A DISSERTATION ON

DEVELOPMENT OF *SOLANUM NIGRUM* L.-BASED NANOFORMULATION TARGETED AGAINST MDA-MB -231 CELL LINE

SUBMITTED TO THE

DEPARTMENT OF BIOENGINEERING

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IN

BIOTECHNOLOGY

BY

Saima Farheen

(M. Tech Biotechnology) (IV Semester)

Roll No. 2101361010

UNDER THE SUPERVISION OF

Dr. Iffat Zareen Ahmad

Professor

Department of Bioengineering

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD

LUCKNOW-226026

DECLARATION FORM

I, **Saima Farheen**, a student of **(M. Tech Biotechnology)** (2nd Year/ IV Semester), Integral University have completed my six months dissertation work entitled **"Development of *Solanum Nigrum* L.-Based Nanoformulation Targeted Against MDA-MB -231 cell lines"** successfully from **Integral University** under the able guidance of **Dr. Iffat Zareen Ahmad.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Saima Farheen

Dr. Salman Akhtar
Associate Professor
Department of Bioengineering
(Course Coordinator)

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY SUPERVISOR

Certificate that Ms **Saima Farheen** (Enrollment Number 1700101607) has carried out the research work presented in this thesis entitled **"Development of *Solanum nigrum* L.-Based Nanoformulation Targeted Against MDA-MB-231 cell lines"** for the award of **M. Tech Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her M**. Tech Biotechnology** degree.

I wish her good luck and bright future.

Dr. Iffat Zareen Ahmad
Professor
Department of Bioengineering

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Saima Farheen,** a student of **M. Tech Biotechnology** (2nd Year/ IV Semester), Integral University has completed her six months dissertation work entitled **"Development of *Solanum nigrum* L.-Based Nanoformulation Targeted Against MDA-MB-231 cell lines"** successfully. She has completed this work from Integral University under the guidance of Dr. Iffat Zareen Ahmad. The dissertation was a compulsory part of her **M. Tech Biotechnology** degree.

I wish her good luck and bright future.

Dr. Ashish
Assistant Professor
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

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SAIMA FARHEEN

TABLE OF CONTENTS

ABBREVIATIONS

CSCs	Cancer stem cells
DAPI	4′,6-diamidino-2-phenylindole
DCFH	2,7-dichlorodihydrofluorescein
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ES	Extract of *S. Nigrum*
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
LCIS	Lobular carcinoma in situ
LPS	Lipopolysaccharides
NE	Nanoemulsion
NES	Nanoemulsion of *S. nigrum* extract
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SN	Solanum Nigrum
SNLs	Solid lipid nanoparticles
WHO	World Health Organization

INTRODUCTION

1. INTRODUCTION

Cancer is one of the most threatening and crucial diseases in humans and presently there is a considerable amount of new anticancer agents from natural products. WHO estimated that the cancer is one of the prominent roots of the death in worldwide, which accounted for 7.6 million deaths (around 13%) of the world's population in 2008 (Llovet *et al*., 2003). Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells in the body. These abnormal cells can form tumors, invade nearby tissues and organs, and spread to other parts of the body through the bloodstream or lymphatic system. Cancer cells can also spread to other parts of the body through the bloodstream or lymphatic system, forming new tumors in different locations, a process known as metastasis. There are many different types of cancer, including lung cancer, breast cancer, prostate cancer, skin cancer, colon cancer, and many others. The causes of cancer are complex and can involve a combination of genetic, environmental, and lifestyle factors. Cancer can affect any part of the body and is caused by a variety. With an estimated 1.67 million new cancer cases reported in 2012 (or 25% of all cancers), breast cancer is the second most common cancer in the world and by far the most common cancer among women. It is the most prevalent form of cancer in females in both more and less developed areas, with a modest increase in cases in less developed areas (883,000 cases) compared to more developed areas (794,000 cases) (Stewart BW, 2014). The prevalence rates range almost four times between different parts of the world, from 27 per 100,000 in Middle Africa and Eastern Asia to 92 in Northern America (https://www.cancer.org/cancer/cancerbasics/what-is-cancer.html). Between 1989 and 2015, there was a significant shift in the population, going from 39% to 20.3% (Ferlay *et al*., 2002). Due to increased awareness and the use of early detection techniques in recent years, the mortality rate has decreased. The anticipated demographic change in the death rate for Indian women from 2012 to 2025 is 16288 in the age group under 65 and 9959 in the age group over 65. Women are 100 times more likely than males to develop breast cancer, and women also experience the disease at a higher rate (Siegel *et al*., 2017). The primary reason breast cancer is incurable is that it is a metastatic cancer that frequently spreads to distant organs such the bone, liver, lung, and brain. A positive prognosis and a high survival percentage can result from the disease's early detection. The likelihood of having breast cancer can be increased by a number of risk factors, including sex, ageing, oestrogen, family history, gene mutations, and poor lifestyle (Majeed *et al*., 2014). Even

while the incidence rate of breast cancer rises annually, the mortality rate falls as a result of widespread early detection and cutting-edge medical treatments. In recent years, biological treatments have been created and have successfully treated breast cancer.

Solanum nigrum, also known as black nightshade, is a plant species also that has historically been utilised in herbal medicine for its conceivable anti-inflammatory, antioxidant, and anticancer effects. While there is some scientific evidence suggesting that extracts of *S. nigrum* may have anti-cancer effects, more research is needed to determine its efficacy and safety as a treatment for Human Mammary Carcinoma (MDA-MB 231). In vitro studies have shown that *S. nigrum* extracts may inhibit the growth and proliferation of cancer cells, including MDA-MB 231 cells, by inducing apoptosis (programmed cell death) and by inhibiting angiogenesis (the formation of new blood vessels that feed tumor). However, these studies have been conducted in laboratory settings and the results may not necessarily translate to humans. *S. nigrum* is an herbal plant commonly known as “black nightshade” or “Mako” in general. It usually grows in temperate and moist climatic environment in different kinds of soils. *S. nigrum* belongs to Solanaceae distributed chiefly in Asia, Europe, North America and extended to South and Central Africa and Australia and cultivated in tropical and subtropical agro climatic region (Särkinen *et al*., 2018). This plant is utterly used in traditional medicine in various parts of world to cure various liver disorders, chronic skin ailments, inflammatory conditions, painful periods, fevers, diarrhoea, eye diseases, jaundice etc. The vital active component found in *S. nigrum* are polysaccharides, glycoproteins, glycoalkaloids, steroidal glycosides, tannin. The leaves are rich in polyphenols, including phenolic acids and flavones. The genus name *Solanum* is taken from Latin word “solar” means to sooth and *Nigrum* means “black” due to the colour of the fruits. In the oriental systems of medicine, *S. nigrum* is used for many purposes which encompasses antioxidant, diuretic, antipyretic, anti-inflammatory, antitumorigenic and hepatoprotective (Ali et al., 2018). Morphologically, The ovate to heart-shaped leaves of *S. nigrum range* in size from 4.0 to 7.5 cm in length and 2 to 5 cm in breadth. This herbal plant's many parts contain a variety of chemical components, including *Solasonine* and *Solamagine* are the bioactive compounds found in leaves. Berries contain steroidal alkaloid, alpha- and beta-glycosides. There are 5 steroidal glycosides in immature berries, SN0, SN1, SN2, SN3, and SN4. *Solamargine*, *Solasonine, Ultroside* A and B are present in the stem and root.

S. nigrum constitutes a minor food crop, with the shoots and berries not only used as vegetables and fruits but also for various medicinal and local uses. Many traditional systems of medicine are dependent on this plant worldwide for numerous numbers of diseases but modern therapeutic applications have not recognized its importance so far (Nawab *et al*., 2012). Natural products have typically and conventionally been examined for encouraging new leads in pharmaceutical developments. In most of the case plants materials and its substances are being utilized in traditional medicine because they are easily available in rural area and comparatively cheaper than many modern therapeutic agents. Out of total 250,000 species of plant authenticate to exist on earth out of which 1 thousand have anticancer activities. *S. nigrum* has been used in traditional folk medicine to treat various kind of cancer. More recently, many research studies have reported that *S. nigrum* showed anti-cancer activity for human breast carcinoma, hepatocellular carcinoma cells, human ovarian carcinoma cells, human colorectal carcinoma cells, and human endometrial carcinoma cells. Solasodine, khasianine, β2 solasonine, solasonine, and solamargine showed cytotoxic activities against a gastric carcinoma cell line, MGC803. alpha-Solanine exhibited cytotoxicity against MDA-MB 231, HepG2, SW480, and MGC803, which are human mammary, human hepatoma, colon adenocarcinoma, and gastric carcinoma cell lines, respectively, and potent infammatory activity against nitric oxide (NO) release in LPS-induced murine macrophage cell line RAW264.7 (Gu *et al*. 2018). Extract of *S. nigrum* cause cell arrest in G0 and G1 phase with minute toxicity among animals. Its total number of isolated alkaloids alter the function and structure of the tumor cells and interrupts the DNA and RNA synthesis and transform the cell cycle events of tumor cells (Artun *et al*., 2016). Experimentally it has been approved that the aqueous extract of *S. nigrum* can be employed with drugs like doxorubicin and cisplatin in chemotherapy of mammary carcinoma patients. (Khan *et al*., 2019).

Based on their capacity to modify one or more particular molecular processes, a variety of novel chemo preventive medicines are being discovered. The identification of potent herbs and the understanding of their underlying processes may result in the creation of an alternative or supplemental approach to the prevention and/or treatment of cancer. Several efficient preventative strategies have been adopted in an effort to significantly lower the incidence rate of breast cancer, which is continuously rising throughout Asia as a result of the pervasively high incidence of breast cancer mortality.

Of all the initiatives that have been made, finding new anticancer substances in foods or plant-based medications is a practical and promising strategy for the treatment and prevention of cancer. MDA-MB 231 is one of the leading causes of cancer-related deaths worldwide. Despite the advances in cancer treatment, MDA-MB 231 remains a challenging disease to treat due to its resistance to chemotherapy and radiation therapy. Thus, there is an urgent need to develop new therapeutic approaches to improve the survival rate of breast cancer patients. One of the promising approaches is the use of natural products and their derivatives for cancer treatment. Among these natural products, *S. nigrum* has been reported to possess anticancer properties due to its phytochemical constituents. However, the use of *S. nigrum* is limited due to its low solubility and bioavailability. Therefore, the development of nano formulations of *S. nigrum* can enhance its solubility and bioavailability, thus improving its therapeutic potential against breast cancer. To increase the oral bioavailability and give it a strong resistance to physical, chemical, and environmental degradation, a variety of techniques, including some nano-based approaches, have been tried in the past. When compared to conventional biomaterials, nanostructured biomaterials offer a variety of benefits, including enhanced cellular contact, high bioavailability, and particular specified functions (Javed S *et al*., 2011, Shi J *et al*., 2010). A nano-sized drug carrier has made substantial progress in the delivery of molecules that are normally impossible to transfer, such as molecules with limited water solubility and genetic biomolecules, and it presents a viable solution to many problems in drug delivery and tissue engineering (Zang P *et al*., 2008, Jain KK, 2012). Incorporating the active ingredient into inert lipid vehicles (Aungst BJ *et al*., 1993) such as oils (Burcham DL *et al*., 1997) surfactant dispersions (Serajuddin A, 1998) self-emulsifying formulations, emulsions, microemulsions, or nano emulsions, is one of the more recent formulation design options for improving bioavailability. In comparison to these drug delivery methods, nanoemulsion has a number of advantages, including a higher solubilization capacity, a quicker onset of action (no extra time for dispersion), less inter subject variability in terms of gastrointestinal fluid volume, a longer shelf life, toxicological safety, a high content of the lipid phase, and the ability to produce on a large scale by high-pressure homogenization. In this study, we offer a nano formulation of leaf extract from *S. nigrum* that is intended to exclusively target breast cancer cells. This revolutionary method makes use of the advantages of nanotechnology to boost the extract's therapeutic effectiveness and selectivity, opening a viable path for the creation of a brand-new breast cancer treatment.

OBJECTIVES

- Development of *Solanum nigrum*- based nanoemulsion.
- Characterization of *S. nigrum*- based nanoemulsion.
- In vitro cancer activity of *S. nigrum* extract and its nano formulations.

REVIEW OF LITRETURE

2. REVIEW OF LITERATURE

A glandular organ that is mostly present in female mammals, including humans, is the breast. It is in charge of generating and supplying milk to feed infants while nursing. Each human breast normally consists of connective tissue, glandular tissue, and adipose (fat) tissue. The glandular and stromal (supporting) tissues make up the majority of the tissues in the breast. While stromal tissues consist of the breast's fatty and fibrous connective tissues, glandular tissues contain the milk-producing glands (lobules) and the ducts (the milk passageways). Additionally, the immune system's lymphatic tissue, which eliminates cellular waste and fluids, is present in the breast (*Breast cancer process india*, 2010). The tissue that makes up the breast is very varied, ranging from extremely fat to extremely thick tissue. A network of lobes may be present inside this tissue. Each lobe is made up of lobules, which are tiny, tube-like structures that house milk glands. Milk is transported from the lobes to the mammilla via tiny ducts that connect the glands, lobules, and lobes. The areola, the area that is darker and contains the mammilla, contains the mammilla at its centre. The blood and bodily fluid vessels in the breast coexist. The cells are fed by the blood. The body fluid system eliminates bodily waste. The body fluid nodes, the tiny, bean-shaped organs that help fight infection, are connected to the body fluid veins. Cancerous or benign neoplasms are also possible. Because a cancerous tumour is malignant, it will spread to various parts of the body as it grows. A tumour is a neoplasm that will continue to grow but not spread. Once the cancer has spread to various bodily parts or when carcinoma cells have travelled to those parts via blood arteries and/or body fluid channels, carcinoma spreads. This process is known as metastasis. Numerous tumour kinds can appear in various breast regions. The majority of breast tumours are the consequence of benign (non-cancerous) alterations. For instance, fibrocystic change, a non-cancerous disorder, causes women to develop lumpiness, fibrosis (the creation of scar-like connective tissue), cysts (accumulated packets of fluid), and areas of thickening, tenderness, or breast pain (*American cancer society*, 2009).

2.1.1. Pathogenesis:

Breast tumours typically begin as ductal hyperproliferation, and after being repeatedly stimulated by numerous carcinogenic stimuli, they progress to benign tumours or even metastatic carcinomas. In the development and spread of breast cancer, tumour microenvironments like stromal effects and macrophages are crucial. When just the

stroma of the rat mammary gland was exposed to carcinogens not the extracellular matrix or the epithelium neoplasms could be generated (Maffini *et al*., 2004, Sonnenschein *et al*., 2017). Between the normal and tumor-associated microenvironments, different DNA methylation patterns have been identified, suggesting that epigenetic changes in the tumour microenvironment can promote carcinogenesis (Polyak K, 2007, Basse *et al*., 2015). Cancer stem cells (CSCs), a new subclass of dangerous cells within tumours, have recently been identified and linked to tumour initiation, escape, and recurrence. This small population of cells has the capacity for self-renewal and is resistant to traditional treatments like chemotherapy and radiotherapy. They may arise from stem cells or progenitor cells in healthy tissues (Baumann *et al*., 2008, Zhang *et al*., 2017).

2.1.2. Types of breast cancer:

- **Invasive Breast Cancer:** Cells that infect the breast's surrounding fatty and connective tissues by penetrating the duct and lobular walls. Cancer can spread to the lymph nodes or other organs but not always be invasive (cells that infect the breast's surrounding fatty and connective tissues by penetrating the duct and lobular walls. Cancer can spread to the lymph nodes or other organs but not always be invasive (Breast cancer. Merck, 2008).

- **Non-invasive Breast Cancer:** Cells that stay in the breast's ducts and do not infiltrate the fatty and connective tissues around them. The most prevalent type of non-invasive breast cancer (DCIS) accounts for 90% of cases. LCIS, a less frequent condition that is thought to raise the risk of breast cancer, is lobular carcinoma in situ.

2.2.3. Etiology of breast cancer:

Breast cancer's complicated and still poorly understood underlying cause. Numerous factors that significantly contribute to the aetiology of breast cancer and raise the likelihood of the disease's development have recently been discovered. Exogenous and endogenous variables are among them. Some of the common factors are-Age, Genetics, Harmone levels, radiation exposure, exposure to carcinomas etc.

2.2.4. Genetic causes:

Family history has long been recognised as a breast cancer risk factor. Important factors include both maternal and paternal relatives. The risk is higher if the affected

relative was a close relative, had cancer in both breasts, or got breast cancer at an early age. The most crucial first-degree relatives to consider when calculating risk are the mother, sister, and daughter. The risk may also increase if there are several second-degree relatives (grandmother, aunt) who have breast cancer. All of a man's close female relatives are at an increased risk if he develops breast cancer. When inherited, the defective genes BRCA1 and BRCA2 significantly raise the lifetime risk of breast cancer, which is estimated to be between 40 and 8%. BRCA1 gene carriers frequently experience breast cancer at a young age (Breast cancer, emedicinehealth, 2010).

2.2.5. Hormonal causes:

Breast cancer may develop sooner as a result of hormonal level changes. It may be accompanied with the onset and cessation of periods (menstrual cycle), early pregnancy, hormone replacement therapy, using oral medications, etc (Fletcher, 2008).

2.2.6. Environmental cause:

Women who deal with low doses of radiation for extended periods of time, such as X-ray technicians, are known to be slightly more at risk (Tiernan, 2003).

2.2.7. Life style and dietary cause:

Breast cancer may be brought on by an active lifestyle, a high-fat diet, and obesity, particularly in postmenopausal women. Alcohol abuse is yet another factor contributing to breast cancer. According to the amount of alcohol consumed, the danger rises. One and a half times as likely to get breast cancer in women who drink two to five alcoholic beverages per day as non-drinkers (Tiernan, 2003).

2.3. Worldwide distribution:

The most typical location for breast cancer in women is increasing in frequency. Breast cancer strikes one in nine American women at some point in their lives. 42,000 women die from this condition each year, and around 1.50,000 women receive new diagnoses (Henderson *et al*., 1980). 20% of all cancers in European women are breast cancers, with an annual incidence of 1,80,000 cases. In the course of their lives, one in every 40 Japanese women develops breast cancer. Oncogene activation is a key step in the multi-step process that leads to the development of solid tumours in humans. Breast cancer is rare before the age of 20, and in rare cases, before the age of 30, but

after that, the incidence climbs quickly until the age of 50, after which the rate slows down, even if the incidence rate keeps rising. While India (23.5%) and Japan (33.4%) have higher incidence rates of breast cancer than Western Europe and North America (15–25 deaths per 100,000 females, or 30–40% of the incidence rate). Africa (29.5%) and China (26.5%). Among one lakh female people in India, the incidence rate of breast cancer was 25.6%. For Mumbai, Bangalore, and Chennai, respectively, in 1985, 15.8% and 20% (Riordan *et al.*, 1985). The periodic compilation of the global incidence of cancer from the hospital-based registries is provided by the International Agency for Research on Cancer (IARC), World Health Organisation (WHO), International Association of Cancer Registries (IACR), and National Cancer Registries (NCR) in India. The best sources of data for descriptive epidemiological research of cancer can be found in these data compilations.

2.4. Stages of breast cancer:

Stage I: Primary tumour with axillary lymph node unaffected and less than 2 cm in diameter.

Stage II: A primary tumour measuring 2.5 to 5 cm in diameter with or without palpable axillary nodes.

Stage III: A main tumour with a diameter of more than 5 cm and axillary nodal involvement with a few nodes. or metastases to the axillary lymph nodes.

Several successful preventive methods have been devised in an effort to significantly lower the incidence and mortality of breast cancer given that the incidence rate of breast cancer is continuously rising in Asia as a result of the persistently high incidence of hepatic illnesses. Out of all the initiatives that have been made, finding new anticancer chemicals in foods or plant medicines is a practical and promising strategy for the prevention and treatment of cancer.

For a very long time, plants have been used to treat human ailments.

Traditional medicine, which derives most of its ingredients from foods and plants, offers a wide range of effective new cancer treatments from substances that were once sourced from natural sources and industrialised (Harvey, 2008, Han *et al.*, 2018). A great variety of medicinal plants used in conventional medical treatments can be found on the Indian subcontinent. The Indian medical system has long been recognised as a

valuable source of information by many westerners. Over 20,000 medicinal plants have been identified in India; however, traditional people still use only 7,000–7,500 of these plants to treat various maladies. The bulk of medicines are made from plant and animal products, minerals, metals, and other such substances. Ayurvedic medications are prepared from plant ingredients, which are used by large pharmaceutical industries. Around I20 medicinal compounds with known structures have been discovered from the small fraction of flowering plant species that have so far been studied, which includes 90 different plant species. Vinblastine, vincristine, Taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, tubocurarine morphine, codeine, aspirin, atropine, pilocarpine, capsaicin, allicin, curcumin, artemisinin, and ephedrine are a few of the helpful plant medications. On the other hand, it is crucial to identify and isolate a drug's active ingredients as well as to clarify its mode of action. These untamed plants are an essential part of the human diet because they provide the body with protein, energy, vitamins, minerals, and some hormone precursors (Onyenuga *et al*., 1995; Fleuret, 1979). Hence, research in both traditional medication combinations and single active molecules is crucial.

2.5. *S. nigrum*

The Solanaceae family includes *S. nigrum*, sometimes known as Maku or black nightshade, which thrives as a weed in a variety of soil types in damp environments. Its two varieties are found one is bearing black fruits and other one is reddish brown colour fruit. Red fruits are edible purpose. One of the largest and most diverse species groupings in the genus is found in the section Solanum, which is centred on the species *S. nigrum* L., sometimes known as the black, garden, or common nightshade. While it is more frequently referred to as the section Morella (Maurella (Dun.) Dumort. or Morella (Dun.) Bitt.), the official name for this section is Solanum since *S. nigrum* is the generic type (Seithe, 1962). Black colour fruit is toxic. Every component of a plant, including the roots, leaves, flowers, and stem, has therapeutic use. It is used in traditional medicine to treat inflammation, edoema, breast and liver cancer because of its diuretic, antipyretic, and detumescent effects (Sultana *et al.,* 1995). *S. nigrum* is a 90 cm tall annual branching plant with dull dark green, juicy, oblong or lanceoid leaves that are toothless to barely toothed on the margins. Solanine, a glycoalkaloids present in most parts of the plant with the high concentration of unripe beers, is a poisonous compound that contributes to Sn's popularity. Although it is considered a

rich source of poisons, it has proven also be reservoir of phytochemicals with pharmacological prospects Due to its recently demonstrated remarkable anticancer activity, this plant has received a lot of attention (Son *et al*., 2003) and hepatoprotective effect (Hsieh *et al*., 2008). The numerous elements found in herbs are what give them their medicinal properties.

The quality and quantity of a plant's chemical components determine its therapeutic effectiveness, and these components might vary depending on a number of different circumstances, one of which is the geographical location, which exhibits quantitative diversity in its chemical components. It has been subjected to numerous attempts to produce antitumor active ingredients. Certain substances that are cancer-fighting, such glycoproteins (Heo *et al.,* 2014) steroidal glycosides (Hu *et al*., 1999) glycoalkaloids (Kuo et al., 2000) and polysaccharides (Li *et al*., 2007), have been isolated from SNL. Phytochemical studies revealed that SNL contained glycoalkaloids (*solanine, solamargine, solasonine and solasodine*) (Saijo, 1982; Elsadig *et al.,* 1997). Traditional medicine has only used *S. nigrum* to treat a number of conditions, including fever, pain, hepatitis, and inflammation (Yang *et al*., 2021). *S. nigrum* is utilised in eastern medicine for a variety of conditions, including those requiring antioxidant, anti-inflammatory, diuretic, hepatoprotective, antitumorigenic, and antipyretic agents (Ali *et al.,* 2018). These varied activities are caused by a variety of biological substances that have been thoroughly identified. *S. nigrum* is used in many traditional medical systems around the world to treat a variety of ailments, but modern therapeutic uses have not yet acknowledged its significance (Nawab *et al*., 2012).

2.5.1. PLANT PROFILE

- Biological source: It is made up of dried, fully developed, solanum nigrum berries.
- Geographical Source: Medicinal Botany, National Institute of Siddha, Chennai, Tamil Nadu, India.
- Family: Solanaceae.
- Common name: Black nightshade, Makoi.

2.5.2. MEDICINAL USES OF *S. NIGRUM*

Leaves used to cure rheumatoid and gouty arthritis, skin conditions, as well as TB, nausea, and neurological illnesses.

- The juice and decoction of the berries are beneficial for treating inflammation, cough, diarrhoea, and skin conditions.
- A steroidal glycoalkaloid that can be used to make 16-DPA progenitor.
- Solanum nigrum extract has also been discovered to have anti-oxidant, anti-inflammatory, and anti-pyretic properties. The dried fruits of *Solanum nigrum* extracted in ethanol demonstrated remarkable hepatoprotective properties against CCL4-induced oxidative damage to breast cells.
- It was a traditional European drug with strong narcotic, analgesic, and sudorific properties that was regarded as a "somewhat dangerous remedy."
- The anti-cancerous property of *S. nigrum* is its most significant characteristic.
- Dysentery, gastrointestinal problems, wound pain, and fever are treated with infusions. Using the plant's juice to treat skin conditions like ulcers.
- The fruits are also used to cure asthma and "excessive thirst," as well as being tonics, laxatives, and hunger stimulants.

2.5.3. *SOLANUM L.* SECTION SOLANUM SPECIES' MORPHOLOGICAL TRAITS

Following are some characteristics of species found in the section Solanum:

Habit: Unarmed herbs, occasionally suffrutescent plants, and sporadically shrubs or epiphytes.

Stems and Leaves: The stems are rough in texture, sparingly pubescent (i.e., puberulent), or infrequently glabrous (i.e., glabrous).

The stalks (also known as petioles), which are 5–30 mm long, bear the alternately placed leaves, which are 2–13 cm long and 1–8 cm broad. Oval (also known as elliptic), elongated (also known as lanceolate), or egg-shaped (also known as ovate) leaf blades taper to a point at the tip (i.e., acute or acuminate apex). They have whole, bluntly toothed (also known as crenate), or somewhat lobed borders (i.e., sinuate). On both surfaces, they are puberulent (sparsely hairy).

Flowers and Fruits: Towards the terminals of the branches, the tiny star-shaped blooms (7–12 mm in diameter) are produced in numerous-flowered clusters in the leaf forks (i.e., axils). These flower clusters feature a peduncle, or main stalk, that is 1-2 cm long, and a pedicel, or individual stalk, that is 7–11 mm long for each bloom. The

blooms are composed of a golden centre cone and five fused, 3.5-4.5 mm long, white or purple-tinged petals. They also feature an ovary with a style and stigma on top, five tiny greenish sepals that are 1.5 to 2.5 mm long, and five yellow stamens that are around 2.5 mm long.

When fully grown, the spherical fruit (also known as berries) changes from green to a drab or purplish-black colour. These fruits (5-8 mm in diameter) are often deflexed (borne facing downward) and have sepals that point outward. They have a lot of little seeds in them (about 1.5 mm long by 1 mm wide).

2.5.4. BOTANICAL DESCRIPTION

Subglabrous to villous annual plants with a height of 70–75 cm that are covered in eglandular or glandular-headed simple multicellular hairs. It has tap roots that can be readily peeled off to reveal pale brown, lateral roots that are abundant and tiny, with few branching. It has an unbranched, glabrous or pubescent, slightly woody stem. Oval, ovatelanceolate, or ovate-rhombic to lanceolate leaves are 2.5–7.0 cm long and 2.0–4.5 cm wide, with whole to sinuatedentate edges. Ripes berries are normally roughly ovoid, dark purple to blackish or yellowish-green, 6-10 mm wide, and fall from calyces. Each fruit contains between 15 and 60 1.7-2.4 mm long seeds. The wood is light yellow and can be seen through the thin, easily peeled bark. The flowers are typically symmetrical and have five petals. Consuming green, unripe fruits that contain the glycoalkaloids *solamargine, solasonine, solanine,* and *solamagrine, solasodine* and *solanidine* (0.09–0.65%) is harmful to humans and livestock. The leaves are varied, alternating, without stipules, and may be whole or divided. Although solanine is present in all plant parts and levels rise as plants grow, it appears that soil type and climate have an impact on solanine levels (Wetter *et al*., 1978). The fruits and leaves of *S. nigrum* have a combined alkaloid content of 0.101 and 0.431, respectively. Fruit that is ripe has very few alkaloids and is safe to consume. The little investigation done on glycoalkaloids, which are claimed to be responsible for anticancer action, suggested that *solasonine* and *solamargine,* from leaves and unripe fruits, are the two most important.

2.5.5. *S. nigrum* secondary metabolites and their biological impact

High quantities of polyphenols such gentisic acid, luteolin, apigenin, kaempferol, and m-coumaric acid may be found in the leaves of *S. nigrum* (Huang *et al*., 2010).

According to HPLC results, anthocyanins such delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were exclusively found in the purple fruits of *S. nigrum*. Throughout time, several phytoconstituents with medicinal qualities, such antidiabetic and antioxidant activity, have been identified and described from *S. nigrum*. Gas chromatography-mass spectrometry (GC-MS) examination of the *S. nigrum* fruit berries' chloroform fractions revealed a total of 29 components that showed strong antioxidant and anticancer activities against breast cancer cell lines (MDA-MB-231 and MCF-7) (Khan *et al*., 2016).

Solanum leaf could be used as a source of functional foods and nutraceuticals for the prevention and management of diseases associated with cognitive impairment, such as Alzheimer's disease, according to a very interesting study that found that dietary inclusions of this vegetable could protect against cognitive and neurochemical impairments brought on by scopolamine. The quality and amount of a plant's chemical components determine its therapeutic effectiveness, and these components might vary based on a number of different circumstances, one of which is the geographical location, which exhibits quantitative diversity in its chemical components.

Traditional medicine has been recognised as a significant source of chemo preventive medications that might lower morbidity and adverse effects associated with conventional chemotherapy. Breast cancer mortality is still high despite improvements in identification and treatment because the development of cancer cells that are resistant to current treatments limits the effectiveness of these treatments (Stockler *et al*., 2000, Schultz *et al*., 1999).

2.6. Nanotechnology

Nanotechnology has emerged as a promising approach to improve the therapeutic potential of natural products by enhancing their solubility and bioavailability. Nano formulations of natural products have been reported to exhibit improved pharmacokinetics and pharmacodynamics, resulting in enhanced therapeutic efficacy. Several types of nano formulations have been developed, including liposomes, solid lipid nanoparticles, polymeric nanoparticles, and nano emulsions. These nano formulations can improve the solubility and bioavailability of natural products, enhance their cellular uptake, and improve their therapeutic efficacy.

To increase the oral bioavailability of *S. nigrum*, a variety of techniques, including some nano-based ones, have been employed in the past and provide them a strong defence against deterioration caused by physical, chemical, and environmental factors. As compared to traditional biomaterials, nanostructured biomaterials provide a number of benefits, including enhanced cellular contact, high bioavailability, and particular specified functions (Javed *et al*., 2011). It provides a promising answer to several problems in tissue engineering and medication delivery. The active ingredient may be added to inert lipid carriers such as oils (Burcham *et al*., 1997) surfactant dispersions, self-emulsifying formulations emulsions, micro or nano emulsions, and liposomes in modern formulation design techniques for bioavailability increase. In comparison to traditional drug delivery methods, nano emulsion has a number of benefits, including a better solubilization capacity, a quicker beginning of action (with no additional time for dispersion), less inter subject variability in terms of gastrointestinal fluid volume, and a longer shelf life (Shafiq *et al*., 2007) and high levels of the lipid phase, toxicological safety, and the potential for industrial-scale production using high-pressure homogenization (Mehnert W *et al*., 2001).

As carriers for medicinal compounds, nano emulsions are colloidal particulate systems of submicron size range. From 10 to 1,000 nm is the range of their sizes. These carriers have a negative charge and an amorphous, lipophilic surface. They are solid spheres. They improve the therapeutic effectiveness of the medicine as a drug delivery mechanism and reduce toxic and adverse effects. Treatment of reticuloendothelial system (RES) infection, liver enzyme replacement therapy, cancer treatment, and immunisation are major applications. A biphasic system known as an emulsion occurs when one phase is intimately distributed in the other phase as tiny droplets with diameters between 0.1 and 100 μm. The inclusion of an emulsifying agent (emulgent or emulsifier) can stabilise the thermodynamically unstable system. Dispersion medium, exterior phase, or continuous phase are other names for the dispersed phase, which is also known as the internal phase or discontinuous phase. Mini emulsions, which are fine oil/water or water/oil dispersions stabilised by an interfacial coating of surfactant molecules with droplet sizes ranging from 20 to 600 nm, are also referred to as nano emulsions. The transparency of nano emulsions is due to their small size. Three different forms of nano emulsions are possible to create:

1. A continuous aqueous phase oil in water nanoemulsion in which the oil is dispersed.

2. Water droplets are spread in a continuous oil phase in a water in oil nanoemulsion.
3. bi-continuous nanoemulsion.

2.6.1. Nanoemulsion benefits

Pharmaceutical drug delivery via nanoemulsion has grown to be quite appealing. Moreover, nanoemulsion has a strong benefit in the cosmetics industry. The following factors contribute to nanoemulsion formulation's appeal in medicines and cosmetics

- Nanoemulsions have tiny droplets with a larger surface area that increase absorption.
- It might serve as a replacement for vesicles and liposomes (Bouchemal *et al*., 2004).
- It is possible to create it in a variety of ways, including foams, creams, liquids, and sprays.
- Drug's bioavailability is increases by nanoemulsion (Kim *et al*. 2001; Wagner *et al*. 1996).
- In cell culture technology, it offers improved absorption of oil-soluble nutrients.
- It facilitates lipophilic drug solubilization.
- Moreover, it has been suggested that nano emulsions may be employed to transport active ingredients to specific targets, particularly in cancer treatment.
- Because of the extremely tiny droplet size of nanoemulsion, creaming and sedimentation issues never appear.
- Nanoemulsions have a far higher dispersibility than microemulsions because the smaller droplet size precludes flocculation, which causes the system to disperse without separation.
- Due to the lack of any thickening agent and colloidal particles, nanoemulsion has a clear and fluid quality that increases formulation patient compliance and makes it safe for administration.

2.6.2. Nanoemulsion components

Oil, emulsifying agents, and aqueous phases are the three primary elements of a nanoemulsion (Gasco *et al*., 1991; Kriwet *et al*., 1995; Trotta ,1999). Oil and water can be combined to create a crude temporary emulsion that, when allowed to stand, will split into two distinct phases as a result of the coalescence of the scattered globules. There are many different types of oils, including castor oil, maize oil, coconut oil, evening primrose oil, linseed oil, mineral oil, olive oil, peanut oil, etc. Such systems can be made more stable by emulsifiers or emulgents. Emulgents are often categorised

as hydrophilic colloids like acacia, surfactants like spans and tweens, and finely split solids like bentonite and veegum. An emulgent has a number of desirable qualities, including:

- It ought to work well at rather low concentrations. Around the scattered globules, emulsifiers create monomolecular, multimolecular, or particulate films (Sharma *et al*., 1985)
- In order to create a full and coherent film and prevent coalescence, it needs to be quickly adsorbed around the scattered phase globule.
- Surface tension need to be lowered to under 10 dynes/cm with its help.
- In order to provide the system with the greatest stability, it should aid in increasing the system's zeta potential and viscosity appropriately.

Several studies have reported the development of nano formulations of *S. nigrum* for cancer treatment. For example, solid lipid nanoparticles (SLNs) of *S. Nigrum* have been developed and evaluated for their anticancer activity against breast cancer cells. The SLNs of *S. nigrum* were found to exhibit enhanced cellular uptake and improved anticancer activity compared to free *S. nigrum*. Similarly, *S. nigrum*-loaded chitosan nanoparticles have been developed and evaluated for their anticancer activity against liver cancer cells. The chitosan nanoparticles were found to exhibit enhanced cellular uptake and improved anticancer activity compared to free *S. nigrum*. Moreover, *S. nigrum*-loaded nano emulsions have been developed and evaluated for their anticancer activity against breast cancer cells. The nano emulsions were found to exhibit enhanced cellular uptake and improved anticancer activity compared to free *S. nigrum*. The anticancer mechanisms of *S. nigrum* and its nano formulations against breast cancer are not fully understood. However, several studies have reported the potential mechanisms of action of SN and its derivatives against breast cancer. For example, *S. nigrum* has been reported to induce apoptosis, cell cycle arrest, and autophagy in breast cancer cells. These effects are mediated by the modulation of various signalling pathways, such as the PI3K/Akt/mTOR pathway, the MAPK pathway, and the JAK/STAT pathway.

MATERIALS AND METHODOLOGY

3. MATERIALS & METHODS

3.1. Sample Collection

S. nigrum plant used in this study was collected from the agricultural farm of Integral University, Lucknow. The samples were collected in a sterile plastic bag and then samples were immediately transported into the laboratory and processed as shown in Figure 3.1.

Figure 3.1. *S. nigrum* plant in the agricultural field

3.2. Chemicals and Reagents used

Tween 80, Sefsol 218, PEG, PBS, DMEM, FBS, Antimycotic solution, MTT, DMSO, DCFH-DA, AnnexinV-FITC,

3.3. Preparation of extract

For preparing the plant extract, leaves of *S. nigrum* were separated from the plant and washed through distilled water until it cleaned properly. Cleaned wet leaves were soaked by using the blotting sheets under room temperature. Drying them in Microwave oven at temperature >50°C for atleast 30 second. By the use of grinder, the dried leaves samples were grinds into the fine powder, total weight of 22 g in Figure 3.2.

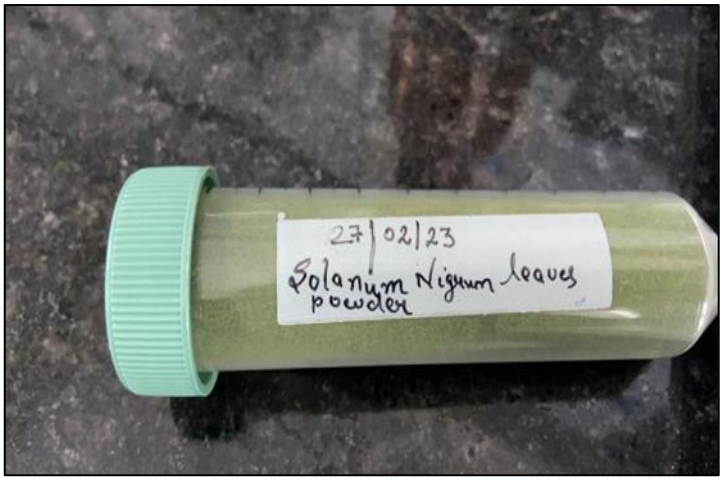


Figure 3.2. Powder of dried leaves samples of *S. nigrum*

Extraction process was initiated by putting the dried leaves of *S. nigrum* into the Soxhlet extractor using 250 ml of distilled water as a solvent for 24 hrs in Figure 3.3.

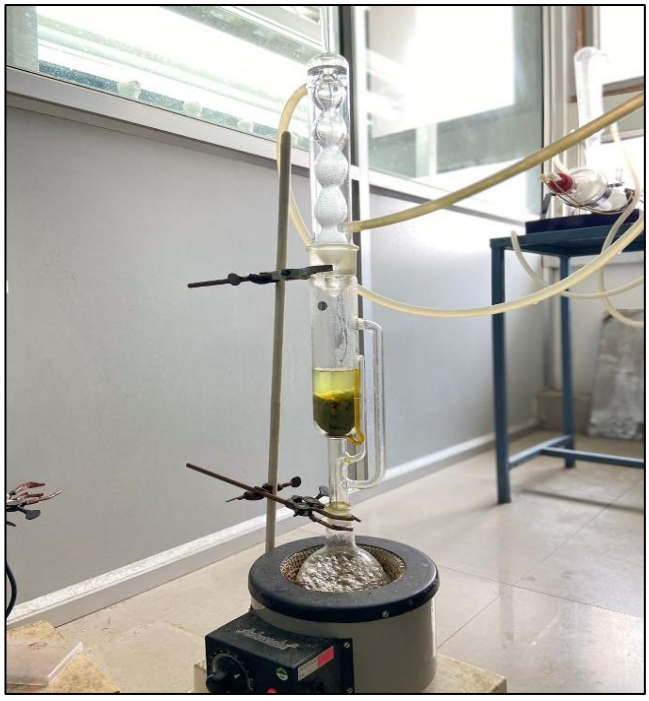


Figure 3.3. Extraction of sample under Soxhlet

The extracted solution of leaves was collected and filtrate by Whatman No. 1 filter paper in reagent bottles as a stock in Figure 3.4.

Figure 3.4. Filtered *S. nigrum* extract

3.4. Nano formulation development and characterization of sample

A stable and reliable formulation was created by doing solubility tests in various oils, building a pseudo ternary phase diagram, and performing thermodynamic stability tests.

3.4.1. Selection of oils, surfactants and co-surfactants for the nanoemulsion formulation

3.4.2. Preparation of blank oil-in-water nanoemulsion

Different batches of drug-free oil-in-water NE include varying ratios of oil_{mix} (including Tween 80: PEG at 1:1, 1:0, 1:2, 2:1) surfactant and co-surfactant prepared.

Here, we used a method to create a nanoemulsion that involved vigorously combining an oil mixture with a cosurfactant and surfactant mixture while employing a vortex mixture.

3.4.3. Nano formulations development

Three components i.e., oil (Sefsol 280, Tween 80) S_{mix} (surfactant-co-surfactant mixture) and distilled water were mixed in different volume ratios in stock of 10 ml to obtain best result**.**

Oil and a specific S_{mix} ratio were mixed properly in different volume ratios i.e., 1:0, 1:1 ,1:2, 2:1 in separate in separate glass vials.

Sixteen different combinations of oil and S_{mix} from each ratio (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:2, 1:3, 1:3.5, 1:5, 1:6, 1:7, 1:8) (Figure 3.6.) were slowly titrated visually inspected for transparency and flow ability and put it inside Sonicator water bath for 30 min.

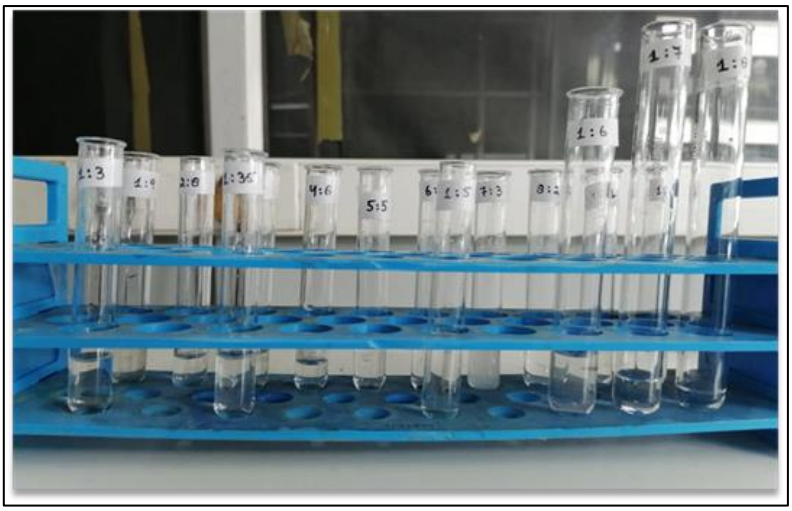


Figure 3.5. Different combination of oil, S_{mix} and water ratio

3.4.4. Pseudo ternary phase diagram study:

By using three components—oil, S_{mix} (surfactant-co-surfactant mixture), and distilled water—the insitu emulsification method (aqueous titration method) was used to construct pseudo ternary phase diagrams. Oil and a certain S_{mix} ratio were mixed appropriately in various volume ratios for each phase diagram. Three axes indicating an aqueous phase, an oil phase, and a S_{mix} phase were used to indicate the nano emulsion's physical condition on the phase diagrams. The nanoemulsion area for each phase diagram was displayed, and the larger region denoted the greater self-nanoemulsifying efficiency. Different formulations were chosen from the nanoemulsion area of each phase diagram, altering the amount of oil while maintaining a minimal concentration of S_{mix}. Selected formulations were subjected to thermostability and dispersibility tests.

3.4.5. Thermostability tests:

Tests for thermostability stress, including centrifugation, freezing and thawing, and heating and cooling cycles, were performed on a subset of formulations.

***Heating-cooling cycle*:** For each temperature cycle, the produced formulations in this investigation were held at 45^{o} C and 0^{o} C for a minimum of 48 hours.

***Centrifugation cycle*:** compositions were centrifuged at 5000 rpm for 30 minutes and looked for signs of phase separation, creaming, or cracking.

***Freeze-thaw cycle*:** For each temperature cycle lasting no longer than 24 hours, the produced formulations were subjected to two distinct temperatures, namely -20^{o}C and 20^{o}C. Three of these cycles were done for each batch of formulation in order to improve the estimation of accelerated stability studies. The formulations with the greatest stability were chosen for additional research.

3.5. Dispersibility tests:

To test each formulation's ability to self-emulsify, one millilitre of each was introduced to 500 ml of 0.1 N HCl and distilled water in a USP Dissolution apparatus Type II at 37 ±0.5^{o}C. The formulation was visually assessed using the following grading system.

Grade A: Forming (within 1 min) nanoemulsion, having a clear appearance.

Grade B: Rapidly forming, slightly less clear emulsion.

Grade C: Fine milky emulsion formed within 2 min.

Grade D: Dull white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).

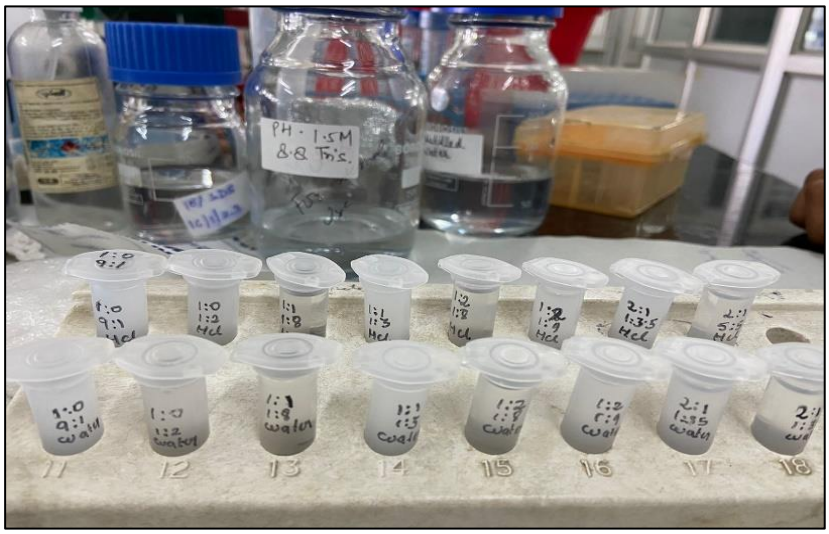


Figure.3.6. Different formulation with distilled water and HCL for dispersibility tests.

3.6. Characterization of sample

3.6.1. Determination of zeta potential:

The potential at the slipping/shear plane of a colloid particle moving under an electric field is known as the ZP or electrokinetic potential. The amount of energy required to accelerate a unit positive charge from infinity to a surface is known as the electric potential of that surface (M. Kaszuba *et al*., 2010). Important tool for understanding the state of the nanoparticle surface & predicting the long-term stability.

3.6.2. Fourier transform infrared spectroscopy (FTIR):

As an analytical technique, the FTIR spectrometer demonstrates that the final formulation maintains its chemical groups with a nucleus that exhibits no variation in medication stability. According to the procedure outlined in (Mirza et al., 2011), the potassium bromide (KBr) disc technique was used to record the FTIR of, *S. nigrum* leaf extract, NE without extract, NE with *S. nigrum* extract excipients and neat medication on the Perkin Elmer calorimeter (PerkinElmer, Inc., Waltham, MA). After correcting the baseline, scanning was carried out from 4500 to 400 cm^{-1}.

3.6.3. Transmission electron microscopy (TEM):

TEM TOPCON 002B was used to examine the surface morphology of NES (CDRI, Lucknow, India). A drop of nanoemulsion was put on a carbon-coated grid, diluted

with 2% phosphotungestic acid (1:100), filtered and left there for 30 seconds. The coated grid was placed on a slide and covered with a cover slip when it had dried. The slide was examined using a light microscope with a 200 KV voltage.

3.6.4. In vitro drug study:

Drug solution, microemulsion, and mucoadhesive microemulsion in vitro release experiments were carried out utilising the dialysis method to clarify the impact of these systems on the release kinetics of the pharmaceuticals (Patel *et al*., 2013).

Stock solution of Quercetin (C15H10O7) prepared for the standard curve.

Preparation of 10 ml each NE formulation (*S. nigrum* extract and *S. nigrum* + *C. Intybus* extract) of the S_{mix} ratio of 2:1 (1:3.5) for the drug release study.

Pre-treated dialysis membranes were soaked before use in distilled water at room temperature for some 12 hrs and kept frozen with PBS before prior to dissolution study to remove preservatives.

The drug release for suspension of the nano formulations of the both extracts were estimated in dialyzing media; phosphate buffer saline (PBS) pH 6.4.

One end of pre-treated dialysis tubing was tied with thread and then 10 ml of each respective nano emulgens was placed in it and tied the other end as well to avoid leakages and was allowed to rotate freely in 60 mL of dialyzing medium and stirred continuously at 100 rpm with magnetic bead on magnetic plate at 37°C± 0.5°C.

Aliquots of 1mL were removed at different time intervals (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 24 hrs).

These samples were quantitatively examined for the drug dialyzed across the membrane using a UV-visible spectrophotometer (317 nm) versus diffusion media as a control.

3.7. Cell culture:

MDA-MB-231 cell line were grown in DMEM culture medium and supplemented with 10% FBS, antibiotics and antimycotic solution (1.5%) at 5% CO2 and 95% relative humidity at 37^0 C.

3.7.1. Cell viability assay by MTT:

Briefly, cells ($1x10^4$) were seeded in 96-wemicroculture plates in 200µL (DMEM) medium for 24 and 48 hrs. Cells treated with Extract of *S. nigrum* (ES) and Nanoemulsion of *S. nigrum* (NES) at a concentration (10µg/ml, 20µg/ml, 40µ/ml, 60µg/ml, 80µg/ml and 100µg/ml) in the culture medium. At the end of incubation, 20 µL of 5 mg/ mL MTT was added to each well and the plates were further incubated for 3 hrs. Thereafter, supernatant from each well was carefully removed and formazan crystals were dissolved in 200 µL of dimethylsulphoxide (DMSO). The absorbance was recorded at 530 nm (Agrawal N *et al.,* 2007).

3.7.2. Intracellular ROS generation by Microscopy:

ROS generation was assessed in MDA-MB-231 cells using 2', 7'-diclorodihydrofluorescein di-acetate (DCFH-DA, Sigma Aldrich, Missouri, USA) dye as fluorescence agent. Briefly, cells ($1x10^6$) were seeded in 6 well plates and treated with at a concentration of µg/ml for 24 h. Cells incubate with 30 $mgmL^{-1}$ of DCFHDA dye for 30 minutes in dark at room temperature. The cells were then washed with PBS buffer. The production of ROS can be measured by changes in fluorescence due to the intracellular accumulation of DCF caused by the oxidation of DCFH. Intracellular ROS, as indicated by DCF fluorescence, was measured with a fluorescence microscope (Hseu YC *et al.,* 2012).

3.7.3. DAPI staining:

Nuclear fragmentation was detected by staining nuclei with DAPI. MDA-MB-231 ($1X10^6$) were seeded in six well plates for 24 hrs. Thereafter, cells were treated with SE and NES for 24 hrs. Cells were washed with PBS and fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100. Cells were stained with DAPI and images were captured using flourescence microscope (Olympus).

3.7.4. Cell cycle analysis:

Cell cycle analysis was carried out to check distribution of the cells in different phases induced by SE and NES using propidium iodide (PI) staining method. MDA-MB-231 were seeded in T-25 culture flask and allowed to grow for 24 h. After 24 h, cells were treated with scytonemin for 24 h. At the end of incubation, cells were harvested, mixed in ice cold 70% ethanol and incubated for 1 h at 4°C. Thereafter, cells were centrifuged and resuspended in 300 mL PBS and incubated with 30 mg of RNAseA and 15 mg of

PI for 30 minutes at room temperature in dark. Samples were analyzed by flow cytometry using FACS Calibur instrument.

3.7.5.ROS generation assay:

ROS generation was measured using 2, 7-dichlorodihydrofluorescein diacetate dye by flow cytometer. MDS-MB-231 cells (1X 10^6) were seeded in 6 well plates for 24 h. Cells were treated with SE and NES for 24 hrs. At the end of incubation, cells were harvested by trypsinization, fixed in cold methanol and incubated with 30 mg/mL of DCFH DA dye for 30 minutes in dark at room temperature. After the end of incubation, cells were centrifuged, resuspended in 300 mL of PBS. Samples were analyzed by flow cytometry using FACS Calibur instrument (BD biosciences).

3.7.6. MMP (mitochondrial membrane potential) assay:

MMP was measured by flow cytometry using JC-1(5,5,6,6- tetrachloro-1,1,3,3-tetraethylbenzimidazolecarbocyanine iodide) dye. MDA-MB-231 (1 × 10^6) were seeded in six well plates for 24 h. Cells were treated with ES and NES for 24 h. At the end of incubation cells were harvested by trypsinization, washed with PBS and incubated with 5 mg/ml JC-1 dye for 30 min in dark at room temperature. Following incubation, cells were resuspended in 300µl of PBS and analyzed using FACS Calibur instrument and FACSuite Software (BD Biosciences). Emission filters of 535 and 595 nm were used to quantify the population of mitochondria with green fluorescence (JC-1 monomers, treated as depolarised mitochondria) and red fluorescence (JC-1 aggregates, treated as a polarized mitochondria), respectively.

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSIONS

4.1. Solubility studies:

The goal of solubility experiments was to find the best oil phase for the development of the *S. nigrum* nanoemulsion in order to get the highest drug loading. It is important for the nanoemulsion to keep the drug in its solubilized state because the molecule is more soluble in the oil phase. Sefsol 280 was shown to have the maximum solubility of *S. nigrum* extract in the oil phase studied followed by tween 80.

4.2. Pseudo ternary phase diagram study:

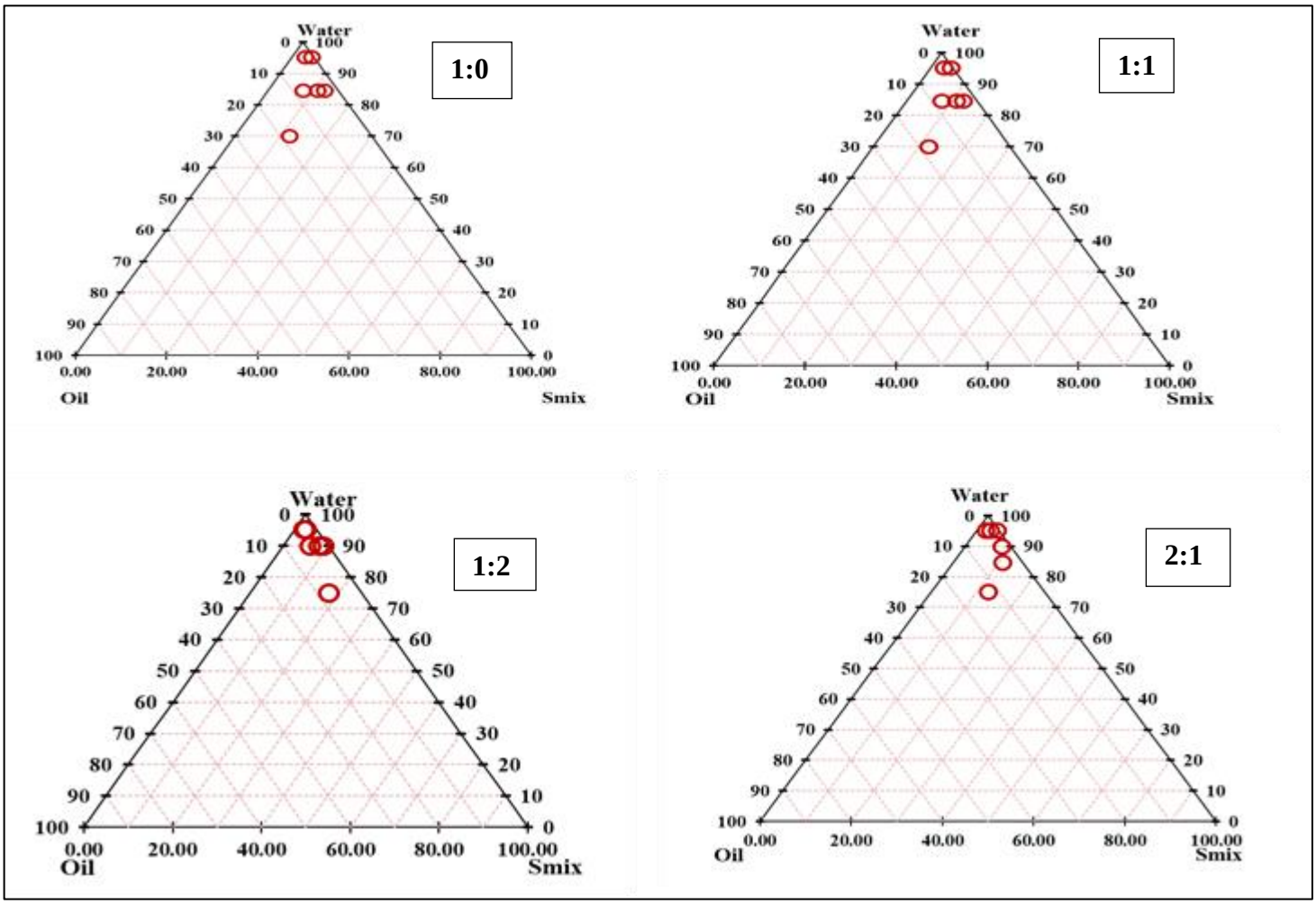


Fig.4.1. Pseudoternary phase diagrams of nano emulsions, F1 (S_{mix} 1:0), F2 (S_{mix} 1:1), F3 (S_{mix} 1:2), and F4 (S_{mix} 2:1).

4.3. Thermostability test:

The formulations were tested for thermodynamic stability following nanoemulsion region optimisation. Nanoemulsions continue to be stable under stressful circumstances. To assess the stability of the formulations, three tests—a heating/cooling cycle, a centrifugation process, and freeze-thaw cycles—were carried out. Observations from the testing are provided in Table 4.1.

Table 4.1. Thermostability tests of prepared nano formulations out of which from 1:2(1:8), 2:1(5:5), 1:0 (9:1),1:1(1:3) passed.

Formulations	Smix ratio	Oil %	Smix %	Water %	Thermodynamic stability tests			Dispersibility tests		Results
					Heating / Cooling	Centrifugation	Heat / Thaw	0.1 N HCL	H_2O	
NE_1	1:0	0.48	4.29	95.24	✓	✓	×	-	-	FAILED
NE_2		3.08	12.31	84.62	×	-	-	-	-	FAILED
NE_3		1.43	3.33	95.24	✓	×	-	-	-	FAILED
NE_4		6.15	9.23	84.62	✓	✓	✓	C	C	FAILED
NE_5		5.00	5.00	90.00	×	-	-	-	-	FAILED
NE_6		2.86	1.90	95.24	✓	✓	×	-	-	FAILED
NE_7		7.00	3.00	90.00	×	-	-	-	-	FAILED
NE_8		3.81	0.95	95.24	✓	✓	×			FAILED
NE_9		13.85	1.54	84.62	✓	✓	✓	A	A	PASSED
NE_{10}		8.33	16.67	75.00	×	-	-	-	-	FAILED
NE_{11}		1.25	3.75	95.00	✓	✓	×	-	-	FAILED
NE_{12}		2.22	7.78	90.00	✓	✓	✓	A	A	PASSED
NE_{13}		0.83	4.13	95.04	×	-	-	-	-	FAILED
NE_{14}		4.13	8.57	90.00	✓	✓	✓	C	C	FAILED
NE_{15}		0.62	4.36	95.02	✓	✓	✓	B	B	FAILED
NE_{16}		3.33	26.67	70.00	×	-	-	-	-	FAILED
NE_1	1:1	0.48	4.29	95.24	×	-	-	-	-	FAILED
NE_2		3.08	12.31	84.62	✓	✓	✓	A	A	PASSED
NE_3		4.62	10.77	84.62	×	-	-	-	-	FAILED
NE_4		1.90	2.86	95.24	✓	×	-	-	-	FAILED
NE_5		7.69	7.69	84.62	✓	✓	✓	A	A	PASSED
NE_6		17.91	11.94	70.15	×	-	-	-	-	FAILED
NE_7		7.00	3.00	90.00	✓	✓	✓	D	D	FAILED
NE_8		8.00	2.00	90.00	✓	×	-	-	-	FAILED
NE_9		13.85	1.54	84.62	✓	×	-	-	-	FAILED
NE_{10}		1.67	3.33	95.00	×	-	-	-	-	FAILED
NE_{11}		1.25	3.75	95.00	✓	✓	✓	A	A	PASSED
NE_{12}		6.67	23.33	70.00	✓	×	-	-	-	FAILED
NE_{13}		1.64	8.20	90.16	×	-	-	-	-	FAILED
NE_{14}		3.57	21.43	75.00	✓	✓	✓	B	B	FAILED
NE_{15}		2.50	17.50	80.00	✓	✓	×	-	-	FAILED
NE_{16}		1.11	8.89	90.00	✓	✓	✓	C	C	FAILED
NE_1	1:2	0.48	4.29	95.24	✓	✓	✓	B	B	FAILED
NE_2		2.00	8.00	90.00	×	-	-	-	-	FAILED
NE_3		4.62	10.77	84.62	✓	✓	×	-	-	PASSED
NE_4		1.90	2.86	95.24	✓	✓	✓	C	C	FAILED
NE_5		12.50	12.50	75.00	×	-	-	-	-	FAILED
NE_6		2.86	1.90	95.24	✓	✓	✓	B	B	FAILED
NE_7		3.33	1.43	95.24	✓	✓	✓	A	A	PASSED
NE_8		16.00	4.00	80.00	×	-	-	-	-	FAILED
NE_9		18.00	2.00	80.00	✓	✓	×	-	-	FAILED
NE_{10}		1.67	3.33	95.00	✓	✓	✓	D	D	FAILED
NE_{11}		3.00	8.00	90.00	✓	✓	✓	A	A	PASSED
NE_{12}		4.44	15.56	80.00	×	-	-	-	-	FAILED
NE_{13}		1.64	8.20	90.16	✓	✓	✓	D	D	FAILED
NE_{14}		1.43	8.57	90.00	✓	✓	×	-	-	FAILED
NE_{15}		0.62	4.36	95.02	×	×	-	-	-	FAILED

4.3. Characterization and evaluation of nanoemulsion:

Selected *S. nigrum* formulations were characterized and evaluated by following parameters.

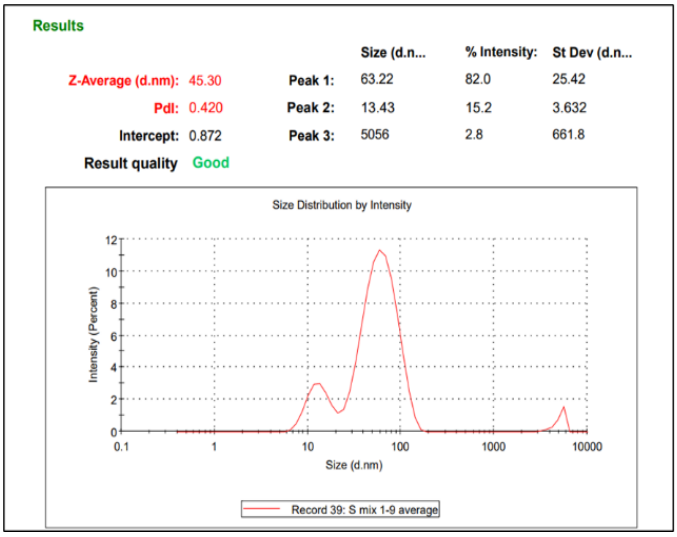
4.3.1. Visual appearance:

SN had no turbidity and was transparent and clear. This test was carried out to distinguish it from macroemulsion, which has a milky appearance.

4.3.2. Analysis of particle size by Zeta sizer:

The selected formulations with an optimized concentration of oil, surfactant, cosurfactant, and water, F2 i.e., 1:2 (1:8) shows good result compare to rest of the ratio were characterized initially by computing their particle size, polydispersity index, and zeta potential by using the DLS apparatus. The computed average sizes of selected ratio were about 45.30 nm along with PDI values of 0.420, respectively, affirming the uniform size distribution of the particles in the nanoemulsion.While neutral nanoparticle potential is between -10 mV and +10 mV, the nanodroplets with great dispersity exhibit zeta potential values between +30 mV and -30 mV. The estimated average zeta potentials of the *S. nigrum* extract nanoemulsions were -12.4 mV, respectively, indicating that they were neutral nanoparticles with significant dispersion.

(a)



(b)

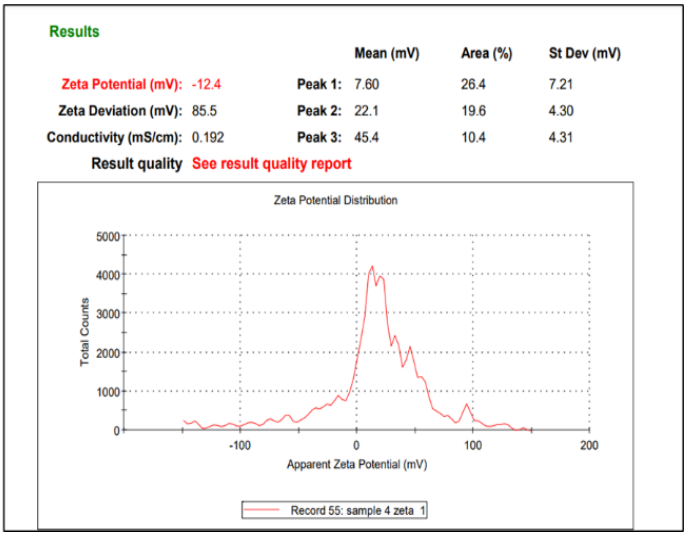


Fig.4.2. Representation of (a) zeta size and (b) zeta potential of selected ratio which is passed through stability and dispersibility test.

4.3.3.TEM:

The morphology of formulation was observed under TEM. It shows that the image of NES ranges at 200 nm as shown in Fig 4.4.

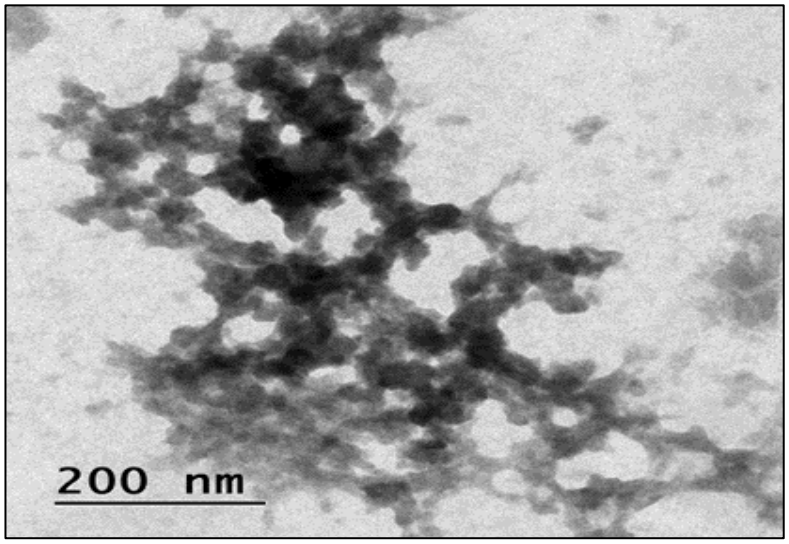


Figure 4.4. TEM images of nanoemulsion of *S. nigrum* leaf extract

4.3.4. FTIR analysis:

There was no additional peak (or widening of peaks) seen on the FTIR graphs of the drug, excipients, and formulation findings, indicating that there is no incompatibility between the drug and excipients. Entrapment of drug study of all three samples by FTIR spectroscopy is shown in Figure.4.5.

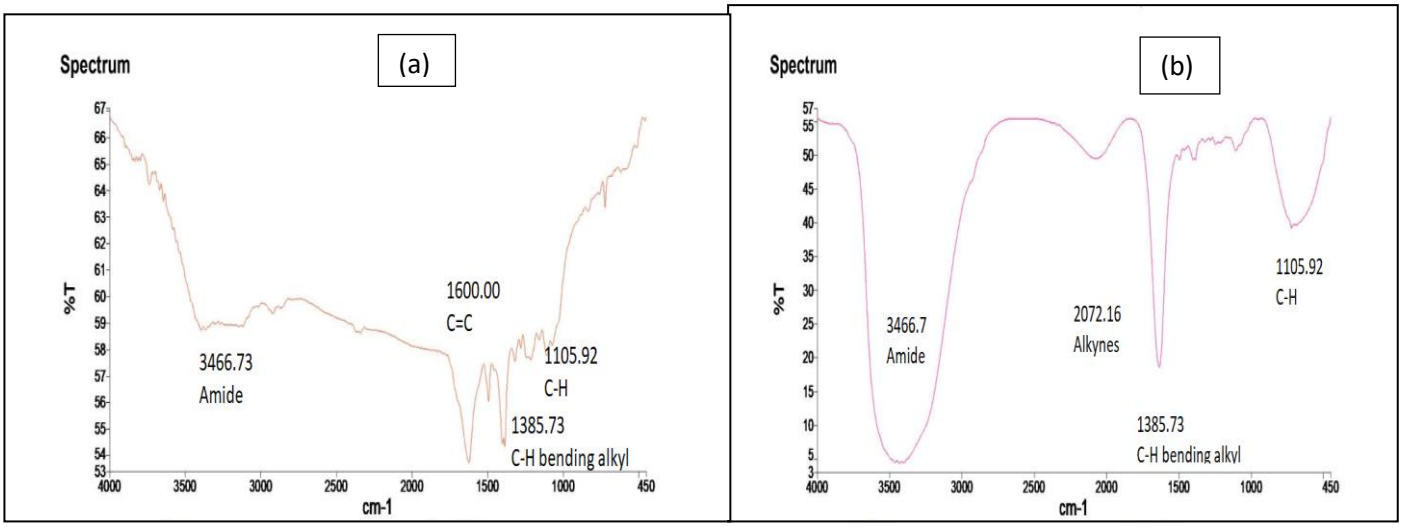


Figure 4.5. FITR analysis of (a) ES (leaf extract) and (b) SNE (nanoemulsion).

4.3.5 *In vitro* drug release study:

The ratio 2:1 nano formulation (NE 12) were evaluated against a conventionally available suspension of *S. nigrum* extract and against *S. nigrum* and *C. intybus* combined. By extrapolating the calibration curve, the concentration was determined, and a graph between time and percent cumulative release was created (Figure.4.5.).

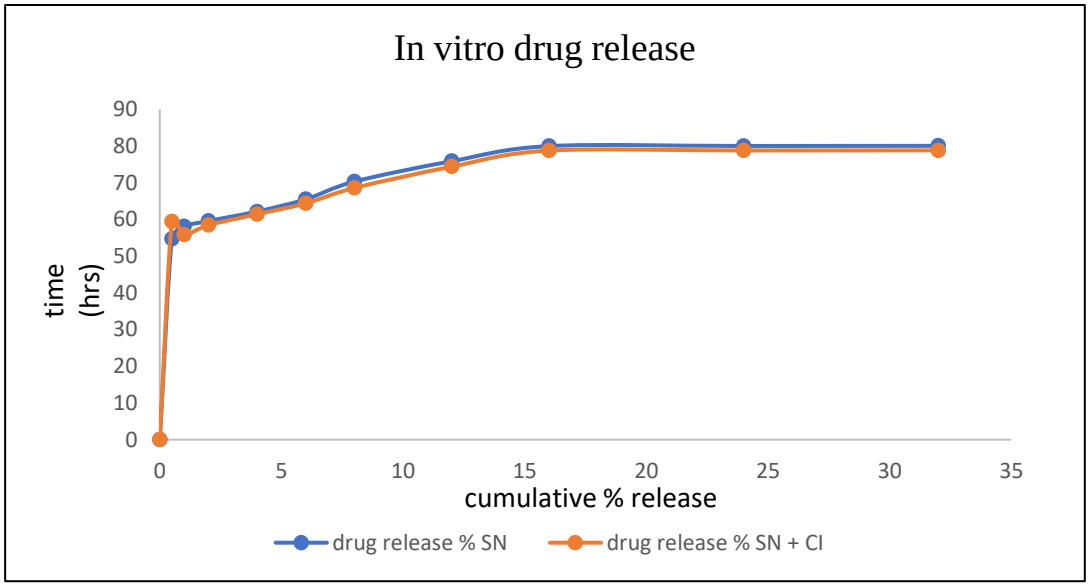


Figure.4.5. Drug release study of *S. nigrum* and *S. nigrum, C. Intybus* combination nanoemulsion.

4.4. *In vitro* Analysis

4.4.1 Cytotoxicity assay:

Cytotoxic activity of SE and NES breast cancer cell lines was evaluated using cell viability assays MTT assay. Nanoemulsion of *S. nigrum* leaf was the most effective against MDA cells (IC50 value 10 µg/mL) and extract of *S. nigrum* with IC50 20 µg /ml. Extract with and nanoemulsion showed dose dependent and time dependent activity while both showed better activity at 24 h. Figure 4.6. depicts the morphological analysis of untreated and treated cells were shown dose dependent effect of extract and nanoemulsion of *S. nigrum* at different concentrations. Altered morphology were shown in a concentration dependent manner analyzed at 20x by inverted contrast microscope. Doxorubicin, as standard drug was tested exhibited dose dependent and time dependent effect in MDA-MB-231 (Table 4.4.)

Table 4.4: %cell viability in terms of (IC50 ± SEM in µg/ml)

Treatments	24h	48h
ES	20±0.05	44±0.04
NES	10±0.03	35±0.07
Doxorubicin	2.0 ±0.04	4.0±0.05

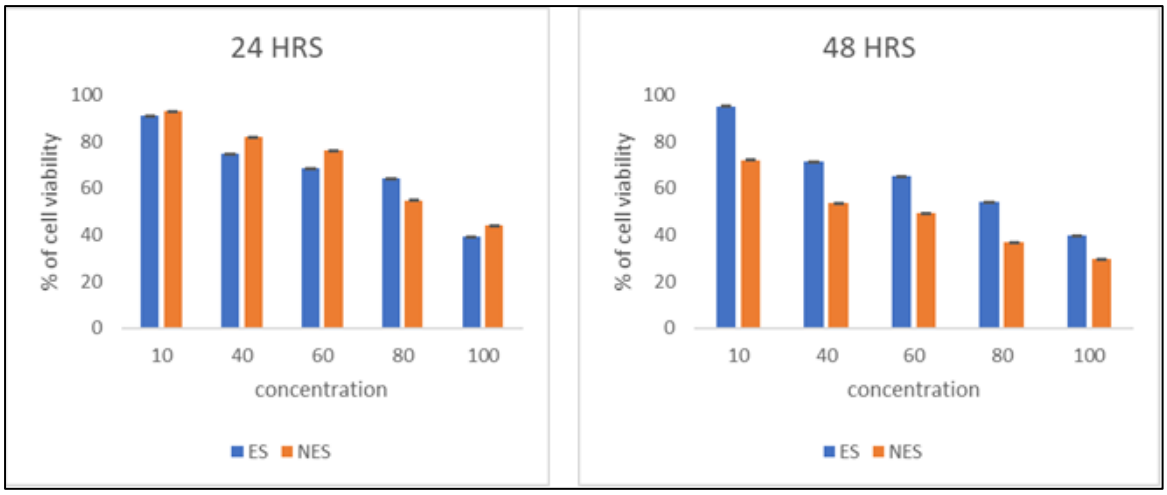


Figure 4.6. MDA-MB 231 cell lines were treated with obtained IC_{50} values of the *S. nigrum* fractions for cell viability at different concentration of ES and NES.

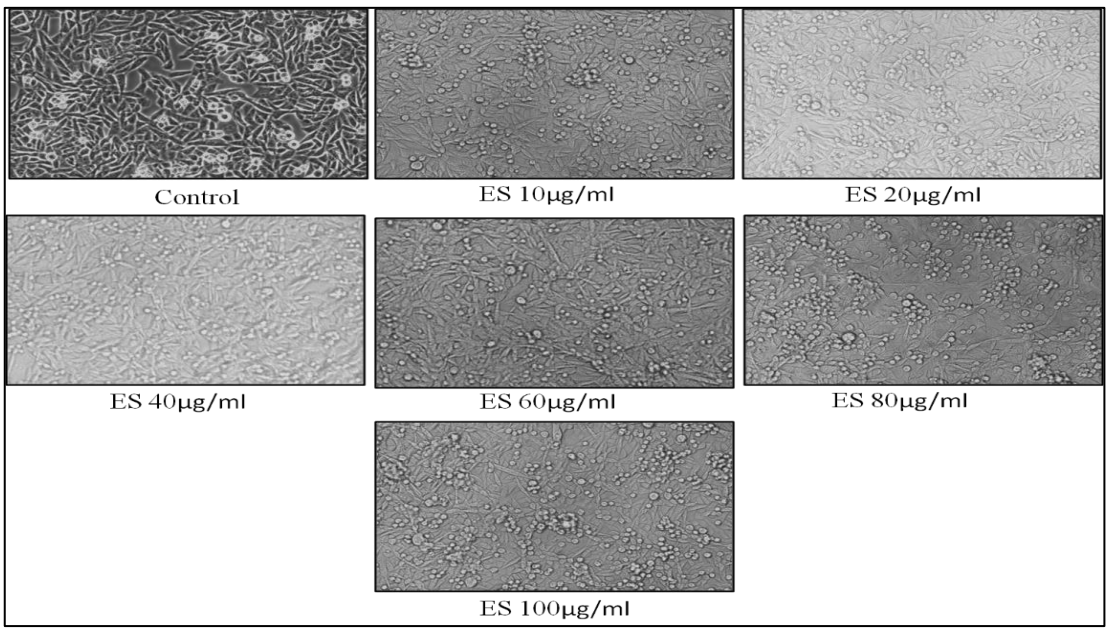


Figure. 4.7. Morphology of treated *S. nigrum* extract in different concentration.

4.4.2. Determination of Intracellular ROS

Several reports suggested that oxidative stress plays important role in anticancer activities of chemotherapeutic drugs and ROS generation has been confirmed to be closely to trigger apoptosis. Reactive oxygen species generation impaired the cell function and ultimately leads to cell death. NES showed more production of ROS as compared to ES as showed in Figure.4.8.

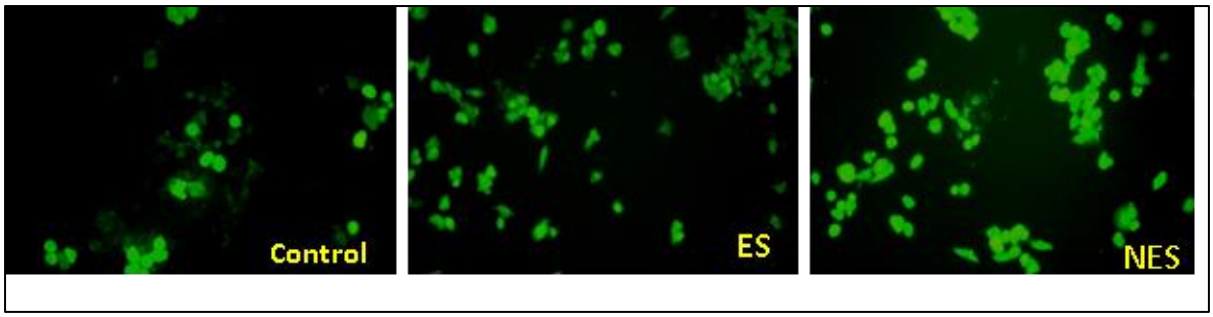


Figure 4.8. ROS production in various fractions

4.4.3. DAPI staining

To evaluate the effects of ES and NES on chromatin condensation, we treated MDA-MB-231 cells with 20 µg/ml for 24 h and examined them for apoptosis using DAPI staining, which distinguishes live from apoptotic cells based on nuclear morphology. The presence of chromatin condensation in treated cells was detected on a fluorescence microscope (×20). DAPI forms fluorescent complexes with DNA, and stained nuclei show a bright fluorescence with a DAPI filter. As shown in Fig.4.9. Cells treated with 10 µg/ml extract and 20 µg/ml of NES. The NES showed more fragmented nuclei in comparison to extract.

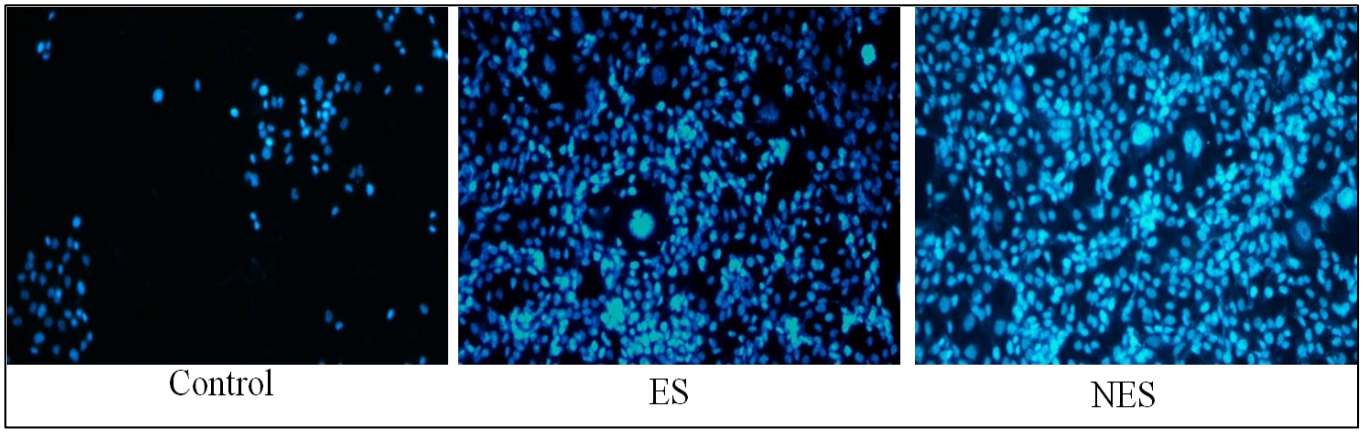


Figure.4.9. Treated cells with *S. nigrum* nanoemulsion using DAPI staining.

4.4.4. Cell cycle analysis

Cell cycle arrest is the main cause of cell death. ES and NES treated cells with concentrations 20 µg/ml and 10 µg/ml for 24 h with MDA-MB-231 were assessed by flow cytometry. ES and NES showed a typical DNA pattern that represented sub-G1, G1, S, and G2/M phases of the cell cycle. NES treated cells firstly showed higher G1 population (80%) as compared to extract (75%) and control (53%).

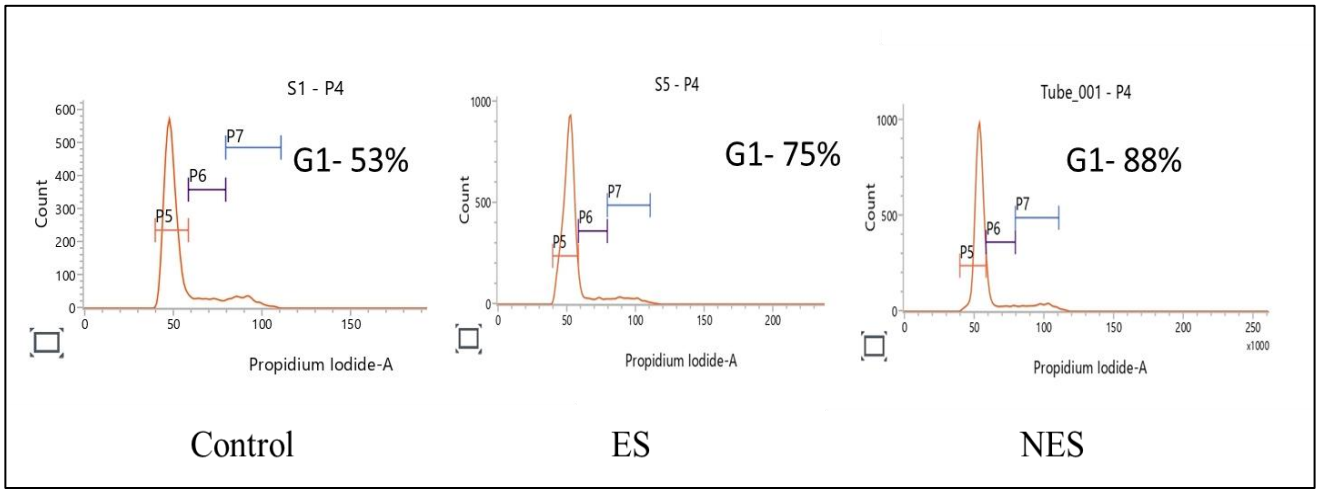


Figure 4.10. NES treated cells firstly showed higher G1 population.

4.4.5. ROS generation assay

Several studies have demonstrated that the chemotherapeutic agents induce ROS generation which consequently leads to oxidative damage and further sensitizes apoptosis. Excessive ROS triggers the apoptosis. We investigated the effect of ROS generation on ES and NES treated MDA-MB-231 cells were analyzed by flow cytometry using 2,7-dichlorofluoresceindiacetate (DCFH-DA), an oxidation-sensitive fluorescent dye. The shift in the peak confirmed increased production of ROS generation in the treated cells while NES treated cells more shift as compared to extract and control Figure 4.11. These results provide evidence that promotes apoptosis by oxidative stress because of production of ROS generation in a dose-dependent manner.

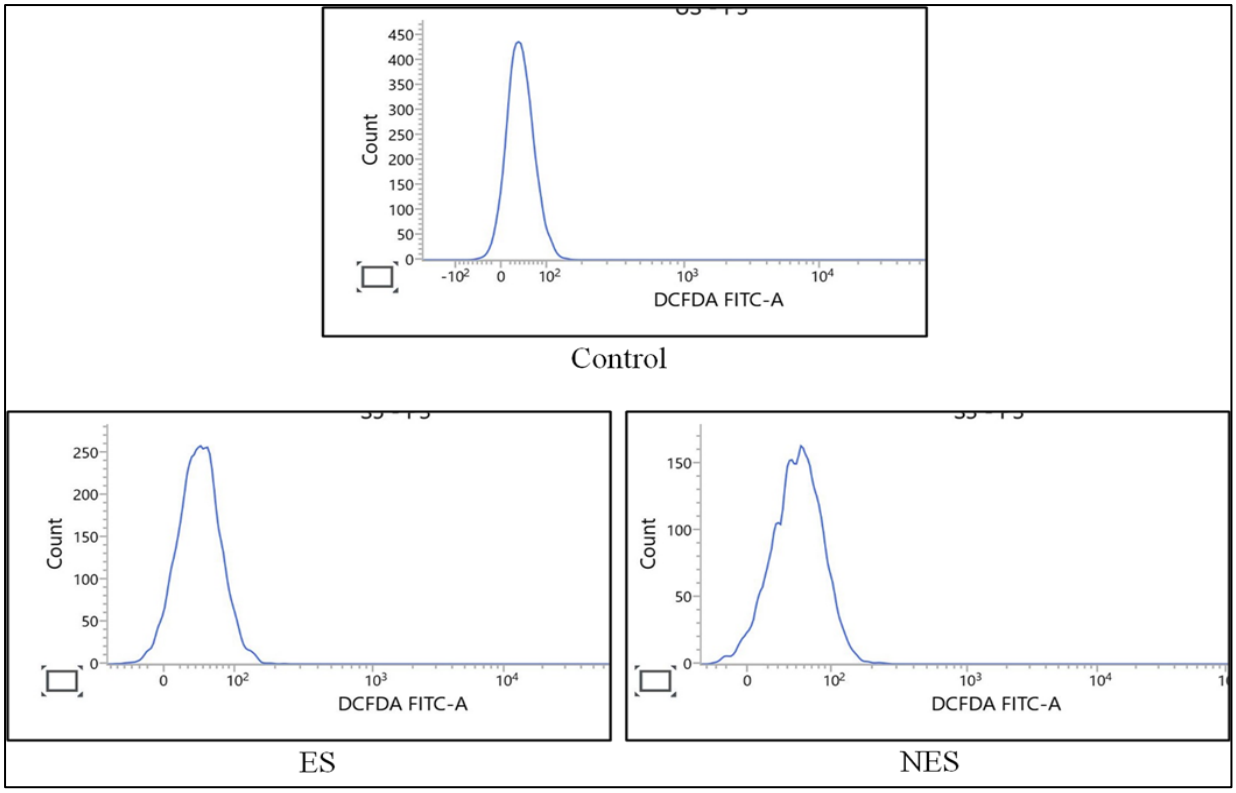


Figure 4.11. Effect of ES and NES on MDA-MB 231 on ROS production by DCFHDA dye using flow cytometry.

4.4.6. Mitochondrial Membrane Potential:

Mitochondrial membrane potential (MMP) ($\Delta\Psi_{mt}$) dissipation is the central mechanism for investigating initiation of drug-induced apoptosis. It has been reported that the intrinsic or mitochondrial pathway through death signals to mitochondria results in the release of mitochondrial intermembrane proteins such as cytochrome *c*, which associate with apoptotic protease-activating factor-1-Apaf-1 to form the apoptosome in order to activate caspase-3. In accordance, the change in the permeability of the mitochondrial membrane was accessed by flow cytometry. The cells treated with 10 µg/ml of NES and 20 µg/ml of ES. Loss of mitochondrial integrity is detected by decrease in fluorescence intensity. As shown in Figure 4.12. MDA-MB-231 treated with ES and NES revealed significant depolarization of mitochondria and loss of MMP in a concentration-dependent manner as indicated by a dramatic loss in the signal intensity.

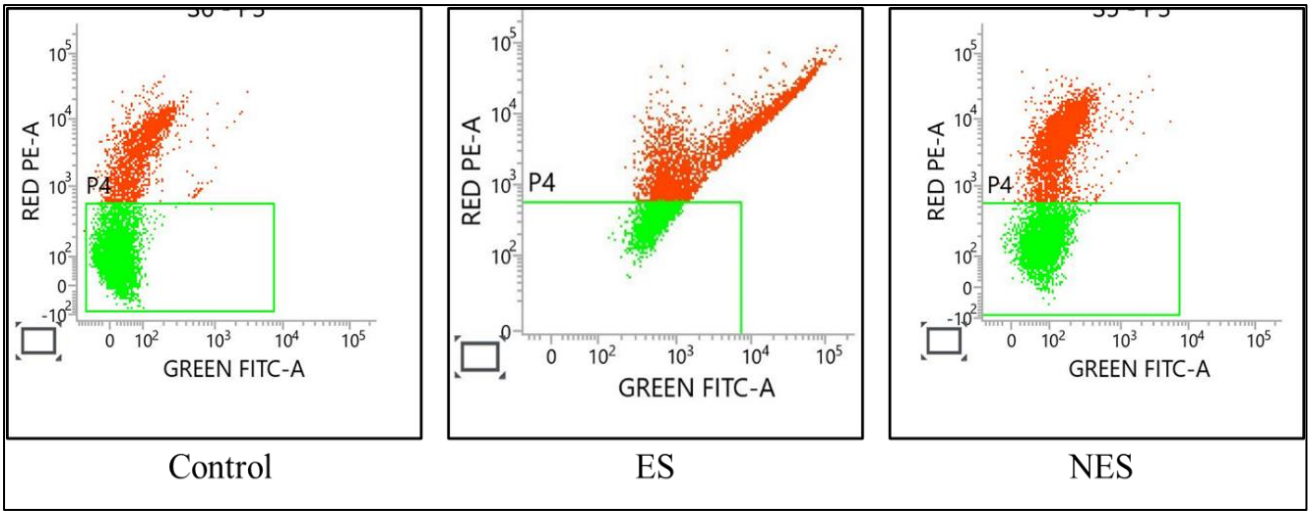


Figure 4.12. Loss of mitochondrial integrity in Control, ES and NES respectively.

5. CONCLUSION

The Soxhlet apparatus was used to effectively manufacture the *S. nigrum* leaf extract. Therapeutically significant chemicals are present in the aqueous extract, according to the metabolic profile. For the effective formulation of the oil-in-water nanoemulsion with an ideal range of necessary components, the aqueous titration technique was used. The three components needed to create a nanoemulsion—water, oil, and Smix—are represented in a ternary diagram to highlight their significance. Utilising the optimal concentration of Sefsol 280 (1.11%), Smix (3.89%), water (95.00%), and extract (10% w/v), *S. nigrum* loaded nanoemulsion (SNE) was successfully synthesised. The formed nanoemulsions were characterised based on their droplet size, PDI, potential, and TEM shape after undergoing a thermostability examination. The chosen nanoemulsion has shown the tiniest nanodroplet. The selected nanoemulsion has demonstrated the smallest nanodroplet size with neutral potential and uniform size dispersion. The nanoemulsion also has the sustained release of the drug along with the physical stability. Thus, the nanoemulsion was further continued for the evaluation of its cytotoxicity and apoptosis potential against breast cancer cell line. The MTT cytotoxicity showed a significant inhibition potential after the treatment with ES and NES in a dose and time dependent manner. From the analysis, it can be concluded that NES has the most potent and therapeutic action with the lowest IC50 value as compared to the NES and the control. The extract and nanoemulsion treatments resulted in a reduction in cell proliferation by halting the cell cycle at the G0/G1 stage. The treatment also demonstrated increased ROS production, altering the potential of the mitochondrial membrane. The DAPI fluorescence also supported the apoptosis potential of the ES and NES by leading to the nuclei condensation and fragmentation of the chromatin. Furthermore, the cumulative results showed that the nanoemulsion (NES) has significant cytotoxicity and apoptotic potential against the MD-MBA231 cell as compared to control and the *S. nigrum* leaf-based extract. In all, these results indicated a potential anticancer impact, providing a promise drug delivery method in the future for the treatment of breast cancer. Our study on the *S. nigrum* extract and its nanoemulsion indicated that comprehending traditional medical systems may be exploited to identify, produce, and commercialized novel, effective, bio-prospect and safe sources of pharmaceuticals.

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