

FORMULATION AND FUNCTIONAL ANALYSIS OF SYNTHETIC FBS FOR ANIMAL CELL CULTURE

Submitted in partial fulfilment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research

By

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**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms.SUMIYA G (42101005)** who carried out project entitled "**FORMULATION AND FUNCTIONAL ANALYSIS OF SYNTHETIC FBS FOR ANIMAL CELL CULTURE**" under the supervision of **Dr.S.JOHNSON RETNARAJ SAMUEL**, Associate professor from September 2023 to April 2024.

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DECLARATION

I, **SUMIYA G (42101005)** hereby declare that the Project Report entitled **"FORMULATION AND FUNCTIONAL ANALYSIS OF SYNTHETIC FBS FOR ANIMAL CELL CULTURE"** done by me under the guidance of **Dr.S.JOHNSON RETNARAJ SAMUEL, Associate Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

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ABSTRACT

Animal cell culture techniques has gained an exponentially significant role in basic and applied biomedical research. Fetal Bovine Serum (FBS) is an inevitable supplement to the basal animal cell culture media. It is essential for the cell attachment, metabolism, growth, proliferation, and differentiation of the cells in vitro. There are several ethical, geographical, scientific and commercial issues revolve around the usage of FBS. Alternatives to animal serum supplementation such as serum alternatives, serum free media and xeno free media has been developed but limitations such as high cost, limited availability, inability to support various cell lines and potential batch to batch variation exists. In our present study, we have developed a synthetic FBS that can aid the attachment, growth and proliferation of C2C12 cell lines. This synthetic FBS consists definite concentrations of different chemical components notably insulin, transferrin and selenium. We have tested phyto-proteins of soya and groundnut milk, in which the groundnut milk supports the overall culture of the cell line at 1.19µg/µl concentration. This synthetic FBS has been shown to be a better alternative to conventional FBS, in accordance with cost-effectiveness, compliance with the 3R's (refine, reduce, and replace), lack of unintended antibody interactions, presence of macro and micronutrients, simple preparation, and availability. Our results concludes that low Concentration of ground nut milk proliferates the growth and attachment of the cells.

Key words : Fetal bovine seurum, Synthetic FBS, Groundnut milk,C2C12 line,FBS alternative.

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LIST OF ABBREVIATIONS

DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
BSA	Bovine serum albumin
TGF	Transforming growth factors
IGF	Insulin-like growth factors
PDGF	Platelet-derived growth factors
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RPMI	Roswell Park Memorial Institute
BSE	Bovine spongiform encephalopathy
SFM	Serum Free media
POD	Peroxidase
GOD	Glucose oxidase
CHOD	Cholesterol oxidase
CHE	Cholesterol Esterase
4AAP	4-aminoantipyrine
PBS	Phosphate buffered saline

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CHAPTER -1

INTRODUCTION

1.1 ANIMAL CELL CULTURE

Animal cell culture refers to the process of growing animal cells in vitro, outside their natural environment, typically in a laboratory setting. This technique is widely used in various fields of biological research, pharmaceutical development, and biotechnology for studying cell physiology, disease mechanisms, drug screening, and the production of biological products. cell culture was first successfully undertaken by Ross harrison in 1907. Animal cell culture is described as the in vitro maintenance and proliferation of animal cells that will continue to grow outside the living organisms if supplied with appropriate nutrients and growth conditions. The process of growing cells under laboratory conditions is termed as Cell Culture. It is carried out in vitro (within glass) as opposed to in vivo (within the living). It deals with the isolation of cells from animal tissue, surgical intervention for removal of tissues or organs from animal and their placement into an environment (media) in order to enhance their survival and proliferation (Fang et al.,2017).

1.1.1 IMPORTANCE OF ANIMAL CELL CULTURE

Animal cell culture is a process of growing animal cells outside of their natural environment. Animal cell culture is important because it can be used to explore disease mechanisms, cellular and molecular processes, and medication discovery. (Fang *et al.*,2017). The study of physiological and biochemical processes in the lab now requires the use of animal cell culture. An essential substitute for the use of homogenized animal tissue in these investigations is the production of homogeneous cell populations by carefully managed development over a number of generations. Biological laboratories can now routinely perform the procedure with minimum equipment, however in the past, these cultures were thought to be difficult to set up and maintain without contamination (Michael and Flickinger *et al.*,2010).

Since mammalian cell cultures are now the systems of choice for large-scale manufacturing operations for required proteins, mostly in the healthcare industry, animal cell technology has grown in importance. Animal cell culture is widely used

in biomedical research for studying diseases, testing drugs, and understanding basic cellular mechanisms. It also plays a crucial role in biotechnology and pharmaceutical industries for producing vaccines, therapeutic proteins, and monoclonal antibodies. (Oh SKW *et al.*,1993).

1.1.2 TYPES OF CELLS IN ANIMAL CELL CULTURE

There are three types of cells: primary cells, secondary cells, and continuous cell lines cultured in invitro (Hu W-S *et al.*,1997).

1.1.2.1 PRIMARY CELLS

Culture of cells dissociated from the parental tissue (such as kidney, skin, or liver). Most of the primary culture cells have a finite lifespan of 5-20 divisions in vitro. Due to their limited life span, one cannot do long-term experiments with these cells. The primary cell culture could be of two types depending upon the kind of cells in the culture (Van Der Valk *et al.*,2010)

ADHERENT CELLS

Adherent cells, also called anchorage-dependent cells, are grown in a cell culture medium while attached to the bottom of a tissue culture flask. Cells are dissociated enzymatically or mechanically. Growth is limited by surface area, which may limit products yields. Used for cytology, harvesting products continuously, and many research applications.

SUSPENSION CELLS

Appropriate for cells adapted to suspension culture and a few other cell lines that are non-adhesive (eg. Blood cells). Does not require enzymatic or mechanical for dissociation. Growth is limited by the concentration of cells in the medium. Used for bulk protein production, batch harvesting, and many research applications.

1.1.2.2 SECONDARY CELLS

When a primary culture is sub-cultured, it becomes secondary culture. Subculture (or passage) refers to the transfer of cells from one culture vessel to another culture vessel. This is periodically required to provide fresh nutrients and growing space for continuously growing space for continuously growing cell lines. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically). Such cultures are called secondary cultures.

1.1.3 CELL LINES

A cell line is a permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space. Lines differ from cell strains in that they become immortalized (Chu L et al.,2001).

1.1.4 FINITE AND CONTINUOUS CELL LINES

Normal cells usually divide only a restricted number of times before losing their ability to proliferate, which is genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur naturally or can be chemically or virally induced. When a finite cell line undergoes a transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line (Trinder et al., 1999).

1.1.5 FBS (FETAL BOVINE SERUM)

Fetal bovine serum (FBS) is a common supplement used in animal cell culture media to provide essential nutrients, growth factors, and hormones necessary for cell proliferation and maintenance. Derived from the blood of bovine fetuses, FBS is rich in various components such as proteins, lipids, vitamins, minerals (Freshney et al.,2010).

FBS is a commonly utilized growth supplement in the in vitro culture of animal and human cells, tissues, and organs. Over the years, increasing demand, high price, batch-to-batch variability in quality and composition, increasing ethical concerns lead to the search for an alternative to FBS. Although other strategies have been proposed and used, none are as popular as FBS, and each supplement has drawbacks of its own. We outlined the significance of FBS in cell culture, talked about the problems with its use, and showcased the recent attempts to replace or minimize FBS in this review. The possible combination of four distinct. Although other strategies have been proposed and used, none are as popular as FBS, and each supplement has drawbacks of its own (Agostini *et al.*, 2017)

CHAPTER – 2

LITERATURE SURVEY

2.1 HISTORY OF ANIMAL CELL CULTURE

The influence of cell culture technology on human society has been immeasurable. Progress in biology in recent years. The development of antibiotics and aseptic techniques in the mid-20th century facilitated the establishment of cell lines and primary cultures from various animal tissues. In most circumstances, the addition of proper cell development requires baseline culture conditions containing animal-derived products, particularly serum, is essential in cell culture for proper cell growth (Fang *et al.*, 2017). The major functions of serum in culture media include providing Cells require hormones for growth and differentiation, transport proteins, necessary nutrients, trace elements, adhesion and extension factors, as well as stabilizing and detoxifying substances to maintain a suitable growth environment (Fang *et al.*, 2017). In animal cell culture a lot of amino acids, vitamins and other inorganic metals trace elements.

2.2 ROLE OF FBS IN ANIMAL CELL CULTURE

The use of FBS as a supplement to supply vital nutrients, growth factors, hormones, and proteins required for cell growth and proliferation is critical in the culture of animal cells. When pregnant cows are slaughtered, the blood of the fetus is taken and used to make foetal bovine serum (FBS). Its rich content, comprising lipids, vitamins, minerals, amino acids, and different growth factors like transforming growth factors (TGFs), insulin-like growth factors (IGFs), and platelet-derived growth factors (PDGFs), makes it a popular choice for cell culture. These constituents facilitate the adhesion, spreading, and proliferation of cells, thereby aiding in the preservation of cell viability and functionality in vitro. (Agostini *et al.*, 2017).

FBS has been the most often utilized cell culture supplement. FBS includes less antibodies and more growth factors than calf and adult bovine serum, enabling for the multiplication of the majority of human and animal cells. Although other synthetic serum-free media have been produced, many researchers continue to rely on FBS. Since it performs well in cell culture systems. The supplementation of Fetal Bovine Serum in animal cell culture is inevitable for the growth and proliferation of

the cells (Melinda Grace Rossan Mathews *et al.*, 2024). Fetal bovine serum plays a crucial role in animal cell culture as it provides essential nutrients, growth factors, hormones, and proteins necessary for the growth and proliferation of cells. FBS contains a variety of components such as amino acids, vitamins, minerals, lipids, and carbohydrates that support cell growth and metabolism. The growth factors present in FBS help stimulate cell division and promote cell survival. Additionally, FBS acts as a buffer to help maintain the pH of the cell culture medium and provides a source of attachment factors that help cells adhere to the culture surface. Overall, FBS is an important supplement in animal cell culture as it helps create an optimal environment for cell growth, proliferation, and maintenance. Its use is common in research laboratories and biotechnology companies to support the growth of various cell types for experiments and production purposes (Freshney *et al.*, 2010). Additionally, FBS contains growth factors such as insulin-like growth factor (IGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) that stimulate cell proliferation and differentiation. These growth factors play a crucial role in regulating cell cycle progression and promoting cell survival. (Agostini *et al.*, 2017).

2.2.1 History of FBS use in cell culture

Ringer's cell culture medium was developed by Sydney Ringer in 1882 and was the first instance of *in vitro* cultivation of animal tissue. Over the next few decades, scientific innovation led to the development of the first animal cell cultures, then synthetic media. The 1940s saw the establishment of cell lines, basal media, and research into protein-free media (Freshney *et al.*, 2010).

In the late 1950s, Theodore Puck first introduced the use of FBS in cell culture with the purpose of encouraging cellular proliferation. Additional studies revealed that, in addition to its ability to stimulate cell proliferation, FBS also contained a multitude of components essential to cell growth and viability (e.g., hormones, vitamins, growth factors, etc.).

For several decades, the use of fetal bovine serum has been ubiquitous across laboratories worldwide its popularity has continued to lay in its indispensability as a supplement in cell and tissue culture applications.

2.2.2 Composition of FBS (FETAL BOVINE SERUM)

FBS sourced from bovine fetuses contains more than 1000 components such as growth factors, hormones and transport proteins that contribute to cell growth when supplemented into cell culture. FBS provides a rich culture system known to support the widest range of cell types, including both cell lines and primary cells.

Proteins

FBS contains a variety of proteins such as albumin, transferrin, and immunoglobulins. These proteins provide essential amino acids and other nutrients necessary for cell growth (Barnes et al., 2010).

Lipids

FBS contains lipids such as fatty acids, cholesterol, and phospholipids, which are important for cell membrane formation and function (Freshney *et al.*, 2010).

Carbohydrates

Sugars and other carbohydrates in FBS serve as an energy source for cells.

Growth factors

FBS contains growth factors such as insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), which stimulate cell proliferation and differentiation (Sunstrom et al., 2000).

Hormones

Hormones like insulin and glucocorticoids are present in FBS and play roles in regulating cellular metabolism and other processes (Butler *et al.*, 2018).

MINERALS AND TRACE ELEMENTS

FBS contains a variety of proteins such as albumin, transferrin, and immunoglobulins. These proteins provide essential amino acids and other nutrients necessary for cell growth (Barnes et al., 2010).

Vitamins

FBS provides vitamins like B vitamins (e.g., B12, folic acid), vitamin C, and vitamin E, which are cofactors for many enzymatic reactions involved in cell metabolism (Ames *et al.*, 1998).

Buffering agents

FBS may contain buffering agents like bicarbonate, HEPES, or phosphate buffers to maintain the pH of the cell culture medium.

Antibiotics/Antimycotics

Some FBS preparations may be supplemented with antibiotics or antimycotics to prevent contamination by bacteria, fungi, or other microorganisms.

2.2.3 FBS DISADVANTAGES

Fetal bovine serum is depended upon by investigators as an indispensable supplement in cell and tissue culture systems. Due to increased demand and limited availability, the price of FBS has increased by more than 300% in the past few years. In addition, there are ethical and scientific controversies about the collection and use of FBS in culture systems. Although there are some disadvantages listed below

1. Unpredictable shortage in the supply of FBS
2. Unintended interactions with test substances
3. Ethical concerns with animal welfare.
4. Components of ill defined.
5. Increasing the price of FBS (it was increased above 300% in the last few years as of 2016).
6. It can be contaminated with prion proteins, endotoxins, different kinds of microbes, immunoglobulin and viruses. (RMBIO *et al.*, 2016).
7. Fetal distress during blood collection to prepare FBS leads to high anger among animal welfare and ethical association around the world (Skardal *et al.*, 2018).

8. The variation between the brands seasonal (winter and spring) and geographical batch-to-batch variation in the quality (variation in composition, possible contamination specific to season and geographic) of FBS questioning the experimental reproducibility (Panchision *et al.*,2009).

9. The presence of non-human N-glycolylneuraminic acid (Neu5Gc) in the animal serum may promote chronic inflammation and influence tumor progression and vascular inflammation (Taylor R.E *et al.*, 2010), which should have been excluding the usage of FBS in human embryonic stem cell (ES) for cellular therapy (Martin M.J *et al.*, 2005) yet, it is widely used (Macauley *et al.*,2011).

2.3 INTRODUCTION TO SYNTHETIC MEDIA

Animal cell culture has advanced significantly with the use of synthetic media, which provide formulations free of animal-derived ingredients. These media are engineered to offer exact control over the ratio of nutrients, growth factors, and other vital elements required for the development and multiplication of cells. Examples that are frequently used are RPMI 1640 and DMEM (Dulbecco's Modified Eagle Medium), which have specific combinations of vitamins, salts, amino acids, and glucose. Their advantages, which include reduced batch-to-batch variance, better-defined culture conditions, and reproducibility, have led to their widespread usage in the cultivation of diverse mammalian cell lines. Particularly, the use of synthetic media decreases the possibility of contamination with adventitious agents and addresses ethical concerns regarding goods originating from animals, which makes them indispensable for bioprocessing and research applications (Tangvoranuntakul *et al.*,2003).

A chemically defined media, sometimes referred to as a synthetic medium, is a growth medium whose chemical composition is known and that is appropriate for the in vitro cultivation of human or animal cells. Standard cell culture media typically comprise a base medium enhanced with animal serum (FBS) to provide nutrients and additional unidentified components. The indeterminate character of serum, the compositional heterogeneity from batch to batch, and the possibility of contamination are its technical drawbacks.

Serum-based media and chemically defined media are clearly distinguished from one another unspecified animal-derived products, including hydrolysates, growth

factors, hormones, carrier proteins, and attachment factors, may be included in serum-based medium. Complex contaminants, like albumin's lipid content, will be present in these unidentified animal-derived products. Conversely, chemically defined media demand that each component be precisely recognized and its concentrations be understood. Fetal bovine serum, cow serum, or human serum cannot be present in a chemically defined medium since it is completely devoid of substances produced from animals. Recombinant forms of albumin and growth factors, typically generated from rice or *E. coli*, or synthetic chemicals like the polymer polyvinyl are frequently added to chemically specified media in order to do this (Brindley *et al.*, 1994).

2.3.1 CLASSES OF MEDIA

Animal culture media can be divided into six subsets based on the level of defined media (Jayme and Smith, 2000).

- Serum-containing media (commonly 10-20% FBS).
- Reduced-serum media (commonly 1-5% FBS).
- Serum-free media (synonymous with Defined media).
- Protein-free media (no protein but contains undefined peptides from plant hydrolysates).
- Chemically-defined media (with only recombinant proteins and/or hormones).
- Protein-free, chemically defined media (contains only low molecular weight constituents, but can contain synthetic peptides/hormones)
- Peptide-free, protein-free chemically defined media (contains only low molecular weight constituents).

2.3.2 SERUM FREE MEDIA

An important development in animal cell culture is the use of serum-free media, which provide formulations free of serum-derived ingredients. Because it contains a wide range of growth factors, hormones, and nutrients necessary for cell development and proliferation, (FBS) has been used extensively in cell culture for a long time. Serum-free alternatives have been developed, nonetheless, due to issues

like contamination risk, ethical issues with animal-derived goods, and batch variability (Mizutani *et al.*,2018).

Serum-free media are made with specific, synthetic, or recombinant components that are carefully combined to supply all the nutrients, growth factors, vitamins, and minerals needed for cell growth. These media have a number of benefits over serum-containing media, such as improved specified culture conditions, decreased batch-to-batch fluctuation, and increased reproducibility. Serum-free medium also reduce contamination risk and ease ethical issues related to using items obtained from animals (Griffiths *et al.*,2006). From fundamental research to the production of bioprocesses, the use of serum-free media has transformed several facets of animal cell culture. Greater consistency, control, and scalability are now possible with a greater variety of culture methods available to researchers and biotechnologists. The significant progress in biomedical sciences can be attributed to the use of serum-free media in cell treatment, regenerative medicine, vaccine production, and biopharmaceutical manufacturing.

2.3.3 ROLE OF SERUM FREE MEDIA IN CELL CULTURE

Serum provides

- Growth factors and hormones
- Binding and transport proteins
- Attachment and spreading factors
- Additional amino acids, vitamins and trace elements
- Fatty acids and lipids
- Protease- inhibitors
- Detoxification (due to binding and inactivation)
- (Colloid) osmotic pressure (Hartung et al., 2002).

2.3.4 ADVANTAGES OF SERUM FREE MEDIA

In addition, there are ethical and scientific controversies about the collection and use of FBS in culture systems. Although there are some disadvantages listed below.

- Low protein content, which can hinder product purification.

- Elimination of the risk of contaminants-viruses, mycoplasma, prions from animal-derived products which may be transmitted to the end product used by humans, e.g., bovine spongiform encephalopathy (BSE) or mad cow disease.
- Elimination of factors that may interfere with hormones or growth factors when studying their interaction with cells.
- Removal of concerns regarding the limited availability of fetal bovine serum, with periods of world shortages.
- Reduced cost, fetal calf serum can account for up to 85% of the overall cost of the medium when calculated for large-scale cultures.
- There are increasing concerns about animal suffering inflicted during serum collection that add an ethical imperative to move away from the use of serum wherever possible.
- Batch to batch variation.

2.3.5 APPLICATIONS OF SERUM FREE MEDIA

- **Recombinant Protein Production**

SFM are widely used for the expression of recombinant proteins in animal cell culture systems, ensuring high yields and consistent product quality.

- **Biopharmaceutical Manufacturing**

- SFM play a crucial role in the development and production of therapeutic biologics such as monoclonal antibodies, vaccines, and cell-based therapies, meeting stringent regulatory requirements for product safety and consistency.

- **Cell Biology Research**

SFM facilitate precise control over cellular behaviour and signalling pathways, enabling researchers to study complex cellular processes with greater accuracy and reproducibility.

- **Stem Cell Culture**

SFM provide a defined environment for the maintenance and differentiation of stem cells, supporting research into regenerative medicine and developmental biology.

- **Virus Production**

SFM are utilized for the production of viral vectors and vaccines, ensuring the purity and safety of viral preparations for gene therapy and vaccine development.

- **Drug Screening**

SFM enable reliable and reproducible drug screening assays by eliminating the variability associated with serum-derived factors, allowing for more accurate assessment of drug efficacy and toxicity.

- **Tissue Engineering**

SFM are used in the culture of cells for tissue engineering applications, providing a controlled environment conducive to tissue formation and regeneration (Barnes & Sato 1980).

- **Transfection and Gene Expression Studies**

SFM support efficient transfection and gene expression studies in animal cell culture, facilitating research into gene function and regulation.

- **In vitro Diagnostics**

SFM are employed in the production of cell-based assays and diagnostic tests, ensuring consistency and reliability in diagnostic applications.

- **Animal Cell Line Adaptation**

SFM facilitate the adaptation of animal cell lines to serum-free conditions, enabling the development of cell lines suitable for specific applications and reducing reliance on serum supplementation.

2.4 INTRODUCTION TO PLANT BASED PROTEIN SOURCES

Plant-based protein refers to protein sources that come from plants, such as legumes (e.g. lentils, chickpeas, black beans), nuts and seeds. It acts as a growth factor supplement for in-vitro cell culture. Plant proteins are highly nutritious – not only as good sources of protein, but also because they provide other nutrients such as fibre, vitamins and minerals (Zhang *et al.*,2008). These protein sources are often used as alternatives to animal-based protein sources. Plant-based proteins are valued for their nutritional benefits, environmental sustainability, and potential health advantages, including being typically lower in saturated fat and cholesterol compared to animal based proteins (Nagaoka *et al.*,2019).

2.5 INTRODUCTION TO SOYABEAN MILK

Soyabean

Soybeans are highly valued for their rich protein content. They are an excellent source of plant-based protein, containing all nine essential amino acids required by the human body. Additionally, they are rich in fiber, vitamins (such as folate, vitamin K, and vitamin C), minerals (including iron, calcium, magnesium, and potassium), and phytonutrients. Soybeans, also known as soya beans, are one of the most versatile and widely consumed legumes worldwide (Nielsen *et al.*,2016) They are commonly processed into various products, including tofu, soy milk, tempeh, miso, soy sauce, and soyabean oil.

Soyabean milk

- Soya bean milk is also known as soy milk.
- It is a plant based protein source.
- It is rich in proteins, vitamins and minerals.
- It is a cost effective compared to BSA.
- By replacing BSA with soya milk researchers can reduce reliance on animal derived products aligning with principles of animal welfare and ethical research practices.
- It reduces the risk of contamination.
- The pH value of soy milk varied between 6.41 and 7.34.
- Its viscosity is measured between 16.5 and 26.4mPa.

2.6 INTRODUCTION TO GROUNDNUT MILK

Groundnut seeds

Groundnut seeds, also known as peanuts, are a popular legume crop that is widely grown in many parts of the world. They are an important source of rich protein. Groundnut seeds are highly nutritious, containing protein, healthy fats (monounsaturated and polyunsaturated), dietary fiber, vitamins (such as niacin, folate, and vitamin E), minerals (including magnesium, phosphorus, potassium, and zinc), and antioxidants (such as resveratrol) (Rutherford *et al.*,2010)

GROUND NUT MILK

- Groundnut milk is high in fat and protein compared to other plant based milk.
- It is a rich source of micro nutrients.
- Groundnut is high in magnesium, vitamins and proteins.
- It can support growth and proliferation of animal cells.
- It's a cost effective compared to BSA and reduce the microbial contamination in cell culture.
- The pH value of Groundnut milk varied between 6.25 to 6.9.

CHAPTER - 3

AIM AND OBJECTIVE

3.1 AIM

- Formulation of synthetic FBS and analysing its efficiency in animal cell culture.

3.2 OBJECTIVES

- To formulate the synthetic FBS.
- To standardize their concentration in accordance with their efficiency of cell growth.
- To study the parameters like shelf life, storage, pH, colour and sterility of synthetic FBS.
- To check the synthetic FBS with different cell types.
- To functionally characterization of synthetic FBS on cell attachment and proliferation.

CHAPTER-4

MATERIALS AND METHODS

4.1 SYNTHETIC MEDIA

Synthetic media or Artificial media Composed of a basal medium and supplements, such as serum, growth factors and hormones The quality of serum varies from batch to batch and deteriorates within one year. Particularly, the use of synthetic media decreases the possibility of contamination. Synthetic media in animal cell culture refers to laboratory-designed formulations of nutrients, growth factors, and other essential components necessary for the growth and maintenance of animal cells outside of their natural environment. synthetic media offer a controlled and reproducible environment for cell culture experiments and bioprocessing applications. This allows researchers and biotechnologists to optimize cell culture conditions, improve experimental reproducibility, and develop innovative therapies and bioproducts with greater precision and efficiency.

4.1.1 SYNTHETIC MEDIA FORMULATION (SERUM FREE MEDIA)

- Autoclaved the Milli-Q water in the reagent bottle at 121°C for 15 minutes under 15 psi pressure to ensure sterility.
- After autoclaved, kept UV sterilization of the reagent bottle for further ensure sterility.
- Measured out 20ml of the autoclaved Milli-Q water and aliquot it into two separate 50ml tubes, each containing 10ml.
- First 10ml of aliquoted Milli-Q water was taken and added all the necessary chemical components and different protein source (SOYA MILK \ GROUNDNUT MILK) for a total volume of 20 ml, were dissolved in it.
- After all the components were dissolved, added the remaining 10ml of Milli-Q water to reach a final volume of 20ml.
- Filtered the solution using a 0.22micron syringe filter to remove any remaining particulate matter or contaminants and stored in -4°C.

4.1.2 SYNTHETIC MEDIA FORMULATION

Table: 4.1 Formulation of synthetic FBS from HI-CF data

Pottassium Chloride	6mg
Sodium chloride	186mg
Sodium bicarbonate	52mg
Cholesterol	2.2mg in 1µl of DH ₂ O (3.7µl)
Protein (groundnut milk)	1.19 µg/µl (1µl)
Vitamins	20mg
ITS (Insulin, transferrin, selenium)	(100x) 200µl
Fetuin	200mg

4.2 ALTERNATIVE TO BSA (PROTEIN) ISOLATE FROM PLANT SOURCE

In the present study an attempt was made to find the feasibility of protein as substitute for BSA, which is a single type of protein that has been purified from fetal bovine serum (FBS) serum. Using plants material sources, Some of the examples of plant-based protein sources include legumes like beans, lentils, and peas, nuts and seeds. In present study soyabean and groundnut seeds (rich in protein) were chosen in the form of milk extracted from the seeds. It acts as a growth factor supplement for in-vitro cell culture. Plant proteins are highly nutritious – not only as good sources of protein, but also because they provide other nutrients such as fibre, vitamins and minerals (Franek *et al.*, 2000; Chun *et al.*, 2007). After seen the effect of formulated protein on cell lines.

4.2.1 Preparation of Soyabean milk

- Weighed 30g-35 g of soyabean and soaked in 250 ml of Milli-Q water overnight, approximately 12-15 hours.
- After soaking, grinded the soybean thoroughly to form a homogenous mixture.
- Filtered the soybean mixture twice through a muslin cloth with a pore size of 2 mm to remove larger particles and sediments.

- Aliquoted the filtered soybean extract into sterile 50 ml tubes for further processing and storage.
- Centrifuged the aliquoted soybean extract at 5000 rpm for 5 minutes at 4°C in a cooling centrifuge to separate the supernatant from the pellet.
- Carefully collected the supernatant and discarded the pellet, again repeated the centrifugation process to ensure better separation to avoid debris.
- Filtered the supernatant through oil-absorbing blotting paper to remove any residual oils or lipids or solids.
- Again filtered the supernatant through Whatman filter paper 2 with a pore size of 8µm to further clarify the solution.
- Followed up by filtering the same solution through Whatman filter paper 42 with a pore size of 2.5 µm to remove smaller particulate matter.
- After filtered the solution with vacuum filter through a 0.22micron filter paper to ensure sterility and remove any remaining contaminants.
- Additionally, filtered the solution again through a 0.22 micron syringe filter for extra assurance of sterility.
- Finally, stored the filtered soybean extract in sterile containers at -20°C for long-term storage.

4.2.2 Preparation of groundnut milk

- Weighed 50g of groundnut and soaked in 200ml of Milli-Q water overnight, approximately 12-15 hours.
- After soaking, grinded the groundnut thoroughly to form a homogenous mixture.
- Filtered the groundnut milk mixture twice through a muslin cloth with a pore size of 2mm to remove larger particles and sediments.
- Aliquoted the filtered groundnut extract into sterile 50ml tubes for further processing and storage.
- Centrifuged the aliquoted groundnut extract at 5000rpm for 5 minutes at 4°C in a cooling centrifuge to separate the supernatant from the pellet.
- Carefully collected the supernatant and discarded the pellet, again repeated the centrifugation process to ensure better separation to avoid debris.

- Filtered the supernatant through oil-absorbing blotting paper to remove any residual oil or lipids.
- Again Filtered the supernatant through a whatman filter paper 2 with a pore size of 8µm to further clarify the solution.
- Followed up by filtering the same solution through Whatman filter paper 42 with a pore size of 2.5µm to remove smaller particulate matter.
- After filtered the solution with vacuum filter through a 0.22micron filter paper to ensure sterility and remove any remaining contaminants.
- Additionally, filtered the solution again through a 0.22micron syringe filter for extra assurance of sterility.
- Finally, stored the filtered groundnut extract in sterile containers at -20°C for long-term storage.

4.3 QUANTITATIVE ANALYSIS

4.3.1 ESTIMATION OF PROTEIN BY LOWRY'S METHOD (SOYA MILK & GROUNDNUT MILK)

The Lowry method is a classic and widely used technique for the quantification of proteins in solution. Here's a general procedure for performing protein estimation using the Lowry method.

Aim: To estimate the protein using Lowry's method.

Preparation of working reagents

- **BSA stock solution**

Take 10 mg of BSA in 1ml distilled water.

- **BSA working solution**

Take 20µl from stock solution and 180µl distilled water (make upto 200µl).

REAGENT A

Measured 0.04g of NaOH was taken in 10ml of DH₂O (distilled water) and added 0.2g of sodium carbonate dissolved in it.

REAGENT B

Measured 0.05g pf copper sulphate mixed with 0.1g of potassium sodium tartarate in 10ml of distilled water and dissolved in it.

REAGENT C

Mixed 9.8µl of reagent A with 200µl of reagent B.

FOLIN'S CIOCALTEU REAGENT

Taken equal volume of reagent + DH₂O

(50µl reagent +50µl of DH₂O)

Preparation of BSA Standards

Added DH₂O initially and then BSA working solution.

S1 - 95µl DH₂O + 5µl BSA

S2 - 90µl DH₂O + 10µl BSA

S3 - 80µl DH₂O + 20µl BSA

S4 -70µl DH₂O + 30µl BSA

S5 -60µl DH₂O + 40µl BSA

S6 - 50µl DH₂O + 50µl BSA

Blank - 100µl DH₂O

Procedure

- Added 98µl of DH₂O in the wells and added 2µl sample make volume upto 100µl.
- Added 200µl of reagent C in all the wells.
- Kept incubation for 10 minutes.
- Then added 20 µl of folin's reagent to all the wells and kept in dark for 30 minutes.
- After incubation, measured the absorbance of each sample, standard, and blank at a wavelength of 660 nm.

CALCULATION

- Plotted a standard curve using the absorbance values of the BSA standards against their respective concentrations.
- Determined the protein concentration of the samples by comparing their absorbance values to the standard curve.

4.3.2 ESTIMATION OF CREATININE

Principle

Creatinine reacts with alkaline picrate to produce a reddish color. This is a non-specific reaction and is given by many other substances. Specificity of the assay has been improved by the introduction of a kinetic method ¹, however the cephalosporin antibiotics are still major interferents.

Reaction

Creatinine +  orange coloured complex
Alkaline Picrate

Reagent composition

Reagent 1: Creatinine Buffer Reagent

Sodium hydroxide : >200 mmol/L

Reagent 2: Creatinine Picrate Reagent

Picric Acid : >15 mmol/L

Reagent 3: Creatinine standard : 2 mg / dl

Working reagent reparation

Working Reagent was prepared by combining equal volume of reagent 1 and reagent 2. Mixed by gently swirling. Allowed the reagent mixture to stand at R. T. For 5 minutes for equilibrium. The working Reagent is ready for use.

Procedure

WAVE LENGTH : 505 nm

CUVETTE : 1 cm

Table : 4.1 Quantification for creatinine protocol

Addition Sequence	standard	sample
Working Reagent	100 µl	100 µl
Standard	5 µl	-
Sample	-	5 µl

Mixed and measured the initial absorbance after 30 sec (A₁), started timer simultaneously and read again exactly after 120 sec (A₂). Measured against reagent blank.

Calculate absorbance change $\Delta AT = (A_2 - A_1) / \text{min}$

Calculation

$$\text{Creatinine Concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 2$$

4.3.3 ESTIMATION OF CALCIUM

Clinical significance

Calcium has numerous functions within the body, not only as a structural factor in bones and teeth, but also in normal neuromuscular function and the clotting of blood.

Principle

Arsenazo III combines with calcium ions at pH 6.5 to form a coloured chromophore, the absorbance of which is measured at 650 nm (650-660 nm) and is proportional to calcium concentration.

Arsenazo III has a high affinity ($k^\circ = 1 \times 10^7$) for calcium ions and shows no interference from other cations normally present in serum, plasma or urine.

Reagent Composition

Reagent 1 : Calcium Reagent

Arsenazo III : < 0.1 mmol/l

Buffer : > 50 mmol/l

Reagent 2 : Calcium Standard : 10mg/dl

Procedure

Wavelength : 630 nm

Cuvette : 1 cm

Table 4.2 Quantification for calcium protocol

Additional sequence	Reagent blank	Standard	Sample
Reagent 1	100 µl	100 µl	100 µl
Standard	-	2µl	-
Sample	-	-	2µl
Distilled Water	2 µl	-	-

Mixed and incubated at RT (Room temperature) for 2 min. Measured the absorbance of standard (Abs. S) and test (Abs. T) against reagent blank at 630 nm.

CALCULATION

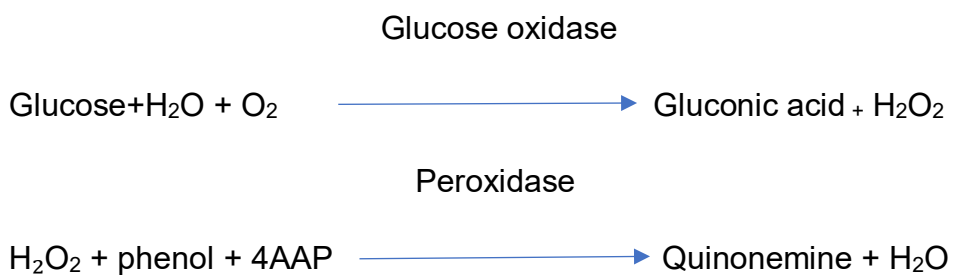
$$\text{Calcium (mg/dl)} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 10$$

4.3.4 ESTIMATION OF GLUCOSE

Principle

Glucose in the sample is oxidised to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4- aminoantipyrine with phenol to yield a coloured quinonemine complex, with absorbance proportional to the concentration of glucose in sample.

Reaction



Reagent composition

Reagent 1 : Glucose Enzyme reagent

Phosphate buffer : >75 mmol/l

Glucose oxidase : > 8500

Peroxidase : > 2000

Phenol : >15 mmol/l

Procedure

Wavelength : 505 nm (493-530)

Cuvette : 1 cm

Table 4.3 Quantification for glucose protocol

Additional Sequence	Reagent Blank	Standard	Sample
Reagent 1	100µl	100µl	100µl
Standard	-	1µl	-
Sample	-	-	1µl
Distilled water	1µl	-	-

Mixed and incubated for 10 min at 37°C. Measured absorbance of the sample Abs. T and standard Abs. S against reagent blank.

CALCULATION

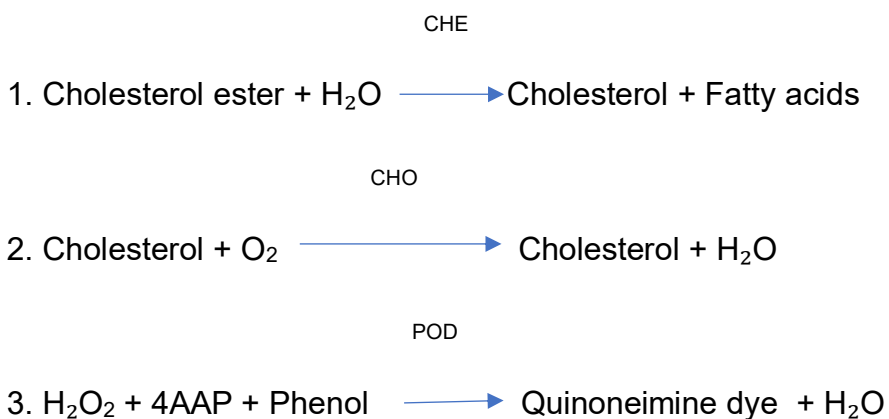
$$\text{Glucose (mg/ml)} = \frac{\text{Abs.T}}{\text{Abs .T}} \times 100$$

4.3.5 ESTIMATION OF CHOLESTEROL

PRINCIPLE

This reagent is based on the formulation of Allain et al and the modification of roeschlau with further improvements to render the reagent stable in solution.

REACTION



Where,

CHE = Cholesterol Esterase

CHO = Cholesterol Oxidase

4AAP = 4-aminoantipyrine

POD = Peroxidase

1. Cholesterol esters are enzymatically hydrolysed by cholesterol esterase cholesterol and free fatty acids.

2.Free cholesterol, including that originally present, then oxidized by Cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide.

3. The hydrogen peroxide combines with 4 aminoantipyrine to form a chromophore (quinoneimine dye) which may be quantitated at 505 nm.

Procedure

Wavelength : 505 nm

Cuvette : 1 cm

Table : 4.4 Quantification for cholesterol protocol

Addition sequence	Reagent blank	Standard	Sample
Reagent 1	100µl	100µl	100µl
Standard	-	1µl	-
Sample	-	-	1µl
Distilled water	1µl	-	-

Mixed and incubated for 5 min at 37°C. Measured absorbance of the sample Abs. T and standard Abs. S against reagent blank. The final color is stable for one hour.

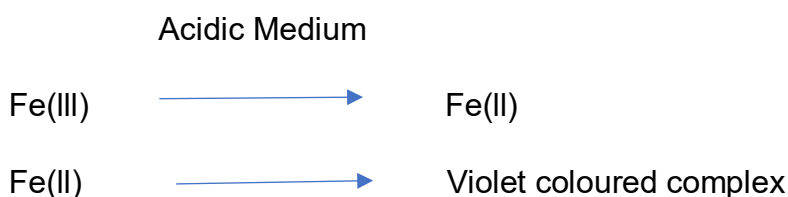
CALCULATION

$$\text{Cholesterol (mg/ml)} = \frac{\text{Abs of T}}{\text{Abs of S}} \times 200$$

4.3.6 ESTIMATION OF IRON

Principle

Iron, bound to Transferrin, is released in an acidic medium and the Ferric ions are reduced to Ferrous ions. The Fe (II) ions react with Ferrozine to form a violet coloured complex. Intensity of the complex formed is directly proportional to the amount of Iron present in the sample.



Procedure

Table : 4.5 Quantification for Iron protocol

Addition sequences	B(ml)	S(ml)	SB(ml)	T(ml)
Iron buffer reagent(L1)	100	100	1.05	100
Distilled water	20	-		-
Sample	-	-	0.2	20
Iron colour reagent (L2)	5	5		5

Mixed well and incubated at R.T. for 5 mins. Measured the absorbances of the Blank (Abs.B), Standard (Abs.S), Sample Blank (Abs.SB) and Test Sample (Abs.T) against D.W.

CALCULATION

$$\text{IRON } (\mu\text{g/dl}) = \frac{\text{Abs T} - (\text{Abs.SB} + \text{Abs B})}{\text{Abs.s} - \text{Abs.B}} \times 200$$

4.4 MAINTENANCE OF CELL CULTURE

4.4.1 Cell Line Information for C2C12

C2C12 cells are a mouse myoblast cell line derived from satellite cells of C3H mice. C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution.

4.4.2 MEDIA AND SUPPLEMENTS

DMEM Media Preparation

DMEM (Dulbecco's Modified Eagle Medium) was used in most of the cells. Sodium bicarbonate at a concentration of 3.75 g/L was added to maintain the pH of the

media. Three antibiotics, namely 1% Streptomycin, 1% Penicillin G sodium salt, and 1% Amphotericin B, were used.

- One litre of Millipore water was taken and sterilized.
- DMEM (17.4 g) medium was added and mixed well until completely dissolved.
- Then, 1g of Streptomycin, 1 g of Penicillin, and 1 ml of Amphotericin B were added and mixed thoroughly.
- sodium bicarbonate (3.75 g) of was added, resulting in a reddish-pink color change in the media.
- The pH was adjusted from 7.2 to 7.4.
- The media was then filtered through a 0.22 μ m Millipore filter using a Filtration unit.
- The media were stored at 4°C for future use.

4.5 PASSAGING OR SUBCULTURE / SEEDING

- A T-25 flask with 70 to 80% confluence was taken.
- The used media was removed, and the flask was washed twice with 1X PBS.
- 2 ml of trypsin was added for the enzymatic technique for cell dissociation.
- The flask was incubated at 37°C for 1 to 2 minutes in a CO₂ incubator.
- The diamond-shaped cell structure changed to an oval shape, after which the trypsin was quickly removed and 2 ml of 10% serum media was added.
- The media was pipetted out to detach the cells from the bottom of the flask.
- The cells with media were transferred into a 15 ml tube and centrifuged at 900 rpm for 3 minutes. The supernatant was removed, and 1 ml of 10% media was added to the cell suspension, gently resuspended until the cells were well mixed.
- Again the cells were centrifuged with 0% media at 900 rpm for 3 mins for inactivation of 10% media.
- The cells were counted using Haemocytometer.
- The cells were seeded into a well plate with different concentrations of synthetic media and protein sources of soyamilk and groundnut milk containing 10% serum media and 0% media.

- **CALCULATION**

$X \text{ Conversion} = \text{Required volume} \times \text{Required Concentration} / \text{Available Concentration}.$

4.6 OBSERVATION OF SYNTHETIC MEDIA WITH BSA(PROTEIN)

For this experiment C2C12 cell line was chosen in 24 well plate in each well 1×10^4 Cells were seeded with different concentrations (as shown in below table) of synthetic media with BSA (1mg/ml from this 32.6 μ l was added) with triplicates. And the cells were grown at 37°C in 5% CO₂ environment and plate is kept in the live cell imaging system (Celloger mini plus) continuously for 3 days of observation. Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed and also high number of residues were seen.

Table : 4.6 Concentration and composition of synthetic media with BSA

CONCENTRATION	COMPOSITION
Control	500 μ l of 10% DMEM media
1% Synthetic media	5 μ l (0%media)
5% Synthetic media	25 μ l (0% media)
10% Synthetic media	50 μ l (0% media)

4.7 OBSERVATION OF SYNTHETIC MEDIA WITH SOYA MILK (PROTEIN)

For this experiment C2C12 cell line was chosen in 12 well plate in each well 5×10^4 Cells were seeded with different concentrations of synthetic media with Soya milk (Protein source) as shown in below table. The cells were grown at 37°C in 5% CO₂ environment and plate is kept in the live cell imaging system continuously for 3 days of observation. Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed.

Table : 4.7 Concentration and composition of synthetic media with soya milk

Concentration	Composition
10% DMEM	750µl 10% DMEM media
1% Synthetic media	2.3µl soya milk + 7.7µl synthetic media + 740µl 0% media
5% Synthetic media	11.5µl soya milk + 38.5µl synthetic media + 700µl DMEM media
10% Synthetic media (100µg)	22.3µl soya milk + 77.7 µl synthetic media + 650µl 0% media
10% Synthetic media (200µg)	44.6µl soya milk + 54.4 µl synthetic media + 650µl 0% media

4.8 OBSERVATION OF SYNTHETIC MEDIA WITH SOYA MILK AND GROUNDNUT MILK (PROTEIN) HIGH CONCENTRATIONS

For this experiment C2C12 cell line was chosen in 96 well plate in each well 5×10^4 Cells were seeded with different concentrations of synthetic media with Soya milk and groundnut milk(Protein source)as shown in below table. The cells were grown at 37°C in 5% CO₂ environment and plate is kept in the live cell imaging system continuously for for 3 days of observation. Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed.

**Table:4.8 SYNTHETIC MEDIA WITH SOYA MILK AND GROUNDNUT MILK
(PROTEIN) HIGH CONCENTRATIONS**

CONCENTRATION	COMPOSITION
Control	750µl 10% media
Soya milk (100 µg)	650 µl 0% media + 100µl synthetic media+ 19.8 µl soya milk
Groundnut milk (100 µg)	650 µl 0% media + 100 µl synthetic media + 84µl groundnut milk

Soya milk (200 µg)	650µL 0% media + 100µl synthetic media + 39.6µl soya milk
Groundnut milk (200 µg)	650µL 0% media + 100µl synthetic media + 16.8µl soya milk
Soya + groundnut milk (200 µg)	650µl 0% media + 100µl synthetic media + 39.6µl soya milk + 16.8µl

4.9 OBSERVATION OF SYNTHETIC MEDIA WITH SOYA MILK AND GROUNDNUT MILK (PROTEIN) WITH LOW CONCENTRATIONS

For this experiment C2C12 cell line was chosen in 96 well plate in each well 1×10^4 Cells were seeded with 1µl, 2µl, 3µl, 5µl, 7µl, 10µl, 15µl concentrations of Soya milk and groundnut milk (Protein source) with 800µl of 10% synthetic media and 7065µl of 0% DMEM media with triplicates. The cells were grown at 37°C in 5% CO₂ environment and observed under the inverted microscope (EVOS FL inverted digital fluorescence microscope, AMG) for 3 days. Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 QUANTIFICATION AND ANALYSIS

5.1.1 ESTIMATION OF PROTEIN BY LOWRY'S METHOD

For protein estimation Lowry method was used lowry's protein assay is based on the biuret reaction. In this experiment the intensity of the colour (Blue colour) is in direct relation to the protein concentration. It is based on the reaction of proteins with copper ions under alkaline conditions, resulting in a colour change that can be measured spectrophotometrically. Here we taken reading at 660 nm for total protein estimation of soyabean milk and groundnut milk.

5.1.2 STANDARD CURVE

Standard curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration.

5.1.3 Soya bean milk and groundnut milk

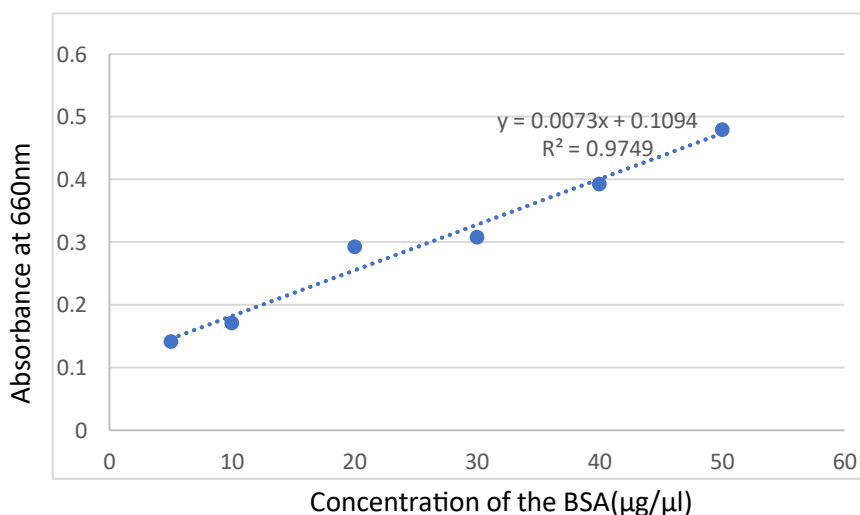


Fig: 5.1: STANDARD CURVE OF PROTEIN(BSA)

CALCULATION FOR PROTEIN IN SOYA MILK:

$$Y=MC+X$$

$$X=(y-c)/m$$

By using this formula we calculated the total amount of protein present in soyabean milk.

$$X = 5.05 \mu\text{g}/\mu\text{L}$$

By using same formula we calculated the total amount of protein in ground nut milk.

$$X = 1.19 \mu\text{g}/\mu\text{L}$$

Total protein content of soya milk is 5.05 $\mu\text{g}/\mu\text{L}$ indicating that soya bean milk is a good protein source having higher concentration of protein compared to ground nut.

5.2 QUANTIFICATION OF CREATINIE

For creatinine estimation we used alkaline picrate method. Here we taken 505 nm of wavelength for creatinine quantification of soybean milk and groundnut milk.

CALCULATION

$$\text{Creatinine Concentration(mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 2$$

By using this formula we calculated the total amount of creatinine in soya milk and groundnut milk

Soyabean milk for 30 sec is 0.088 mg/dl

Soya milk for 120 sec is 0.087 mg/dl

Groundnut milk for 30 sec is 0.072 mg/dl

Groundnut milk for 120 sec is 0.071mg/dl

5.3 QUANTIFICATION OF CALCIUM

For Calcium estimation we used Arsenazo II method. Here we taken 630 nm of wavelength for quantification total calcium of soybean milk and groundnut milk.

CALCULATION

$$\text{Calcium (mg/dl)} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 10$$

BY using this formula we calculated the total amount of calcium in soy milk and groundnut milk.

Groundnut milk = 6.625 mg /dl

Soybean milk = 10.411 mg /dl

Calcium is a essential nutrient for the growth of cells and also involved in many cellular pathways. Soyabean milk shows higher concentration of calcium compared to groundnut milk.

5.4 QUANTIFICATION OF GLUCOSE

For glucose estimation we used GOP /POD method. Here we taken 505 nm of wavelength for quantification total glucose of soybean milk and groundnut milk.

CALCULATION

$$\text{Glucose(mg/dL)} = \frac{\text{Abs.T}}{\text{Abs.T}} \times 100$$

By using this formula we calculated the total amount of glucose in soy milk and groundnut milk.

Groundnut milk = 52.54 mg/dL

Soyabean milk = 46.89mg/dL

Glucose is a essential molecule provides enery to the cells. Glucose was estimated and shows 52.54 mg/dl and 46.89 mg/dl in groundnut milk and soyabean milk. respectively based on the results. Glucose is present slightly more in groundnut milk

5.5 QUANTIFICATION OF CHOLESTEROL

For cholesterol estimation we used CHOD/POD method Ferrozine/ Magnesium carbonate method. Here we taken 505 nm of wavelength for quantification of total cholesterol of soybean milk and groundnut milk.

CALCULATION

$$\text{Cholesterol(mg/dl)} = \frac{\text{Abs of T}}{\text{Abs of S}} \times 200$$

By using this formula we calculated the total amount of cholesterol in soy milk and groundnut milk.

Groundnut milk = 37.27 mg/dl

Soyabean milk = 36.22mg/dl

Cholesterol is a important component for cellular function.Cholesterol is present in soyabean milk and groundnut milk as 37.27 mg/dl and 36.22 mg/dl respectively.The concentration of difference is very high amount in both the samples.

5.6 QUANTIFICATION OF IRON USING IRON & TIBC KIT

For Iron estimation we used Ferrozine/ Magnesium carbonate method. Here we taken 570 nm of wavelength for quantification total Iron of soybean milk and groundnut milk.

CALCULATION

$$\text{Iron (}\mu\text{g/dl)} = \frac{\text{Abs T} - (\text{Abs.SB} + \text{Abs B})}{\text{Abs.s} - \text{Abs.b}} \times 100$$

By using this formula we calculated the total amount of Iron in soy milk and groundnut milk.

Groundnut milk =160.71 $\mu\text{g/dl}$

Soybean mik = 407.14 $\mu\text{g/dl}$

Soyabean milk contain high content of iron compared to groundnut milk , which implies that soyabean milk is a good source not only for protein content but also for iron.

5.7 OBSERVATION OF SYNTHETIC MEDIA WITH BSA (PROTEIN)

In this experiment C2C12 cells were used for the comparison of synthetic media with soybean milk (as a protein source) with different concentrations (Table 4.6 refers) and the cells were grown at 37°C in 5% CO₂ environment and observed in live cell imager (celloger mini plus) continuously for 3 to 4 days. Attachment, growth, and morphological characteristics of the cells, such as shape, size, and appearance were observed. As shown in fig.5.2

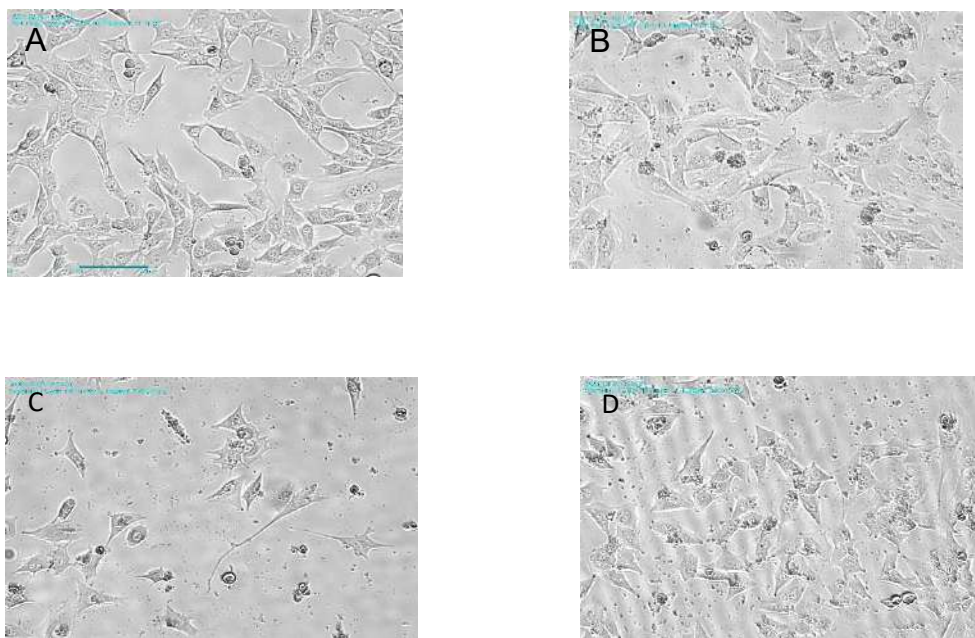


Fig: 5.2: Observation of synthetic media with BSA(A) Control, (B) 1% synthetic media, (C) 5% synthetic media, (D)10%synthetic media).

5.8 OBSERVATION OF SYNTHETIC MEDIA WITH SOYA BEAN MILK

In this experiment C2C12 cells were used for the comparison of synthetic media and soybean milk (as a protein source) with different concentrations (Table 4.7 refers) and the cells were grown at 37°C in 5% CO₂ environment and observed in live cell imager (celloger mini plus) continuously for 3 to 4 days. Attachment, growth, and morphological characteristics of the cells, such as shape, size, and appearance

were observed. As shown in 5.3. In this experiment we conclude that 10% synthetic media showed some better results.

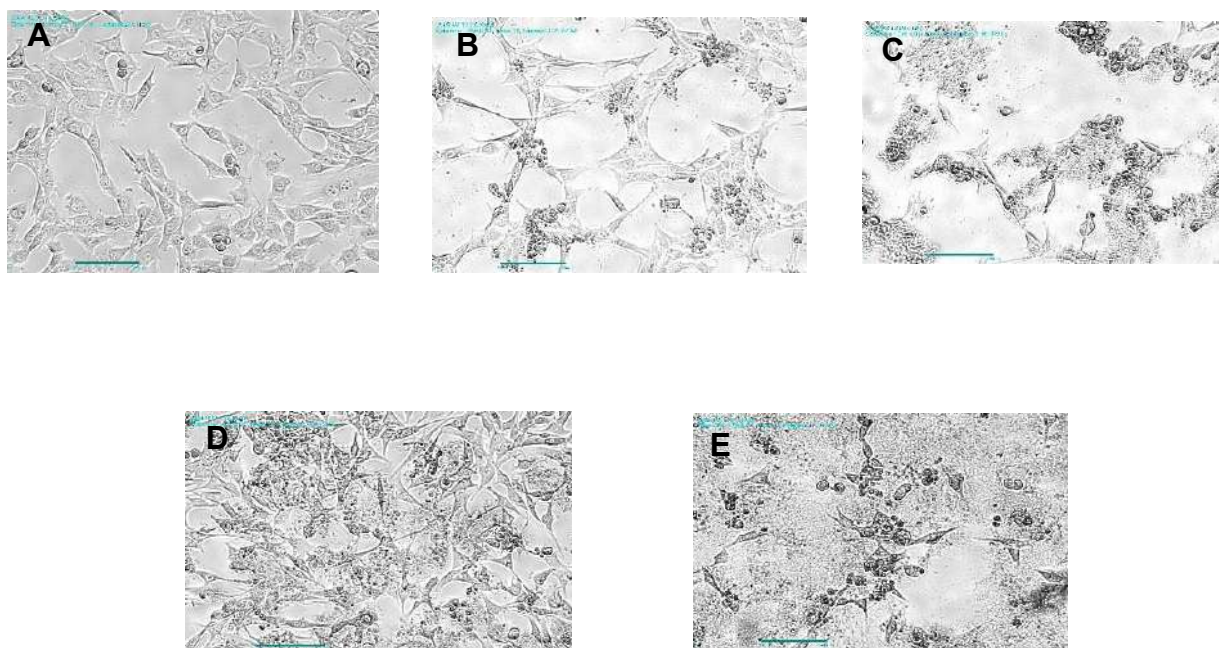


Fig: 5.3: observation of synthetic media with soyabean milk (A) Control, (B) 1% synthetic media, (C) 5% synthetic media, (D) 10% synthetic media (100µg), (E) 10% synthetic media 200µg

5.9 OBSERVATION OF SYNTHETIC MEDIA WITH SOYA BEAN MILK AND GROUNDNUT MILK

In this experiment C2C12 cells were used for the comparison of synthetic media and soybean milk, Groundnut milk (as a protein source) with different high concentrations (Table 4.8 refers) and the cells were grown at 37°C in 5% CO₂ environment and observed in live cell imager (Celloger mini plus) continuously for 3 to 4 days. Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed. As shown in fig 5.4.

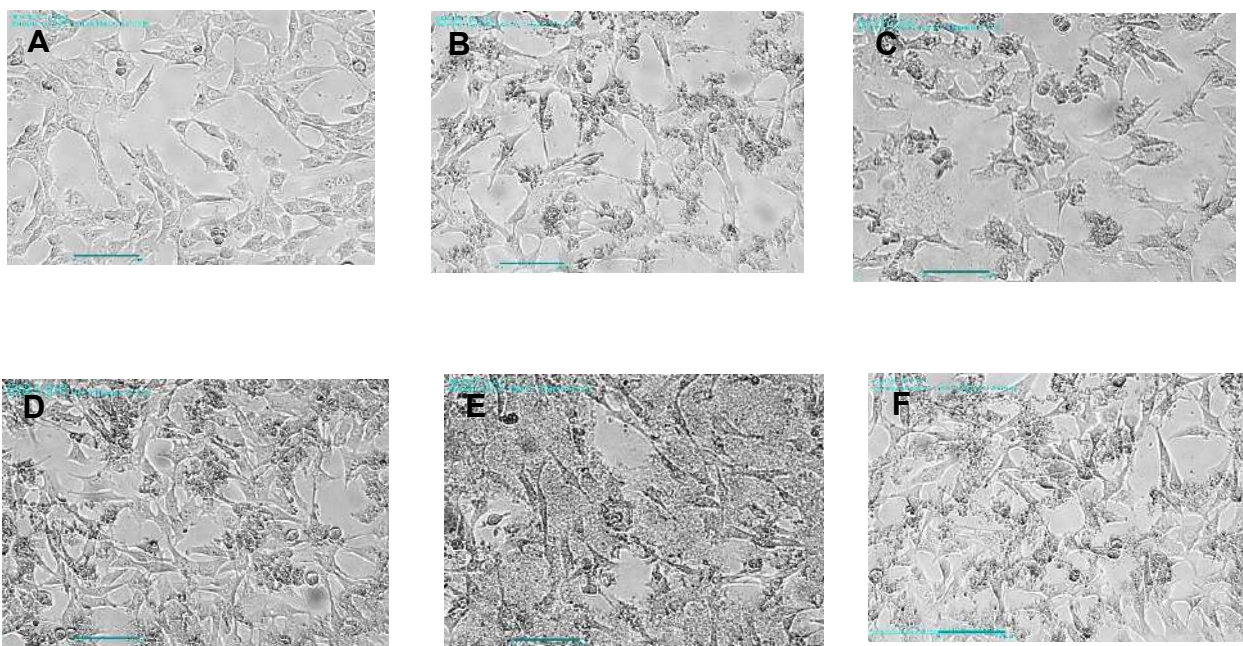


Fig : 5.4: Observation of 10% synthetic media with soy milk and groundnut milk (A) Control, (B) Soya milk 100µg , (C)Groundnut milk 100µg, (D) Groundnut milk 200µg,(E) soya + groundnut milk 200µg ,(F) Soya milk 200µg

5.10 OBSERVATION OF SYNTHETIC MEDIA WITH LOW CONCENTRATION OF SOYA MILK AND GROUNDNUT MILK.

In this experiment C2C12 cells were used for the comparison of synthetic media and soybean milk, Groundnut milk (as a protein source) with different low concentrations and the cells were grown at 37°C in 5% CO₂ environment and observed under the inverted microscope (EVOS FL inverted digital fluorescence microscope, AMG). For 4 days . Attachment, growth and morphological characteristics of the cell such as shape, size, and appearance were observed .As shown in fig 5.5.

LOW CONCENTRATIONS OF SOYA MILK

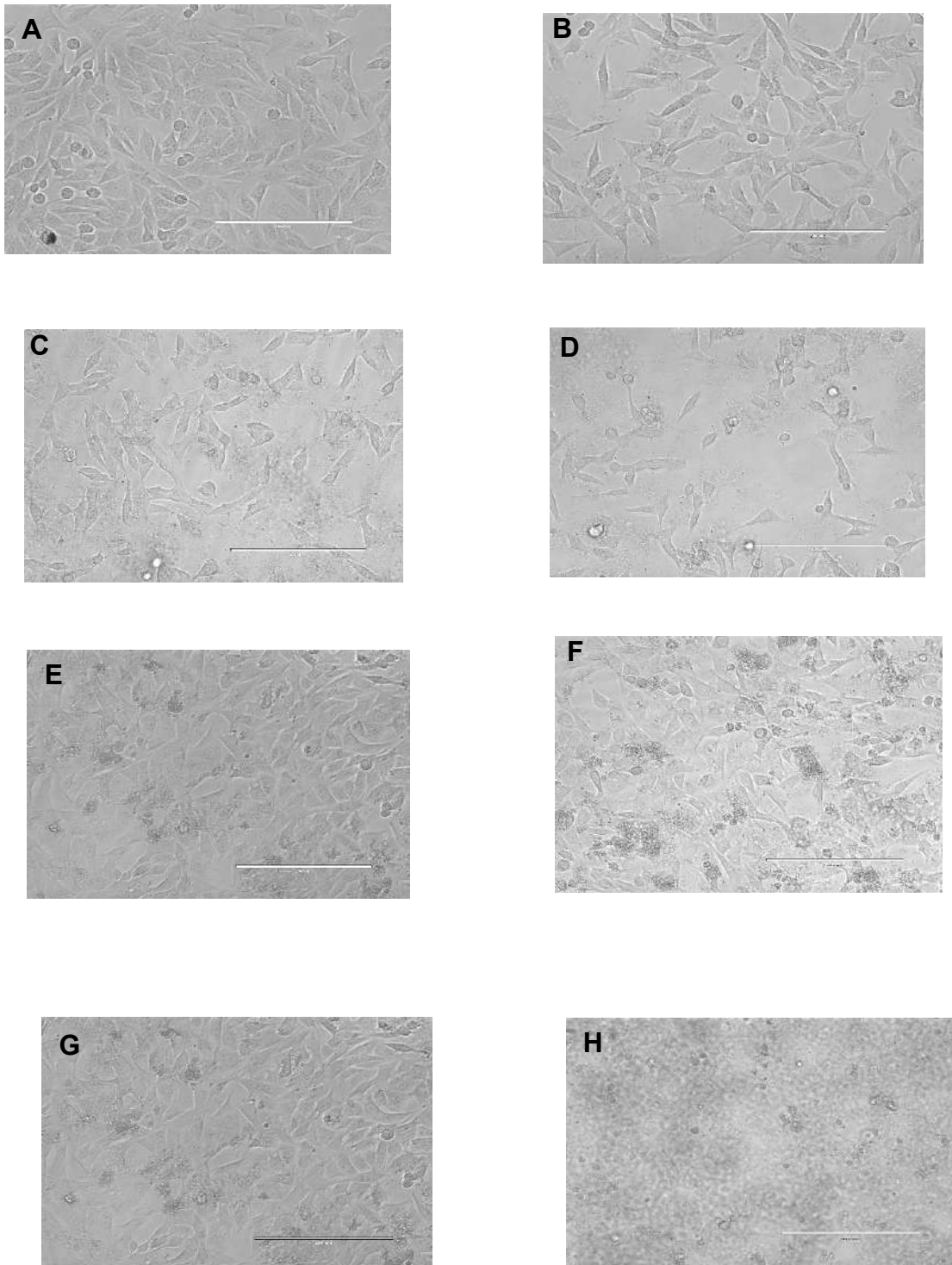


Fig : 5.5: Observation of 10% synthetic media with low concentrations of soya milk(A)Control, (B) Soya milk- 1 μ l, (C) Soya milk -2 μ l, (D) Soya milk -3 μ l, (E) Soya milk-5 μ l, (F) Soya milk-7 μ l, (G) Soya milk-10 μ l, (H) Soya milk-15 μ l)

LOW CONCENTRATIONS OF GROUNDNUT MILK

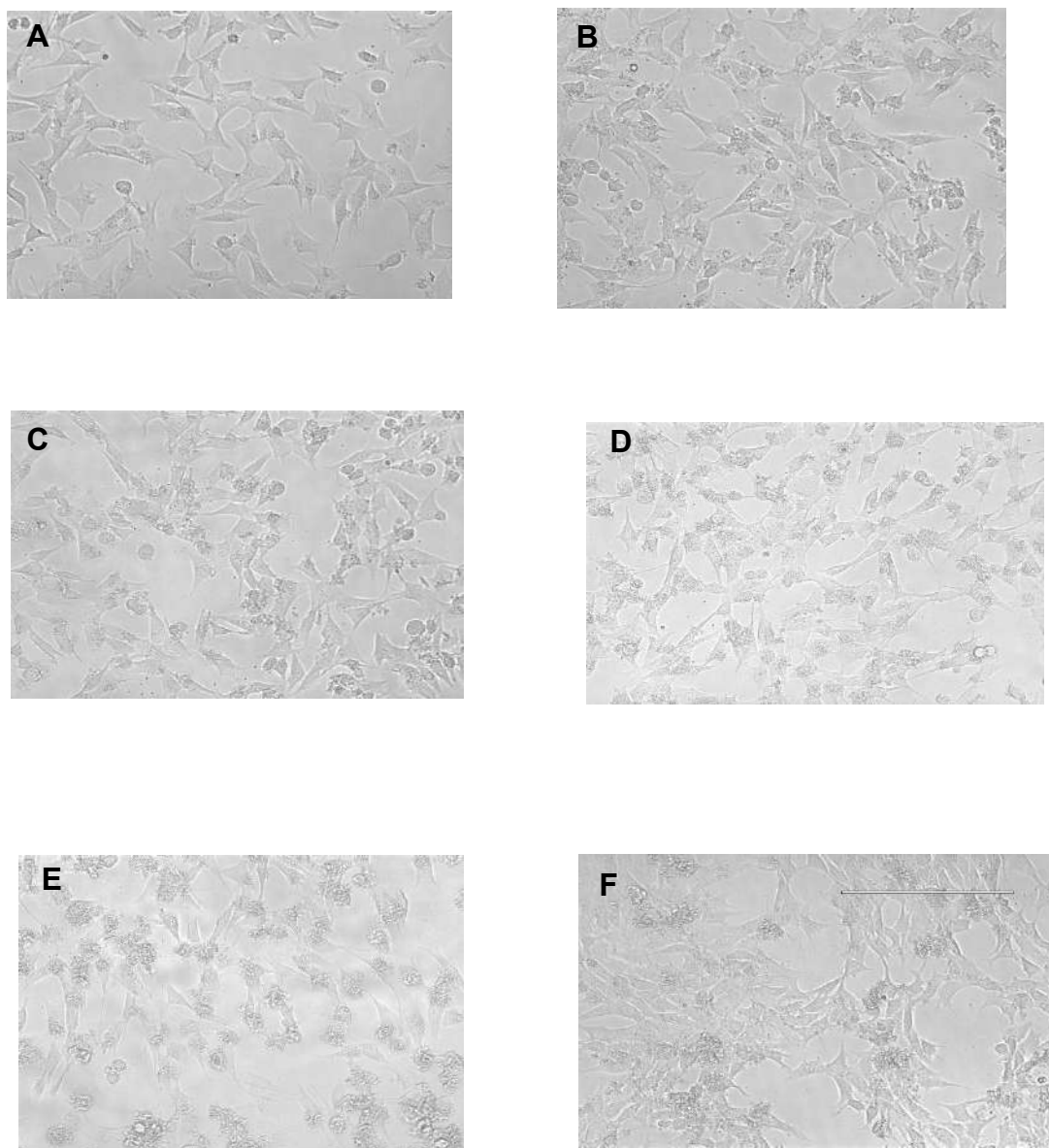


Fig : 5.5: Observation of 10% synthetic media with low concentrations of Groundnut milk. (A)Groundnut milk -1 μ l,(B)Groundnut milk-3 μ l, (C) Groundnut milk - 5 μ l, (D) Groundnut milk-7 μ l, (E)Groundnut milk -10 μ l, (F) Groundnut milk -15 μ l.

COMPONENTS	METHOD USED	ESTIMATED AMOUNT IN SOYA MILK (mg/dL)	ESTIMATED AMOUNT IN GROUND NUT MILK	ESTIMATED AMOUNT IN CONVENTIONAL FBS (Lindl, 2002)
Glucose	GOP/POD Method	46.89 mg/dL	52.54 mg/dL	1.25µg/ml
Cholesterol	CHOD/POD method	36.22 mg/dL	37.27 mg/dL	310µg/ ml
Calcium	Arsenazo II method	10.411 mg/dL	6.625 mg/dL	160µg/ ml
Creatinine	Alkaline picrate method	0.088 mg/dL (30sec) 0.087 mg/dL (120sec)	0.072 mg/dL (30sec) 0.071 mg/dL(120sec)	31µg/ ml
Total Protein	Lowry's method	5.05 µg/ µl	1.19 µg/ µl	38µg/ ml
Iron	Ferrozine/ magnesium carbonate method	407.14 µg/dL	160.71 µg/dL	-

Table : 4.9 Estimation of components

The synthetic FBS contains protein, glucose, cholesterol, calcium, iron and other micronutrients that can aid in the attachment, growth and proliferation of the cell line. Even though the quantity of the elemental composition could not substantiate the quantity present in conventional FBS, yet it functionally equates its potential in animal cell culture. Also, the protein source of soya milk is higher than that of groundnut milk but due to the presence and accumulation of large number of debris even after multiple filtration processes, groundnut milk at the concentration of 1.19 µg/µl is considered to be good to support the animal cell culture.

SUMMARY AND CONCLUSION

Fetal Bovine Serum is important for animal cell culture. Many alternatives developed but not up to the mark due to the following reasons. Cost of the medium, not support versatile cells, ethical issues, efficiency of the medium to overcome these issues we have developed animal free serum alternative for cell culture applications. Our synthetic medium composed of known concentrations of components along with insulin, transferrin and selenium. For protein source plant material was used, notably soya milk and groundnut milk, both has performed well in the development of animal free serum media. Alternative the composition such as glucose, cholesterol, protein, iron, calcium and creatinine was assessed in soya milk and groundnut milk for cell culture applications after formulation of synthetic FBS, we have checked with cell studies using live cell imager our results confirms that groundnut milk at 1.19 $\mu\text{g}/\mu\text{l}$. is optimum for culturing different cell types. based on the data we conclude that, our animal free serum alternative maybe used for various cell culture applications.

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