosis in hGF cells at all the concentrations.

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 AE. CHx exposure showed cytotoxicity as at 10% CHx exposure, the cytotoxic potential could be visualized with rounded shrunken cells in the wound area. Subsequently, widening of wound at 50% to 100%. 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[89]. In contrast to our work, study by Schiott[90-94] et al, concluded that two years daily mouth wash of CHx in human patients showed no systemic effects and no oral difference from the control. 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 % AE exposure, at 50 % AE exposure, improved closure of wound area with aggregated fibroblasts was observed. Rehabilitation of the damage to the cells owing to 100 % AE exposure was done. It can also be reinforced that AE enhance the wound closure, beneficial for reduced risk of infection. Further, similar experiments evaluating CUR, aloe vera, taurine and vitamin C have been proposed[95].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 *A. nilotica*..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.

OBSERVATIONS & RESULTS

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