The man of science has learned to believe in justification, not by faith, but by verification.

Thomas Henry Huxley
The milk whey media showed *L. lactis* growth in all transfers and nisin activity was detected only till third transfer. Initially 100% concentration of milk whey showed maximum activity, later on gradual decline was observed till third transfer. However at 25% concentration of whey rise in nisin activity was seen from first to third transfer. This change in nisin activity was due to change in pH values. The initial pH was around 6.7 for all concentrations but after every transfer pH value, decreased which possibly affects the production of Nisin. However maximum stability and solubility of nisin is reported in acidic pH values (*Sayyed T, et al., 2010*), but production of nisin is very low. The optimal initial pH values for maximum nisin production were around 6.5 and 7.5 which is confirmed by *Jozala et al., (2005)*. As it was seen that at 25% concentration, the culture media was more dilute as compared to 50%, 75% and 100%. The growth of *L. lactis* and thereby the production of nisin was low. This could be due to scarcity of some nutrients necessary for the organism’s development in small concentrations. Even though the 100% whey concentration was the best conditions for the development of *L. lactis* and nisin release initially, later on after every transfer reduction in the nisin activity was noted which could be associated with decrease in nutrients in the culture media also reported in the work of *Jozala et al., (2007)* who observed that the concentration equilibrium of the nutrients in a medium was essential for the higher nisin productivity by *L. lactis* cells. Besides, the lactic acid bacteria are fastidious and require culture media with high nutritional value, which increases their growth and bacteriocin production.

*Guerra and Pastrana (2002)* observed that nutrient sources were not adequate to increase bacteriocin production on diluted whey. In addition, the same authors alternatively supplemented yeast extract, lactose and glucose for supplements into the milk whey for bacteriocin production. They observed that the use of feeding substrates containing glucose instead of lactose could be an appropriate alternative for increasing fed batch production of pediocin. According to the research, the maximum nisin values obtained in that study in which milk whey not filtered was 22 fold higher than the milk whey diluted. Since the culture media is of low cost, the higher nutritional content of milk and milk whey gave an excellent growth conditions for the *L. lactis* and nisin expression (*Jozala et al., 2011*).

Observing the results obtained in milk whey, we choose to use nutritional supplements. By the analysis of the bibliography, we tried to put culture media that promote the growth and nisin excretion by the organism. The ideal culture media for the development of *L. lactis* cells contain yeast extract, tomato juice and milk. Based on this information,
culture media containing only tomato extract as supplement along with milk whey was utilized as base culture media. The supplementation of media gave a better adaptation for the nisin producing cells. It was observed that no nisin activity was detected in first transfer however *L. lactis* growth was seen. This could be associated with the time taken for adapting to the supplementation in the media. Second transfer onwards nisin activity was detected till fifth transfer, with 25% media concentration showing maximum activity and 100% concentration showing minimum activity. This could be attributed to the faster decrease in pH at 100% concentration than at 25% concentration.

Nisin production is affected by several cultural factors such as producer strain, nutrient composition of media, pH, temperature, agitation and aeration and also by other factors such as substrate and product inhibition, adsorption of nisin onto the producer cells and enzymatic degradation (*Jozala et al.*, 2011).

### 4.2 Antimicrobial study

Plant essential oils and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies (*Anyanwu et al.*, 2014). Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the essential oils and extracts inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of many essential oils has been previously reviewed and classified as strong, medium or weak (*Zaika*, 1988). The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents even against some antibiotic resistant strains (*Kone et al.*, 2004). In this study, using agar diffusion method it was observed that extracts of Cinnamon produce antibacterial activity against gram negative and gram positive organisms. Results of the present study confirmed the observation of earlier studies (*Yuste and Fung*, 2004). However, it was seen that *Curcuma longa* exhibited no antibacterial activity against the test organism at the specific dose which is contradictory to previous observations (*Singh et al.*, 2002). This variation may be because of the dose used in this study, the method of extraction of medicinal plant, the genetic variation of plant, age of the plant or environment. It is worthy of note that antimicrobial activity results of the same plant part tested most of the time varied from researcher to researcher. This is possible because concentration of plant constituents of the same plant organ can vary from one geographical location to another depending on the age of the plant, differences
in topographical factors, the nutrient concentrations of the soil, extraction method as well as method used for antimicrobial study (Doughlari, 2012).

The Cinnamon extract (CE) was found to be effective against *E.coli*, *P.aeruginosa*, *S.aureus*, *C.albicans* and *A.niger*. This effect is in agreement with other research work regarding the antibacterial effect; however there is a difference in the concentration of cinnamon extract at which we found antibacterial activity (Mau et al., 2001; Yuste and Fung, 2004). Cinnamon extraction by methanol and ethanol demonstrated antimicrobial activity against all five test organisms but differences were observed in the zone of inhibition against them. Of the five organisms *C.albicans* was found to be more sensitive to methanolic extract than *A.niger*, *E.coli*, *S.aureus* and *P.aeruginosa*. It was observed that all the five organisms had lower MIC values with ethanolic extract than with methanolic extract. This is due to the nature of active principles in cinnamon which are more soluble in ethanol than in methanol (Senhaji et al., 2004). Cowan (1999) reported that ethanol is capable of extracting tannins, polyphenols, flavonol, terpenoids, alkaloids and low amounts of essential oils. Phenolics, flavonoids and alkaloids inhibited bacterial cell wall synthesis (Sarmauli et al., 2008). The degree of changes and damage to the cell structures are affected by the type of antibacterial agent, type of bacteria and the antibacterial concentration being applied to the cells (Parhusip, 2006).

Essential oils are volatile liquids with strong odor. They contain complex compounds formed by aromatic plants as secondary metabolites. These oils are synthesized in all plant parts such as buds, flowers, leaves, stem, wood, bark. Essential oils of some spices are utilized in food industry as preservatives and in perfumery and medical industries. Antimicrobial activity of some plant essential oils such as cinnamon, anise, mace, oregano, clove and zedoary has been reported. The effectiveness of essential oils is not always attributed to one oil but some previous studies have reported that the combination of essential oils caused synergistic inhibitory effect against several microorganisms (Suree, 2011). Cinnamon oil contains benzoic acid, benzaldehyde and cinnamic acid of which the lipophytic moiety of these compounds has been recognized as responsible for its antimicrobial property (Ramos-Nino et al., 1996, Gupta et al., 2008). The results of the antibacterial activity revealed that the essential oil of cinnamon showed high antibacterial activity against gram positive and gram negative organism tested in this study. The results in this study showed contradiction with Bowels et al. (1995), who recorded that *S.aureus* was highly sensitive to cinnamon oil. Babu et al (2011) found out that antibacterial activity of cinnamon oil was most active against *S.aureus* followed by
E.coli and C.jejuni. Prabhuseenivasan et al, (2006) recorded that P.aeruginosa was more sensitive to Cinnamon oil whereas S.aureus and K.pneumoniae was less sensitive to cinnamon oil (Ali .2011). Our results show that A.niger was most sensitive to cinnamon oil followed by C.albicans> E.coli> S.aureus> P.aeruginosa. These results showed that essential oil of cinnamon is most powerful against the test organism as compared to ethanolic and methanolic extracts.

Among various natural antimicrobial agents, nisin which is a polypeptide produced by Lactococcus lactis subsp. lactis has been most widely studied for its activities. It is produced by lactic acid bacteria, which are often found in the digestive tract of man and is recognized as a probiotic (beneficial microorganism). Nisin is effective against a wide range of spoilage and pathogenic gram-positive bacteria. Many recent studies reported that nisin inhibited growth of gram-positive bacteria, such as Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Lactobacillus plantarum, Micrococcus luteus, Micrococcus flavus and Brochothrix thermosphaeta. Our results also showed that nisin inhibited the gram-positive bacteria, S.aureus corresponding to previous studies (Panitee et al., 2007). However past research indicates that nisin is ineffective against gram negative organisms, yeasts and moulds. Nisin is a well known broad spectrum bacteriocin, active against gram positive organisms associated with foods. Its use as a food biopreservative is limited by the lack of effect against gram negative bacteria and moreover the development of nisin resistance has been reported in sensitive gram positive organisms (Ming and Daeschel,1993). The combination of bacteriocins with other preservative mechanisms has been reported to reduce the selection for resistance to bacteriocins in target strains or to extend its inhibitory activity to gram negative species (Stevens et al.,1991). Recent studies have shown that the spectrum of activity of nisin may also be extended to gram negative bacteria by using it in combination with other agents. Many reports have been published on the synergistic antimicrobial effects of nisin with sucrose fatty acid ester, the lactoperoxidase system, thymol and carbondioxide . It was also found that garlic shoot juice with various combinations of nisin has a great influence as an antilisterial effect in whole ,low fat and skimmed milk (Eun et al., 2008). Therefore this study aimed to study the synergistic effect of Nisin with Cinnamon and Curcuma longa against cosmetic degrading microbes ,as a potential preservative in cosmetic industry. However it was seen that combination of Nisin and Curcuma longa extracts showed no inhibitory activity against cosmetic degrading microbes. Moreover Cinnamon and Turmeric combination also showed no inhibitory activity against the test
microbes. Thereby only nisin and cinnamon in combination were studied. MIC Values obtained for Cinnamon extracts against the test microbes were tried in various permutations and combinations with varying concentrations of nisin. It was observed that Cinnamon methanolic extract and Cinnamon ethanolic extract in combination with nisin showed additive effect whereas Cinnamon oil and nisin showed synergism. The results of the MIC showed that Cinnamon is potent against test organism at low concentrations when used separately and when used synergistically with nisin. Combined antimicrobials are preferred as microbial tolerance is likely to develop against substances having more than one type of modes of action (Gutierrez et al., 2008). Combinations of spices have demonstrated synergistic or additive effects on microorganisms and showed lower FICs in literature review. Synergistic or additive effects support the use of these spices in combination instead of use in isolation. According to Cain et al. (2003) synergistic activity suggests different modes of action of the combining compounds. None of the combinations showed antagonistic effect. Toroglu (2011) showed how some spice essential oils showed synergistic activities with antibiotics. The effective spice combinations may be engaged in cosmetic preservation and may lead to new choices for antimicrobial agents (Das et al., 2012).

4.3 Cytotoxicity study

Nisin producing lactococci occur naturally in raw milk and cheese. Inadvertently and apparently harmlessly, humans and animals probably have consumed this bacteriocin for centuries. Toxicological studies by many groups have now confirmed that nisin is not toxic at levels much higher than those used in food (Naidu, 2000). Nisin is the only bacteriocin produced by lactococcus species which is used as a food preservative in 45 countries and is generally recognized as safe. It is an antimicrobial peptide with extensive action against microorganisms. Some works suggest nisin could be therapeutically used. For this reason, developing cytotoxicity assays are necessary and important to aid nisin assays for future therapeutic application. Fibroblasts are sensitive to toxic substances and, therefore, used as a standard cell line to cytotoxicity. MTT results showed values above 70% of cells viability after 24 and 48 hours, indicating that nisin has no toxic potential for this cell line in the concentrations assayed. Nisin at pH 4.5 was not toxic to cells, allowing further studies as a potential application for biomaterials (Jozala, Angela, 2012).
Cinnamon oil is commonly used in aromatherapy as a rub to promote blood circulation. It is also used as an external poultice to treat minor bacterial and fungal infections of the skin (Tisserand, 1995). Cinnamon essential oil should not be ingested, due to its potential toxicity (Hoskins, 1984). Its abuse has led to intoxication, especially in children and adolescents (Perry et al., 1990). Some people may be hypersensitive to the essential oil used topically to treat skin infections or as a rubefacient in aromatherapy. Undiluted essential oil should not be applied topically (Skidmore-Roth, 2003; Tisserand and Balacs, 1995). Cinnamon constituents may be irritating to the oral mucous membranes (Stuart, 2005). Cinnamon is used as a spice in food material in Asia so its safety is quite obvious. Budavari et al. have reported acute toxicity of Cinnamon in the animals is very low i.e. Benzaldehyde (LD50 orally, 1300 mg/kg rat), cinnamaldehyde (LD50 orally, 2220 mg/kg rat), linalool (LD50 orally, 2790 mg/kg rat), and salicylaldehyde (LD50 orally, 520 mg/kg rat) 17. Satoshi found that its toxigenicity is low so utilization of this compound may be expected as an antifungal agent in foods and as a treatment of dermatomycosis. Cytotoxicity of cinnamon oil was tested on normal fibroblast cell lines (F2408) and ras active (5RP7) cell lines. The cytotoxicity of the oil was quite strong with IC50 values less than 20 μg/mL for both cell lines. 5RP7 cells were affected more than normal cells. The study showed the potential antimicrobial and anti carcinogenic properties of the essential oil of cinnamon bark, indicating the possibilities of its potential use in the formula of natural remedies for the topical treatment of infections and neoplasms (Unlu et al., 2010).

Nisin acts against Gram positive and also Gram negative bacteria in food with combination of other antimicrobial factors or essential oils extracts of plants. Antibacterial effects of nisin together with cinnamon and oregano essential oil have been studied on the quality of fish and meat products (Govaris et al., 2010; Lu et al., 2010). Yuste and Fung (2003) studied the combined antimicrobial activity of nisin and cinnamon on E. coli O157:H7 in apple juice. Nisin alone was ineffective on Salmonella typhimurium. (Masoud, 2012). The combination of nisin and cinnamon accelerates death of Salmonella typhimurium and E. coli O157:H7 in apple juice and so enhances the safety of the product.

Various studies revealed that nisin and cinnamon are non cytotoxic to humans and are potentially used as a food preservative worldwide. But some studies revealed cinnamon oil to be toxic. However, since our study is based on use of nisin and cinnamon in cosmetology as a preservative we evaluated the cytotoxicity of this best synergistic
antimicrobial combination. Cytotoxicity of this combination was studied on 3T3 Murine fibroblast cell lines. *In vitro* cytotoxicity is necessary to define basal cytotoxicity such as the intrinsic ability of a compound to cause cell death as a result of damage to several cellular functions. This assay is also necessary to define the concentration range for further *in vitro* testing to provide information on parameters such as genotoxicity or programmed cell death (Bouaziz et al., 2006). The effect of chemicals on the capability of cells to replicate is used as an index of toxicity. The concentration of the substances at which 50 per cent of the cells desired activity is inhibited is called the IC$_{50}$ value. Result obtained showed IC$_{50}$ value around 0.42% (42mg/ml). This indicates that above this value the combination is cytotoxic to the fibroblast cells. This could be due to the resultant effect of the plant extract and bacteriocin. Other possibility might be the use of the solvent to dissolve the mixture. In many cases, the selectivity of action of these agents depends mainly on the fact that normal proliferating tissues have distinctive physiological or biochemical characteristics that affect drug actions (Ekwall et al., 1990). It is noted that the concentration of preservative used in cosmetics is lower than the IC$_{50}$ value obtained. Thereby it can be inferred that the synergistic combination may prove to be noncytotoxic at the concentration prescribed for standard preservatives in cosmetics.

### 4.4 Efficacy Study

The fundamental principle of the microbial challenge is based on the concept of measuring the survival ability of selected microorganisms that are purposely introduced into a preserved test product system. Conventional preservative efficacy tests or preservative challenge test methods generally require microbial assays at multiple test points over extended periods of time. Test durations typically range from a minimum of 28 days to 12 or more weeks.

The prolonged four-week test cycle originally evolved via the United States Pharmacopeia (USP) for application in the pharmaceutical industry. It has since been adopted in one form or another for evaluating the efficacy of preservatives in cosmetic and other consumer product formulations. Our studies performed the inoculation test for the synergistic combination. This 28 day challenge test showed positive results. It was observed that all organisms showed 10 log reduction from 0 day to 7th day. This reduction in microbial load was observed till 28th day. However, positive control which was set with Standard preservative Parabens showed initial decline in the microbial growth but later on increase in the microbial load was seen till 28th day. Negative control which was
set without preservative, initially showed consistent microbial load till 14\textsuperscript{th} day but then soon afterwards gradual increase in microbial concentration was noted till 28\textsuperscript{th} day. The preservative is considered to be effective in the product examined if:

✓ The concentration of the viable bacteria are not more than 0.1\% of the initial concentrations by the 14\textsuperscript{th} day

✓ The concentrations of viable yeasts and moulds remain at or below the initial concentration during the first 14 days and

✓ The concentration of each test organism remains at or below these designated levels during the remainder of the 28 day test period. (IP 2007).

Since our combination proved to surpass the criteria of microbial challenge test, the blend may be used as an preservative in cosmetic formulation.

Thus, in order to prevent the contamination that can occur during production, manufacturers are required to manufacture products in compliance with wholesome manufacturing practices and, considering consumer health, it is necessary to add an effective preservative as determined by regulations. The market requirements for natural preservatives are continuously growing worldwide because of the awareness among the consumers regarding the problems associated with synthetic preservatives after long term use. In response to the trend of using natural ingredients in cosmetics & personal care, manufacturers are seeking natural ingredients for preservation.

This invention here by relates to the preservation of cosmetics compositions, which contain a dermatologically acceptable natural combination, against five microorganisms, namely \textit{Escherichia coli} form, \textit{Staphylococcus aureus}, \textit{Pseudomonas aeruginosa}, \textit{Candida albicans} and \textit{Aspergillus niger}. For years research has been conducted in an effort to produce a cosmetic preparation that possesses microbicidal properties, an antimicrobial cosmetic composition. Cosmetics have been formulated with a variety of bactericides which are effective against above five organisms mentioned. Such bactericides are generally synthetic compounds such as Methyl or Propyl Paraben, Dowicil 200 or various Quaternary compounds. Care must be taken that the antimicrobial agents are nontoxic and do not irritate the skin to which the cosmetic is applied. Hence, the antimicrobial composition of this invention comprises of a dermatologically acceptable vehicle or a carrier and as a preservative or antimicrobial agent, an effective amount of the two natural compounds, nisin and cinnamon oil, was found to be active against all five organisms noted above. In consequence the two compounds specified are safe, mild, and efficient preservative agents compatible with conventional, known cosmetic ingredients.