INVESTIGATING THE IMPACT OF DIOSGENIN ON COLORECTAL CANCER DRUG RESISTANCE AND STEM CELL BEHAVIOUR

A dissertation submitted by

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Under the Guidance of

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Scientist-C

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DECLARATION

I, Nandana M, hereby declare that the project report, **"Investigating the impact of diosgenin on colorectal cancer drug resistance and stem cell behaviour,"** submitted by me to St.Teresa's College, Ernakulam, affiliated to Mahatma Gandhi University, Kottayam, Kerala, for the fulfillment of my degree in Master's of Vocational Studies in Food Processing Technology contents of this report are a record of my original work and have not been submitted for any kind of previous assessment, any other university from the period of my study. This record is carried out by me under the guidance of Dr. Ramesh Pothuraju, Ph.D. Scientist-C, Cancer Research Program, Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram-695014, Kerala, INDIA. This declaration confirms my academic integrity in this report and conveys the authenticity and originality.

Place: Thiruvananthapuram

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ABBREVIATIONS	EXPANSIONS
CSC	Cancer Stem Cell
CRC	Colorectal Cancer
5-FU	5-Fluorouracil
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
MAPK	Mitogen-Activated Protein Kinase
CIN	Chromosomal Instability
MSI	Microsatellite Instability
CINP	CpG Island Methylator Phenotype
KRAS	Kirsten rat sarcoma viral oncogene homolog
APC	Adenomatous Polyposis Coli
BRAF	v-raf murine sarcoma viral oncogene homolog B1 mutation
MGMT	O6-methylguanine-DNA methyltransferase gene promoter
MLH1	MutL homolog 1 gene promoter
MMR	DNA mismatch repair gene

ALDH	Aldehyde dehydrogenase
kDa	Kilodalton
TGF-β	Transforming Growth Factor-beta
BMP	Bone Morphogenetic Protein
CTNNB1	Catenin beta 1
MDM2	Murine Double Minute 2
COX	Cyclooxygenase
OVA	Ovalbumin
IgE	Immunoglobulin E
LPS	Lipopolysaccharide
HPLC	High-Performance Liquid Chromatography
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
DSG	Diosgenin
RPM	Revolutions Per Minute
PBS	Phosphate-Buffered Saline
RIPA	Radioimmunoprecipitation Assay.
BSA	Bovine Serum Albumin
Tris HCL	Tris(hydroxymethyl)aminomethane hydrochloride

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
APS	Alkaline Phosphatase
PVDF	Polyvinylidene Fluoride
PBST	Phosphate-Buffered Saline with Tween-20
ECL	Electrochemiluminescence
RLT	RNA lysis buffer
RPE	RNA Pre-Elution Buffer
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
AOAC	Association of Official Analytical Chemists

ABSTRACT

Formidable treatment obstacles arise from tumor progression as colorectal cancer precipitates cancer-related mortality worldwide with unrelenting ferocity. Cancer stem cells play a crucial, notoriously confounding role in tumor progression and therapy resistance within colorectal cancer research fields. Diosgenin exhibits significant anticancer properties modulating CSC pathways, and amplifies 5-Fluorouracil potency quite effectively in Dioscorea alata extracts. Diosgenin effectively scrutinizes CRC drug resistance and stem cell behavior by regulating tumor-promoting genes such as EpCAM or peculiar markers like CD133. Soxhlet extraction enabled fairly successful diosgenin extraction, and subsequent acid hydrolysis yielded thoroughly purified compounds for downright rigorous biological evaluation afterwards. Diosgenin downregulated CSC markers remarkably in tandem with 5-FU while upregulating P53; thus, apoptosis was significantly enhanced, and tumor cell proliferation was reduced drastically. Diosgenin exhibits beneficial effects in tandem with 5-FU, thereby hinting at some potentially novel therapeutic strategy against stubborn CRC treatment resistance. Diosgenin-based treatments for colorectal cancer are underscored rather positively by this research study, highlighting natural substances' quite effective efficacy in cancer therapy.

CHAPTER 1

INTRODUCTION

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Colorectal cancer ranks among the leading causes of cancer-related deaths worldwide, with rising rates and increasing resistance posing significant challenges (Siegel et al., 2020). Advancements in chemotherapy, including the use of 5-fluorouracil, often fail to overcome the emergence of stubborn drug resistance in cancer stem cells (Lao et al., 2021). Cancer stem cells avoid apoptosis rather handily and survive cytotoxic treatments, which play a substantial albeit confounding role in tumor initiation and metastasis (Reya et al., 2001). The emergence of natural compounds like diosgenin has gained popularity somewhat rapidly owing largely to their role in targeting CSCs and overcoming stubborn drug resistance (Wang et al., 2019). Diosgenin's impact on CRC is investigated here with special emphasis on effects related to CSC pathways and acquired drug resistance mechanisms.

Diosgenin, a steroidal sapogenin, occurs naturally in Dioscorea species and fenugreek and has been studied deliberately for various pretty significant health benefits, including anti-inflammatory, antioxidant and anti-cancerous properties (Patel et al., 2020). Diosgenin's anti-cancerous properties have been revealed quite strikingly in several malignancies where it heavily modulates key signaling pathways involved in cell proliferation, metastasis and cell death (Raju & Mehta, 2009). It is found that the Wnt/β-catenin signaling pathway, which is a specific controller of CSC maintenance and chemotherapy resistance in CRC has been especially inhibited by diosgenin (Zhou et al., 2021). Diosgenin’s ability to downregulate CSC markers such as CD44, CD133, and EpCAM and to upregulate tumor suppressor gene P53 has further emphasized its pharmaceutic potential (Shishodia et al., 2006).

5-FU is considered a keystone in CRC chemotherapy, chiefly presenting its effects by hindering thymidylate synthase and including its metabolites in RNA and DNA, causing the arrest of cell cycle and apoptosis (Longley et al., 2003). Although, a major hurdle to successful treatment, is the 5-FU drug resistance, which alters apoptotic pathways, enhances DNA repair mechanisms, and the existence of drug-resistant CSCs (Meyers et al., 2018). As the cancer cells show resistance to the 5-FU drug, a pathway called the Wnt/β-catenin pathway is often involved, in which the upregulation of β-catenin aids CSC viability and tumor growth (Koveitypour et al., 2019). Diosgenin helps block this pathway and suggests that it could be a better way to use diosgenin along with 5-FU to overcome the drug resistance, improving the drug's effectiveness.

Malignant cells with aberrant self-renewal capacities and stubborn therapy resistance exist as a peculiar subpopulation harboring cancer stem cells (Batlle & Clevers, 2017). Various surface markers are often harnessed quite liberally nowadays to pinpoint cancer stem cells fuelling colorectal cancer's nasty progression, metastasis, and stubborn therapy resistance, including CD44 or EpCAM (Zhang et al., 2017). The downregulation of those markers is correlated with a decrease in tumorigenicity and an enhanced therapy response (Zeuner et al., 2014). Previous studies suggested that diosgenin inhibits the properties of CSCs by downregulating these markers and inhibiting important signal pathways (Cheng et al., 2019). In addition, p53, a well-known tumor suppressor, is an important modulator of apoptosis and genomic stability. Often it is linked with improved chemosensitivity and tumor suppression and is upregulated (Vousden & Lu, 2002). This research is focused on the changes of major molecular markers med to check if diosgenin together with 5-FU can alter expression, thus possibly overcoming mechanisms of resistance instigated by CSCs.

Prior studies have shown multiple molecular pathways through which diosgenin carries out its anticancer activities. Diosgenin inhibits the Wnt/β-catenin pathway and modulates NF-κB, PI3K/Akt and MAPK pathways that regulate cancer progression quite extensively and chemoresistance (Shanmugam et al., 2018). Diosgenin triggers apoptosis through these pathways and dampens inflammation while utterly suppressing cell proliferation rather vigorously (Liu et al., 2017). Diosgenin induces mitochondrial damage and reactive oxygen species production thereby significantly contributing to cytotoxic effects on various cancer cells rather robustly (Wang et al., 2015). The combination of these mechanisms of action furthers the case for including diosgenin as an adjuvant in colorectal cancer treatment, especially when used in conjunction with traditional chemotherapy drugs such as 5-FU.

Challenges posed by traditional CRC treatments like stubborn cancer stem cells and acquired chemoresistance necessitate a radical shift in therapeutic approach altogether nowadays. The research herein focuses sharply on the impact of diosgenin treatment on CSC activity and the enhancement of 5-FU effects profoundly on colorectal cancer cells. This study aims to explore the underlying molecular mechanisms leading to the anticancer effect of diosgenin, by examining the expression levels of major oncogenes (CD133, CD44, EpCAM, and β-catenin) as well as the tumor suppressor oncogene P53. This study helps to offer additional supporting information for the possible role of diosgenin as an adjuvant in the treatment for colorectal cancer.

Colorectal cancer is a major challenge in chemotherapy because of its high recurrence rate and resistance to traditional treatments. Diosgenin, a bioactive phytochemical with adequate anticancer potential, has also become a promising candidate for the management of CSC-mediated chemoresistance. Diosgenin targeting of key signaling pathways and modulation of CSC marker expression hold promise to improve therapeutic outcomes in CRC. Growing evidence supports natural compounds in cancer therapy quite extensively and future translational research will likely stem from such findings.

1.1 OBJECTIVES

- To investigate the effects of diosgenin on drug resistance in colorectal cancer cells. This involves whether to distinguish if diosgenin can increase or enhance responsiveness to the resistant cancer cells to chemotherapy.
- To evaluate the influence of diosgenin on the colorectal cancer stem cell behaviour. This involves looking at the self-sustainability and differentiation capacity of the cancer stem cells.

1.2 SCOPE OF THE STUDY

The scope of this research focuses on the evaluation of the therapeutic potentials of diosgenin in regulating drug resistance and cancer stem cell behaviour in colorectal cancer. The research is limited to vitro analysis using the established colorectal cancer cell. Key areas in this research includes, Investigation of drug resistance markers, Evaluation of CSC markers and Evaluation of diosgenin's cytotoxic and anti-proliferative effects on colorectal cancer cells.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 COLORECTAL CANCER

Colorectal cancer originates in either the colon or rectum and is classified as colon cancer or rectal cancer, respectively. These cancers often get lumped together owing largely to similarities between them. Clinical presentation manifests differently, owing largely to tumor site size and the presence of metastasis in various bodily regions. Symptoms manifest quite irregularly, including severe abdominal pain and nausea alongside rather persistent alterations in bowel habits and stool consistency. Tumors in the distal colon frequently manifest with overt rectal bleeding, whereas proximal tumors sometimes result in occult bleeding, precipitating anemia as a secondary complication.(Granados-Romero et al., 2017).

2.2 EPIDEMIOLOGY

Colorectal cancer ranks third among cancers diagnosed in both sexes, excluding non-melanoma skin cancer, accounting for 9.7% of all cancer cases. Over half of the cases are found in more developed regions. Colorectal cancer incidence rates vary globally, with men experiencing higher rates of 20.6 per 100000 and women having lower rates of 14.3 per 100000 individuals (Kuipers et al., 2015). Mortality rates rank second, with nearly 9.2% of deaths attributed largely to CRC. Projections indicate a rather stark 71.5% surge in colon cancer deaths and a 60% hike in rectal cancer fatalities by 2035 (Douaiher et al., 2017). This increase in morbidity is influenced by factors such as lifestyle, body fatness, and dietary habits. Strong evidence is that physical activity provides a protective effect, while frequent consumption of red and processed meats and alcohol raises the risk of developing the disease(Sawicki et al., 2021). Diet patterns in terms of the westernization of lifestyle are one of the factors that have changed along with the socioeconomic advances in civilization and the development of the economy. The global nutrition transition describes the upsurge in the consumption of animal fats, processed meats, refined grains, and sweets, decreased dietary, fruit and vegetable intake, and physical inactivity. Such a lifestyle produces obesity, a risk factor for most chronic diseases, particularly in men. Especially visceral obesity has been reported to influence CRC prognosis(Murphy et al., 2019; Sawicki et al., 2021).

2.3 MECHANISM

Tumorigenesis is promoted thoroughly by genetic and epigenetic alterations in colorectal cancer. Genetic instability in CRC manifests through assorted, notably aberrant pathways, namely chromosomal instability, microsatellite instability, and CpG island methylator phenotype pathways (Malki et al., 2021a).

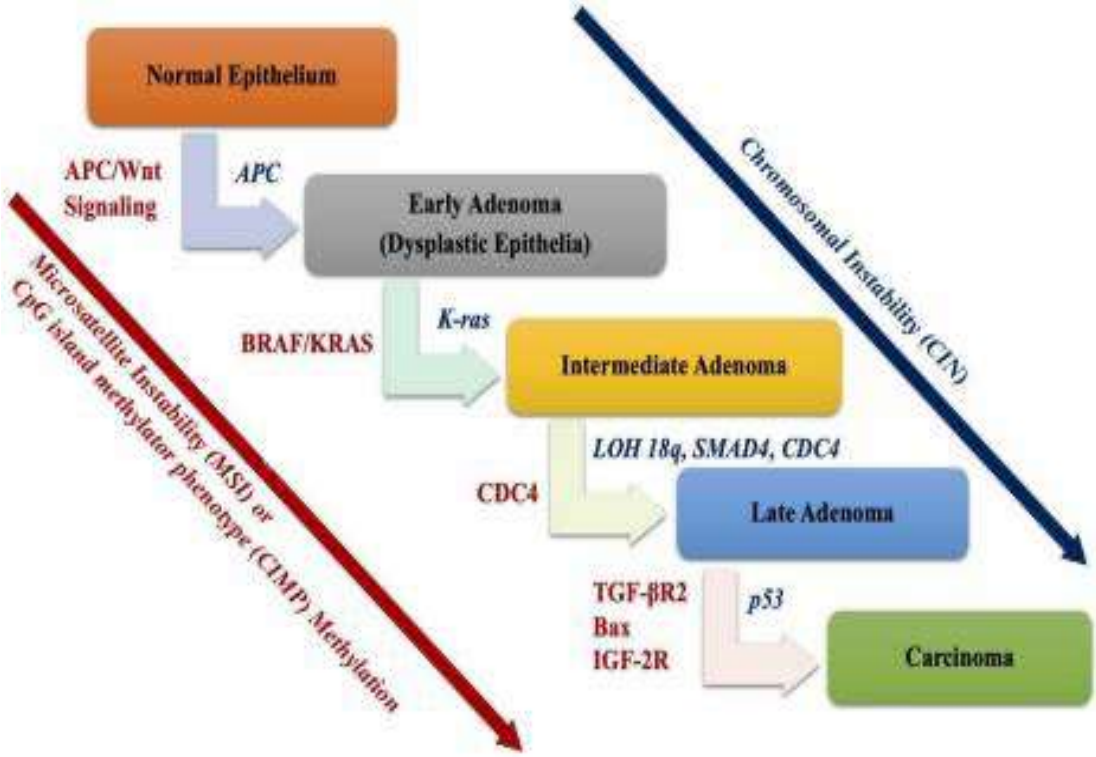


Figure 1

The progression of colorectal adenocarcinoma follows a multistep genetic model regulated by three key pathways: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) hypermethylation. Source: (Malki et al., 2021b)

Colorectal cancers arise through one or a combination of three primary mechanisms: chromosomal instability, CpG island methylator phenotype, and microsatellite instability. Mutations in the adenomatous polyposis coli gene kickstart the classical CIN pathway, followed by KRAS oncogene activation and TP53 tumor suppressor gene inactivation subsequently. CIN tumors, accounting for roughly 85 percent of sporadic CRC cases, exhibit marked aneuploidy and significant loss of heterozygosity. Familial adenomatous polyposis involving germline APC mutations is also linked with them rather extensively in many such cases. Hypermethylation of tumor suppressor gene promoters, especially MGMT and MLH1, characterizes the CIMP pathway frequently linked to BRAF mutations and MSI. Meanwhile MSI pathway arises from the inactivation of DNA mismatch repair genes, a hallmark feature of familial Lynch syndrome. This mechanism occurs in roughly 15% of sporadic CRCs and is

influenced by hypermethylation of MMR genes quite frequently. MSI tumors tend to be poorly differentiated, particularly in the proximal colon, and are generally associated with quite a favorable prognosis. These three pathways frequently overlap somewhat in CRC cases found pretty convincingly already (Tariq & Ghias, 2016).

2.4 CANCER STEM CELLS

Cancer stem cells play a pivotal albeit somewhat obscure role in metastasis and recurrence of colorectal cancer, rather mysteriously. CSCs embody a peculiar subset of malignant cells marked by self-renewal capabilities, extremely high division rates, and erratic differentiation patterns. Colorectal CSCs are identified by specific surface markers, including CD44 and CD133, and EpCAM or LGR5 and ALDH activity. They exhibit high tumorigenicity and resistance to chemotherapy and radiation, making them crucial players in cancer progression, relapse, and disease-free survival overall. This review furnishes a revised snapshot of colorectal CSCs, focusing heavily on involvement in tumor initiation and resistance to certain drugs (Y. Zhou et al., 2018).

2.4.1 CD44

The CD44 gene encodes a receptor known as CD44, which binds hyaluronic acid quite readily in various cellular contexts. This transmembrane glycoprotein plays a crucial role, quite remarkably, in regulating cell interactions and cell migration extensively in various contexts (Spring et al., 1988). CD44-positive colon cancer cells proliferate prolifically and form robust colonies while exhibiting profound resistance to apoptosis and remarkable insensitivity to chemotherapy and radiotherapy versus CD44-negative cells (Wang et al., 2012). CD44 silencing via short hairpin RNA noticeably reduces cell proliferation, migration, and invasion while concurrently inhibiting apoptosis somewhat effectively. Bax expression surged in HCT116 colon cancer cells with CD44 silenced, and Bcl-2 levels plummeted, resulting in cleavage of caspase-3 and PARP. CD44 potentially serves as a therapeutic target for colorectal cancer. Yonder usually resides quietly underneath rather obscure aliases (S. Y. Lee et al., 2017).

2.4.2 CD133

AC133 or prominin-1 is a transmembrane glycoprotein specifically found in various cell types, including hematopoietic cells, endothelial cells, and neuroepithelial cells (Li et al., 2012). CD133-positive colorectal cancer cells exhibit key stem cell properties, including self-renewal and differentiation potential in multiple directions quite readily (Dalerba et al., 2007). CD133 expression has been associated with the differentiation of colorectal cancer cells and larger tumor size in primary colorectal CSCs specifically (Kazama et al., 2018). CD133-positive

colorectal cancer cells display stubborn resistance against chemotherapy and radiotherapy quite effectively under certain clinical conditions (Chinese Journal of Cancer, n.d.). Some studies have reported conflicting findings, suggesting that CD133-negative cells exhibit greater aggressiveness, quite remarkably, in certain circumstances (O'Brien et al., 2007; Shmelkov et al., 2008).

2.4.3 EpCAM

EpCAM is a 40 kDa single transmembrane protein encoded by the tumor-associated calcium signal transducer-1 gene quite irregularly in various contexts. Cell migration and proliferation are heavily influenced by it, playing a crucial role in signal transduction mediated by intercellular adhesion differently (Trzpis et al., 2007). EpCAM fosters carcinogenesis in epithelial cells pretty significantly by activating proto-oncogene expressions like c-myc and cyclin A/E (Maetzel et al., 2009). EpCAM inhibits antigen presentation by dendritic cells pretty effectively, enabling evasion of CD4+ T cell-dependent immune surveillance somehow (Y. Zhou et al., 2016). EpCAM amplifies canonical WNT/β-catenin signaling quite vigorously through intra-membrane proteolysis, facilitating nuclear translocation of its intracellular C-terminal domain. Interaction fosters crosstalk amongst Notch, Hedgehog, and TGFβ/BMP signaling pathways, forming regulatory networks influencing stem cell signals and modulating the expression of key cancer stem cell markers deeply (Kumar et al., n.d.).

2.4.4 β-Catenin

β-Catenin functions as both an adhesion and signaling molecule, playing a crucial role in colorectal tumor development when dysregulated. β-Catenin serves as an adhesion molecule and signaling entity, playing a crucial albeit largely dysregulated role in the development of colorectal tumors. β-catenin, a 92-kDa protein encoded by the CTNNB1 gene, contains 13 Armadillo repeats, facilitating various molecular interactions to a greater extent. β-catenin oddly exhibits profound conservation across species, showing surprisingly over 80% homology with Armadillo in Drosophila, and regulates intercellular adhesion in normal cells by mysteriously forming a complex with E-cadherin and α-catenin at adherens junctions. It plays a role in Wnt signaling, where the APC tumor suppressor tightly regulates its levels within the cytoplasm. Mutations in APC, often found in colorectal cancer, impair β-catenin degradation, leading quickly to accumulation and translocation into the nucleus. β-catenin interacts with Lef-Tcf transcription factors in the nucleus, activating genes that promote proliferation and tumor invasion quite rapidly. Mutations in the CTNNB1 gene anomalously disrupt the

regulation of β-catenin, thereby vigorously enhancing Wnt signaling pathways and driving rapid tumorigenesis (Ilyas et al., 1997).

2.4.5 p53

p53 acts as a key tumor suppressor, coordinating various cellular processes like DNA repair, cell cycle arrest, and apoptosis fairly effectively. p53 levels typically stay remarkably low owing largely to polyubiquitination affected by E3 ubiquitin ligase MDM2 under fairly normal circumstances. In response to stress signals like DNA damage and aberrant growth cues, p53 interacts with MDM2, gets severely disrupted, leading rapidly to stabilization of p53. p53 gets regulated variously by transactivating target genes, primarily thereby allowing diverse cellular responses. The effects of p53 activation are determined by dynamic regulation and interactions with various proteins under post-translational modifications. p53 function gets frequently compromised in human cancers owing largely to its crucial tumor suppression role. TP53 mutations are present in 43% of colorectal cancer tumors, while many remaining cases exhibit impaired p53 activity due to alterations in ATM or DNA-PKcs. TP53 mutations in CRC are mostly missense mutations leading to loss of wild-type p53 function and sometimes acquiring novel oncogenic properties, gain-of-function. Mutations can weirdly boost cancer cell stemness and proliferation, vigorously driving cancer progression, ultimately through enhanced invasion and far-reaching metastasis (Liebl & Hofmann, 2021).

2.5 DIOSCOREA

Figure 2 Dioscorea

Dioscorea species belong to the family Dioscoreaceae and are commonly known as yams worldwide in various tropical regions. This genus encompasses over 600 species scattered haphazardly across Africa and Asia, and the Caribbean islands in the South Pacific. Yams serve as vital carbohydrate sources and supplements, owing partly to their unique organoleptic characteristics.

Aerial and underground tubers supply vital nutrients, including proteins and vitamins, making them a staple food for many people in West Africa. Dioscorea plants are rich in secondary metabolites like steroids and clerodane diterpenes, and various other compounds, including nitrogen-containing phenolics. Bioactive compounds in yams possess rather significant medicinal properties and exhibit profound pharmacological effects quite remarkably. Notable Dioscorea species comprise Dioscorea oppositifolia, also known as Chinese Yam, used in traditional medicine for digestive health, and Dioscorea alata (Purple Yam), known for its antioxidant-rich purple flesh. Meanwhile, Dioscorea esculenta (Lesser Yam) is a smaller and easily digestible variety, and Dioscorea bulbifera (Air Potato) produces aerial tubers and finds use in herbal medicine. Dioscorea villosa (Wild Yam) serves as the source of diosgenin a precursor for steroid hormone synthesis. Major food yams cultivated in West Africa are Dioscorea rotundata and Dioscorea cayenensis, obviously (Salehi et al., 2019).

Figure 3 Dioscorea alata

Dioscorea alata, also known as water yam, purple yam, ube, or greater yam, is globally significant, belonging to the Dioscoreaceae family pretty much everywhere around the tropics nowadays. Tropical South America, Africa, Australia, and the Southeastern U. S. cultivate it extensively, and people know it for its very diverse healing properties. D is loaded with saponins, proteins, flavonoids, and phenolic compounds alongside glycosides, alkaloids, and tannins. Alata exhibits antioxidant and anti-inflammatory properties, somewhat surprisingly

alongside markedly anti-apoptotic and cardioprotective activities in various instances. Diosgenin figures prominently among active constituents exhibiting markedly anti-diabetic properties and heart-protective effects alongside anti-hypertensive and anti-cancer activities. Oxidative stress gets substantially reduced while glutathione superoxide dismutase and catalase activity get markedly enhanced, and caspase-9 activation gets suppressed, thereby inhibiting apoptosis. Anthocyanins and diosgenin, alongside dietary fiber, help regulate blood lipid levels, thereby managing hyperlipidemia effectively under certain circumstances. Further research seems necessary for establishing the effects of various cardiovascular disorders pretty thoroughly nowadays (Kaur et al., 2021).

Kingdom	Plantae
Phylum	Tracheophyta
Class	Liliopsida
Order	Dioscoreales
Family	Dioscoreaceae
Genus	Dioscorea
Species	*Dioscorea alata*

Table 1 Taxonomical classification of Dioscorea alata

2.6 DIOSGENIN

Diosgenin, a steroidal sapogenin and isoprostane derivative, is produced via acidic hydrolysis or enzymatic breakdown of dioscin and protodioscin (Arya et al., 2023). As an important precursor for steroidal drugs, this drug is gaining interest and attention from researchers and industries all over the world. The upsurge in need is due to the beneficial impacts it provides when treating chronic ailments like high strokes, cholesterol (Y. Zhang et al., 2009), depression, Alzheimer's disease, tumors (B. Cai et al., 2020a), diabetes, inflammation, leukemia, climacteric syndrome, thrombus, malignancy, and many other forms of metabolic diseases (Arya et al., 2023). Diosgenin inhibits cancer cell growth or slows it significantly in mechanistic in vitro studies conducted extensively with varied parameters. Only a few studies have sporadically explored its potential in treating various forms of cancer fairly extensively so far. The average annual demand for diosgenin globally exceeds four thousand tons heavily every single year (Yu et al., 2019). Dioscorea species, Heterosmilax, and Trigonella foenum-graecum serve as primary sources of diosgenin, existing mainly in saponin form naturally (B. Cai et al., 2020a). The presence of glucose or rhamnose forms a C-O glycosidic bond with aglycone (Yu et al., 2019), and diosgenin gets released via the hydrolysis of steroidal saponins

rapidly (H. Yang et al., 2016). Advanced extraction techniques like UAE and MAE have been highlighted lately in studies for diosgenin extraction from Trigonella foenum-graecum seed batches effectively. Diosgenin was spray-dried rather effectively using a binary blend comprising maltodextrin and whey protein concentrate for creating effective delivery systems in varied food matrices (Arya & Kumar, 2022a). Diosgenin's therapeutic application remains quite challenging, owing largely to poor water solubility and rapid physiological transformation occurring rather quickly (Pawar, 2020).

2.6.1 DIOSGENIN: CHEMISTRY AND BIOSYNTHETIC PATHWAY

Steroids comprise four fused rings altogether, having 17 carbon atoms featuring diverse functional groups appended rather haphazardly around a saturated tetracyclic hydrocarbon skeleton. Steroidal sapogenins in plant matrices exhibit similarities with triterpenoids but occur mostly within the monocotyledonous Dioscoreaceae family rather than haphazardly (Sparg et al., 2004). Steroidal sapogenins serve as side chains of cholesterol molecules undergoing various modifications, forming two fundamental structures, namely furostane, having 26 carbon units in a five-ring structure, and spirostane, boasting a larger six-ring structure with 27 carbon units. Structural variations of spiro stanols depend heavily on multifaceted chemical modifications and positioning of attachments, mostly at C-5 or C-25 (Arya et al., 2023).

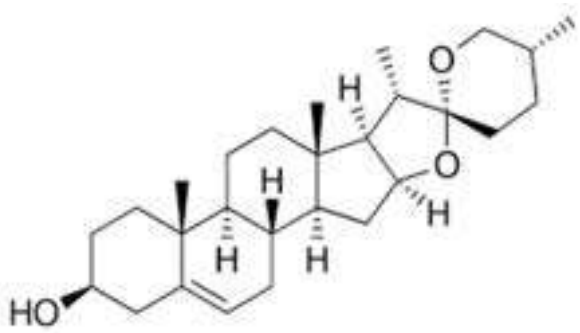


Figure 4: Structure of Diosgenin

Source: (Patel et al., 2012)

Sugar molecules attached haphazardly to the aglycone backbone heavily influence the formation and quirky characteristics of functional groups, yielding diverse compounds rapidly. Steroids get lumped loosely into categories like sterols and brassinosteroids, and sometimes sapogenins are thrown in for good measure. Spirostanols arise from sugar molecules binding at carbon-3, and spiroketal arrangements emerge from bindings at carbon-22 rather curiously.

Furostanol glycosides have an open sugar chain attached at C-26 rather than being stuck on C-3, typically anyway. Further classification hinges on the number and structure of sugar chains attached rather haphazardly to the main structure in types like monodesmosidic and tridesmosidic. Saponin type depends largely on the placement of sugar chains, producing monodesmosidic saponins with one sugar chain at C-3 and bidesmosidic saponins having two sugar chains (Arya et al., 2023). Diosgenin shares structural similarities with cholesterol molecules, somewhat mysteriously in its steroidal configuration. A sterane skeleton attaches at positions 16 and 17 with a double bond that is weirdly situated between positions 5 and 6. Diosgenin usually binds quite readily with some hydroxyl group, thereby facilitating linkage pretty effectively with various sugar molecules (Ondevilla et al., 2021).

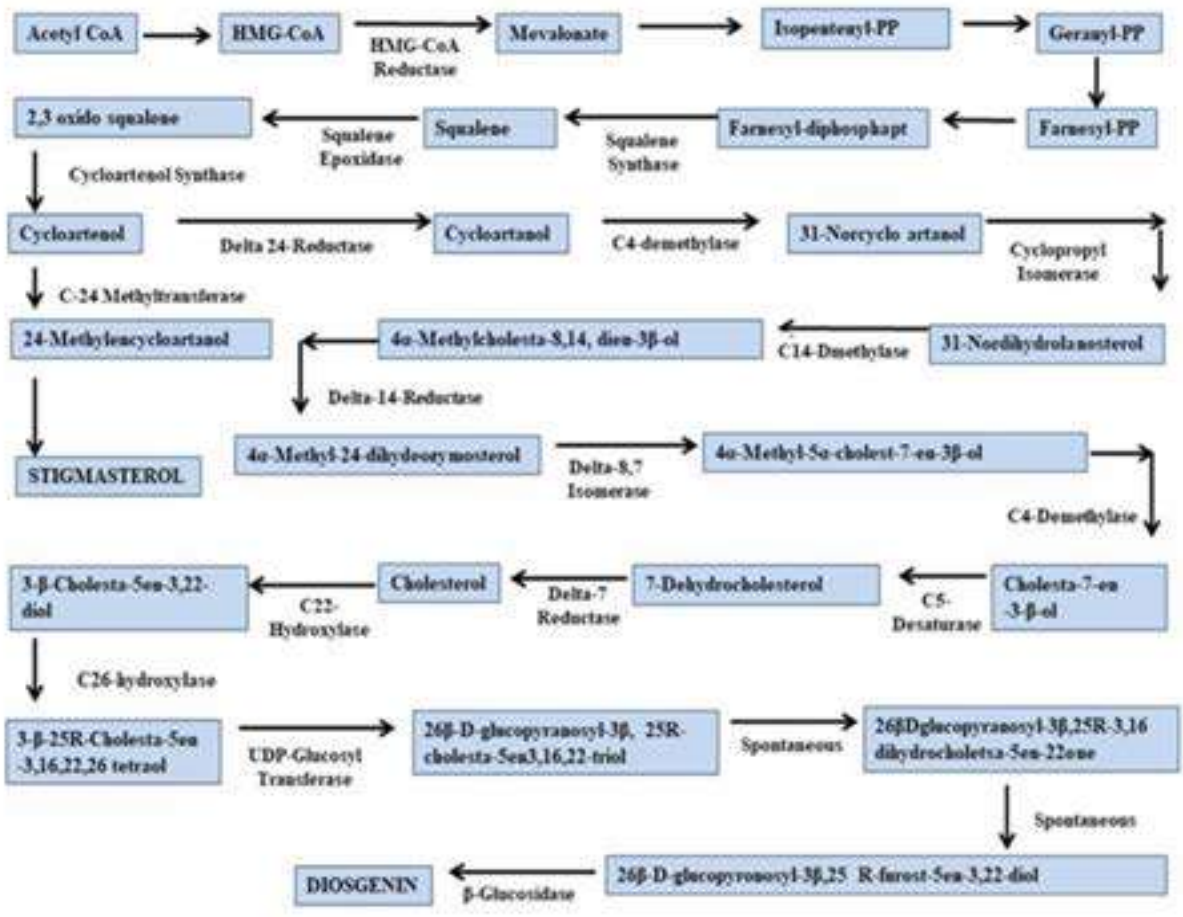


Figure 5: A structured biosynthetic pathway for diosgenin production.

Source:(Arya et al., 2023)

Diosgenin emerges as a steroidal compound synthesized via a mevalonate pathway beginning with acetyl coenzyme A or acetyl-CoA quite rapidly (Czabotar et al., 2014). Diosgenin

biosynthesis proceeds mainly through the conversion of cycloartenol (Mortenson et al., 2007) or lanosterol into cholesterol via two somewhat distinct primary pathways (Vitagliano et al., 2013). Diosgenin production gets optimized fairly quickly via sundry biosynthetic routes encompassing lanosterol pathways and cytosterol and cycloartenol intermediary routes (Chaudhary et al., 2015).

Diosgenin biosynthesis entails crucial enzymes like lanosterol synthase and cycloartenol synthase, belonging rather peculiarly to oxidosqualene cyclase family (Ciura et al., 2017). Squalene epoxidase and squalene synthase genes play crucial roles in converting farnesyl pyrophosphate molecules into squalene with remarkable efficacy, rather mysteriously (P. Kumar et al., 2008). . Diosgenin biosynthesis lately involves a rather convoluted multistep conversion process of acetyl-CoA, ultimately yielding cycloartenol, largely facilitated by the delta-24-reductase gene (Mohammadi et al., 2020). The mevalonate and 2-C-methyl-D-erythritol 4-phosphate pathways pretty much yield isopentenyl diphosphate in cells through somewhat differing biochemical routes (Augustin et al., 2011).

Isopentenyl diphosphate and dimethylallyl diphosphate undergo condensation fairly quickly, catalyzed vigorously by isopentenyl diphosphate isomerase, initiating diosgenin synthesis rather mysteriously. This reaction precipitates the formation of farnesyl phosphate quite rapidly a recognized precursor obviously of diosgenin biosynthesis in certain cells (Vincken et al., 2007). The breakdown of furostanol glycoside by 26-O-β-glucosidase facilitates the final step in diosgenin production, transforming deglycosylated furostanol rather quickly into diosgenin (Jean, 2005).

Diosgenin biosynthesis occurs naturally in fenugreek and Dioscorea zingiberensis apparently via some rather convoluted biochemical pathway. Early diosgenin formation involves stereospecific hydroxylation at C22 by certain 16-oxygenases, which was confirmed through mediation of 1S 22S-dihydroxy cholesterol (C. Zhou et al., 2022). Another study shows that diosgenin biosynthesis occurs in Himalayan Paris and fenugreek, involving cytochrome P450, which catalyzes the oxidative 5,6-spiroketalization of cholesterol vigorously (Christ et al., 2019). Multiple genes and enzymes ostensibly catalyze the conversion of cholesterol into diosgenin subsequently as crudely illustrated in Fig.5

2.6.2 THERAPEUTIC APPLICATIONS OF DIOSGENIN

Diosgenin has been extensively researched by multiple scientists for various pharmacological purposes in several recent studies published in the scientific literature (Parama et al., 2020). Diosgenin has garnered quite a bit of attention lately, owing largely to its remarkable bioactivity in treating assorted medical conditions effectively nowadays. Beneficial effects have been observed in managing allergic diseases and obesity, as well as diabetes, with some menopausal symptoms and skin aging occurring simultaneously. It protects very effectively against cardiovascular maladies, most significantly in most cases alongside atherosclerosis and notably cancer and thrombosis (Jesus et al., 2016). Diosgenin is applied nowadays fairly frequently in the treatment of major diseases. They comprise a list starting with:

2.6.2.1 MELANOGENESIS

Excessive melanin accumulates rapidly, leading to various hyperpigmentation conditions like melasma or post-inflammatory melanoderma and solar lentigines simultaneously elsewhere. Recently, fairly significant inhibition of melanin production by diosgenin derived from yam tubers and fenugreek seeds has been found. Diosgenin supplementation naturally and synthetically bolsters assorted anti-aging strategies pretty significantly nowadays within obscure research realms (Nigam, n.d.). Depigmenting properties radically suppress melanogenesis via modulation of the phosphatidylinositol-3-kinase pathway in a manner that is ordinarily quite effective somehow. Diosgenin pretty effectively thwarts the reduction of GSk3β and Akt phosphorylation otherwise induced by LY294002, a PI3K inhibitor somehow in cells. Diosgenin significantly boosts tyrosinase and microphthalmia-associated transcription factors, further pretty effectively inhibiting melanogenesis quite heavily and hyperpigmentation (J. Lee et al., 2007).

2.6.2.2 DIABETES

Yam tubers and fenugreek seeds bursting with diosgenin exhibit remarkably potent anti-diabetic properties in fairly recent cutting-edge medical research studies. Diosgenin seems pretty useful in regulating metabolic parameters such as blood glucose levels, making it a rather promising candidate for diabetes management nowadays, effectively. Studies reveal that anti-diabetic properties significantly lower plasma glucose levels in diabetic rats very effectively under certain conditions (Raju & Rao, n.d.). Commercially available diosgenin intake aids diabetes prevention by drastically reducing the activity of disaccharides, somewhat effectively in many instances. Diosgenin noticeably ramps down sucrose activity and ramps up lactase

activity and maltase activity, benefiting diabetes treatment significantly every day (Nigam, n.d.).

2.6.2.3 ANTI-CANCER ACTIVITIES

Cytotoxic agents ravage tumor cells, disrupting cell cycle machinery pretty quickly, leading to apoptosis or cell death. Diosgenin manifests markedly anti-cancerous properties by thoroughly inhibiting cell proliferation and reducing the viability of breast cancer cells MEF-7 somewhat effectively. Apoptosis is induced via the caspase pathway, while enzymes such as cyclooxygenase COX heavily influence the conversion of arachidonic acid into prostanoids quite significantly involved in apoptosis and inflammation. Diosgenin's impact on COX expression and activity has been probed fairly extensively in osteosarcoma cells lately, with intriguing results (Nigam, n.d.). Diosgenin showed remarkably potent anticancer activity against S-180 Hep A in mice during various animal studies. It suppresses the proliferation of 1547 cells by arresting the cell cycle in the G1 phase and inducing apoptosis heavily downstream somehow. Diosgenin suppresses receptor-activated nuclear factor-kappa B, thereby regulating gene expression and markedly boosting apoptosis induced by sundry cytokines or chemotherapeutic agents (Patel et al., 2012). Diosgenin exhibits anti-tumor effects in glioblastoma cells, reducing migration and angiogenesis significantly while promoting differentiation pretty effectively (Khathayer & Ray, 2020). It exhibits anti-inflammatory properties by heavily suppressing intestinal inflammation, including diarrhea and mast cell infiltration, and degranulation in the duodenum. Diosgenin markedly reduces OVA-specific IgE generation and total serum IgE according to various clinical studies (Patel et al., 2012). Anti-cancer properties of diosgenin have been seen against breast, liver, and colon cancer by suppressing S-phase kinase-associated protein-2 and modulating cell receptors p38 and tumor suppressor p53 (Arya & Kumar, 2022b). Diosgenin exhibits antimicrobial activity against various human pathogenic yeasts like Candida albicans and other species of Candida rather extensively. Glabrata and C lurked ominously. Tropicalis sounds kinda quirky and deeply embedded in utterly tropical settings, or so it rather vaguely seems. It inhibits hepatitis C virus replication at low micromolecular concentrations, drastically reducing viral RNA and protein levels somewhat too. Dioscorea yams have been linked rather tenuously to the regulation of inflammatory oxidative autophagic processes in ischemia/reperfusion injury management through some targeted protein interactions. This aligns rather neatly with food-based therapeutic approaches and broader notions of pretty green health-conscious treatment strategies, surprisingly (X. Zhang et al., 2021).

2.6.2.4 VASODILATING ACTIVITY

Diosgenin acts as a precursor in the industrial synthesis of hormones like norethisterone and progesterone, mostly from some specific plant sources. Research on its effects has shown its ability to trigger the swift relaxation of coronary arteries independently of the endothelium pretty rapidly nowadays (Dias et al., 2007). Diosgenin downregulates inflammatory response somewhat mysteriously in vascular endothelial cells by triggering L-type calcium channels to mediate extracellular Ca^{2+} influx through the $1,25D_3$-MARRS receptor/ERp57 pathway (W. S. Yang et al., 2017).

2.6.2.5 NEUROPROTECTIVE ACTIVITY

Prevalence of HIV infection has been rising steadily, contributing to various dementing illnesses, alleviated somewhat with controlled doses of diosgenin orally. Research on various novel antioxidants indicates L-deprenyl and diosgenin offer considerable neuroprotection against neurotoxicity induced by Tat and morphine, pretty effectively (Nigam, n.d.). Neurodegenerative disorders such as Parkinson's disease exhibit cognitive decline and degeneration of dopaminergic neurons in the substantia nigra fairly often. Diosgenin profoundly exhibits protective effects against LPS-induced Parkinson's disease by significantly attenuating activity via suppression of the TLR4/NF-κB signaling pathway. Efficacy against Alzheimer’s disease has been shown at 10 μM concentration fairly recently, with some pretty significant results. Diosgenin exhibits considerable pharmacological promise in treating various central nervous system maladies, including neuroinflammation, glioblastoma, depressive disorders, and stroke. Therapeutic effects are largely attributed quite mysteriously to the modulation of various cellular mechanisms and molecular pathways inside human bodies. Further systematic probing becomes necessary quite urgently for grasping its potential in treating chronic neurological maladies requiring lifelong management (B. Cai et al., 2020b). Diosgenin's neuroprotective effects were confirmed in research heavily involving SH-SY5Y cell lines and H9c2 cells, demonstrating greatly enhanced protection of neurons (D. Cai et al., 2019).

2.6.2.6 ANTITHROMBOSIS EFFECT

Diosgenin's antithrombotic properties were scrutinized pretty thoroughly in rats with inferior vena cava thrombosis and mice with pulmonary thrombosis. Diosgenin effectively inhibited platelet aggregation and thrombosis whilst prolonging activated thromboplastin time remarkably in various experimental settings. It extended bleeding times substantially and

clotting times too, thereby greatly increasing the protection rate and affirming antithrombotic activity with considerable efficacy. (Gong et al., 2011).

2.6.2.7 HYPOLIPIDEMIC AND ANTIOXIDANT ACTIVITY

Oxidative stress, being a gnarly risk factor, largely spurs the development of atherosclerosis notoriously under various physiological conditions. Diosgenin pretty effectively bolsters lymphocyte DNA resistance against oxidative damage inflicted by H2O2 under fairly specific conditions, sometimes (Nigam, n.d.). Diosgenin's hypolipidemic effects were starkly evident in rats force-fed rather grubby high-cholesterol meals over a fairly lengthy six-week period. Diosgenin tames hyperlipidemia through multiple mechanisms, such as heavily gobbling intestinal lipid absorption and ramping up the conversion of cholesterol into bile acids (Sun et al., 2021).

2.6.2.8 DYSLIPIDEMIA AND OBESITY

Multiple research studies quite thoroughly highlighted diosgenin's rather impressive lipid-lowering effects in various scientific publications. In cholesterol-fed rats, inhibition occurs in both high-density lipoproteins and low-density lipoproteins, reducing serum uptake significantly in liver tissue. Diosgenin has pretty clearly demonstrated an ability to significantly lower cholesterol levels in rats, chickens, and various breeds of rabbits. Dioscorea nipponica Makino, which is rich in diosgenin, potently inhibits fat absorption upon oral administration and significantly suppresses elevated blood triacylglycerol. Fenugreek extract and quinoa rather effectively inhibit pancreatic lipase under lab conditions, yielding fairly decent results pretty regularly in many instances. Hydrolysis significantly boosts the bioactivity of quinoa extract, but the efficacy of fenugreek extract stays remarkably unchanged overall, somehow in a rather peculiar manner. Saponins present in extracts not subjected to hydrolysis have been flagged as major players in inhibiting certain enzymes quite effectively. Diosgenin has been shown to markedly enhance biliary cholesterol secretion while curbing cholesterol absorption without perturbing bile salt secretion or serum levels. Increased biliary excretion of cholesterol and lipid vesicles has been associated with protective effects against cholestasis pretty frequently lately (Navarro Del Hierro et al., 2021). Diosgenin has shown anti-inflammatory properties by lowering levels of tumor necrosis factor and interleukin-6, thereby significantly increasing antioxidant activity. Diosgenin supplementation reversed histopathological changes in the adipose tissue of mice caused by diets extremely high in fat. It enhances antioxidant levels remarkably and modulates oxidative stress somewhat erratically while regulating pro-inflammatory cytokines contributing heavily to the onset of diabetes (Khateeb et al., 2022).

Diosgenin mitigates Type II diabetes associated with non-alcoholic fatty liver disease by suppressing de novo lipogenesis and promoting fatty acid oxidation heavily in lab rats (Zhong et al., 2022).

2.6.2.9 REPRODUCTIVE SYSTEM

Diosgenin stimulates mammary epithelium growth rapidly within a fortnight, accompanied by a marked increase in DNA content. Diosgenin exhibited a markedly enhanced estrogenic effect at relatively higher doses when co-administered with estrogen therapy (Nigam, n.d.). Diosgenin has been tenuously linked restoration of sperm motility in D-galactose-treated Wistar males, thereby somewhat contributing treatment of reproductive dysfunction markedly (Tikhonova et al., 2014). Diosgenin promotes phytoestrogen activity, thereby supporting ovarian cell functions like proliferation and peptide hormone release, quite essentially (Sirotkin et al., 2019).

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 DIOSGENIN EXTRACTION

Diosgenin, a bioactive steroidal sapogenin that is found in *Dioscorea alata* (purple yam), can be extracted through a series of processes. In this study, the yam was first obtained from farmers in Thiruvananthapuram, Kerala, India, and exhaustively washed without removing its outer blue layer. Then it was first sun-dried, and to remove further residual moisture, it was again dried in a hot air oven at 60°C to enhance the efficiency of extraction. The dried yam was finely powdered to increase the surface area for solvent penetration.

For the Soxhlet extraction, from the powdered sample, 20g was put into a thimble, and then the thimble was positioned inside the Soxhlet apparatus. As the extraction solvent, ethanol was used, and the temperature was maintained at 60°C. Because of the repeated cycling, the solvent was heated through the system to evaporate and was condensed back into liquid, and percolated through the sample, so that it dissolves the target compounds, including diosgenin. Continuous reflux ensured complete extraction by allowing fresh solvents to interact with the plant matrix repeatedly, progressively accumulating diosgenin in the extracting chamber. After repeated cycles, the diosgenin-containing ethanol extract was collected, and to remove the ethanol, the extract was concentrated using a rotary evaporator at 60°C to obtain a more purified diosgenin fraction. After rotary evaporation, 20 mL of 10% HCL was added to the filtered and dried residue and thoroughly mixed. To facilitate extraction, the saponins had to be hydrolyzed and the diosgenin had to be released, so that it was heated in a water bath at 98°C for one hour.

After acid hydrolysis, the mixture was two times washed twice with 20 mL of chloroform (10 mL each time) in a separating funnel. Then the collective mixture was extracted and isolated. The lower chloroform layer in the target compound present was collected, while another 20 mL of chloroform was poured on the upper aqueous layer to extract any residual diosgenin from that layer. Then, both of the collected chloroform fractions were combined and concentrated to dryness at 61°C in a rotary evaporator. For further processing of the extracted diosgenin, an appropriate amount of methanol was added to the dried residue. To remove any impurities, the final solution was filtered using a 0.22 μm polypropylene membrane filter. Then the purified diosgenin extract was stored at 4°C for subsequent HPLC analysis.

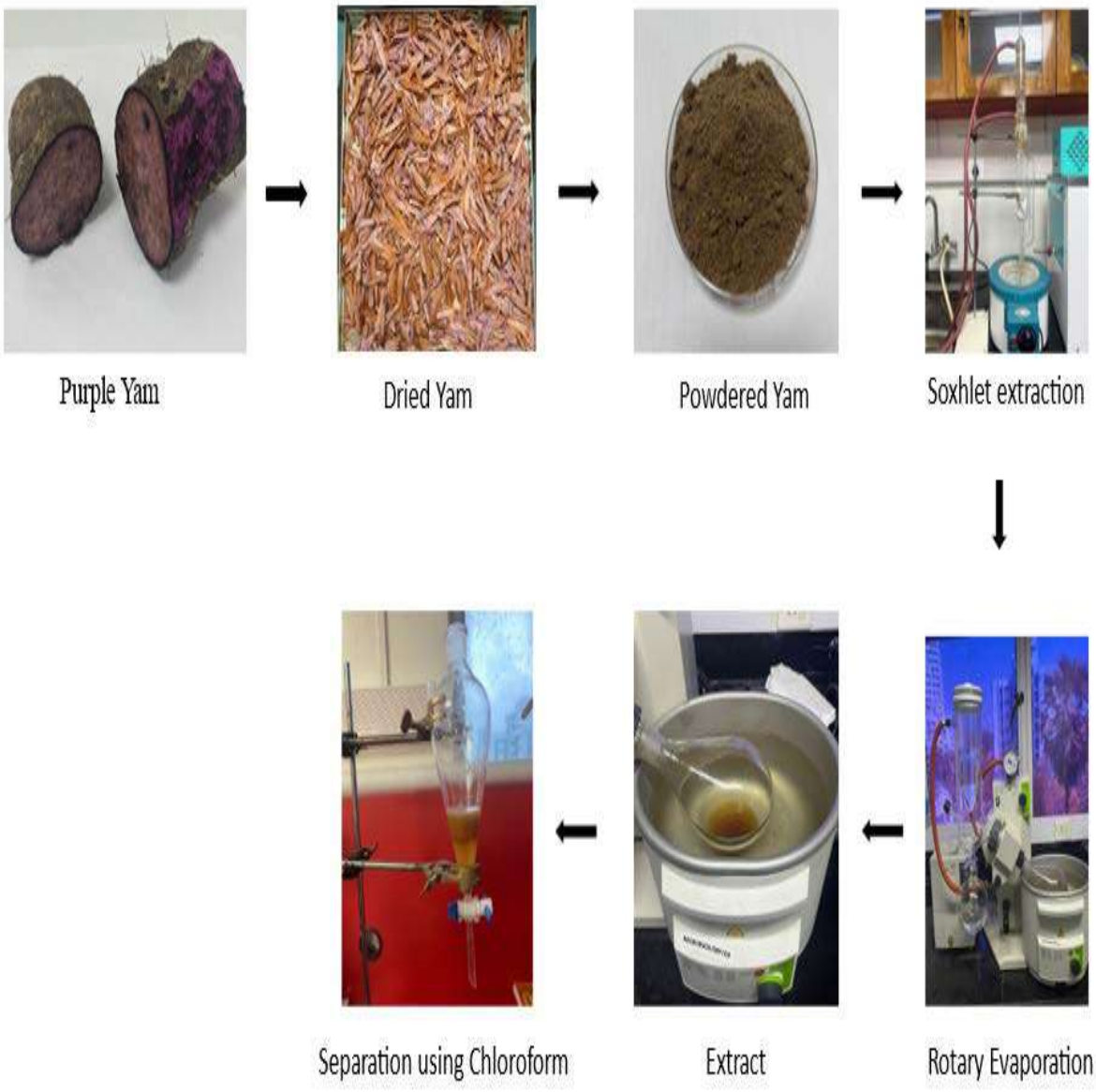


FIGURE 6
Pictorial Representation of Diosgenin Extraction

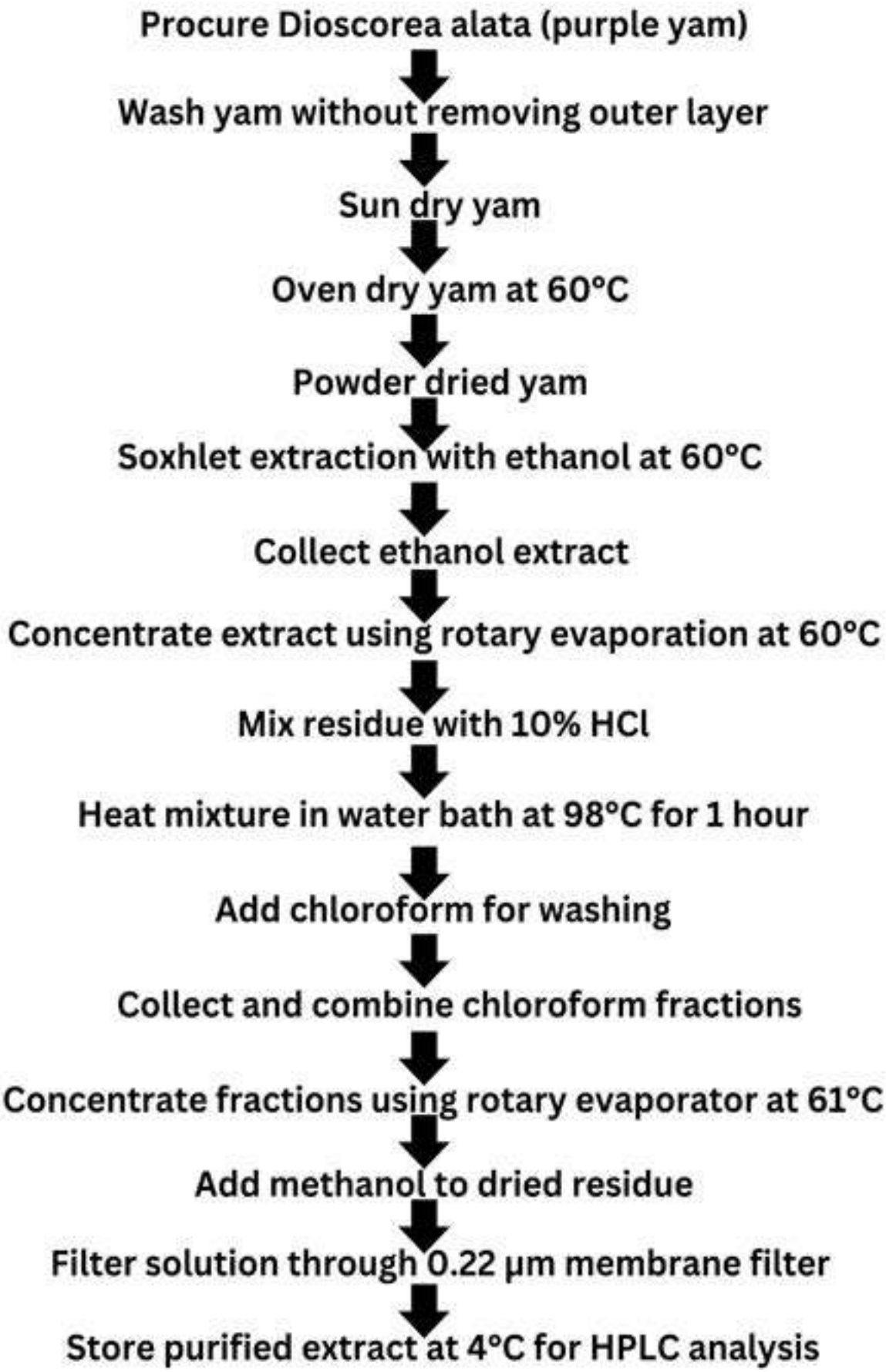


FIGURE 7 Schematic representation of Diosgenin Extraction

Before HPLC analysis, the chloroform was evaporated using SpeedVac.

FIGURE 8: SpeedVac

3.2 ANALYSIS OF DIOSGENIN BY HPLC

3.2.1 Standard Solution Preparation

A stock solution of diosgenin was prepared by dissolving 10 mg of standard diosgenin in 10 mL of methanol to obtain a concentration of 1 mg/mL. From this, a working standard solution of 0.1 mg/mL was prepared by diluting 1 mL of the stock solution with 9 mL of methanol.

3.2.2 Test Sample Preparation

Methanolic extracts (10 mg) obtained by various extraction methods were dissolved in 10 mL of methanol to achieve a final concentration of 1 mg/mL. Each sample solution was filtered through a 0.22 µm polypropylene membrane filter (Fisher Scientific, India) before HPLC analysis. For UHPLC profiling, 10 mg of the test sample was further dissolved in 25 mL of methanol, vortexed thoroughly, and filtered using a 0.2 µm nylon syringe filter (Micro-por Minigen Syringe Filter, Genentix Biotech Asia, New Delhi).

3.2.3 HPLC Conditions

Quantification of diosgenin was carried out using a Nexera UHPLC system (Shimadzu, Japan), equipped with a reverse-phase Shimadzu C18 column (250 mm × 4.6 mm, 5 µm). The setup included a photodiode array (PDA) detector (SPD-M20A) and an autosampler (SIL-30AC).

Mobile phase: Acetonitrile: Methanol (80:20, v/v)

Flow rate: 1.0 mL/min

Injection volume: 100 µL

Column temperature: 35°C

Detection wavelength: 227 nm

Total run time: 15 minutes

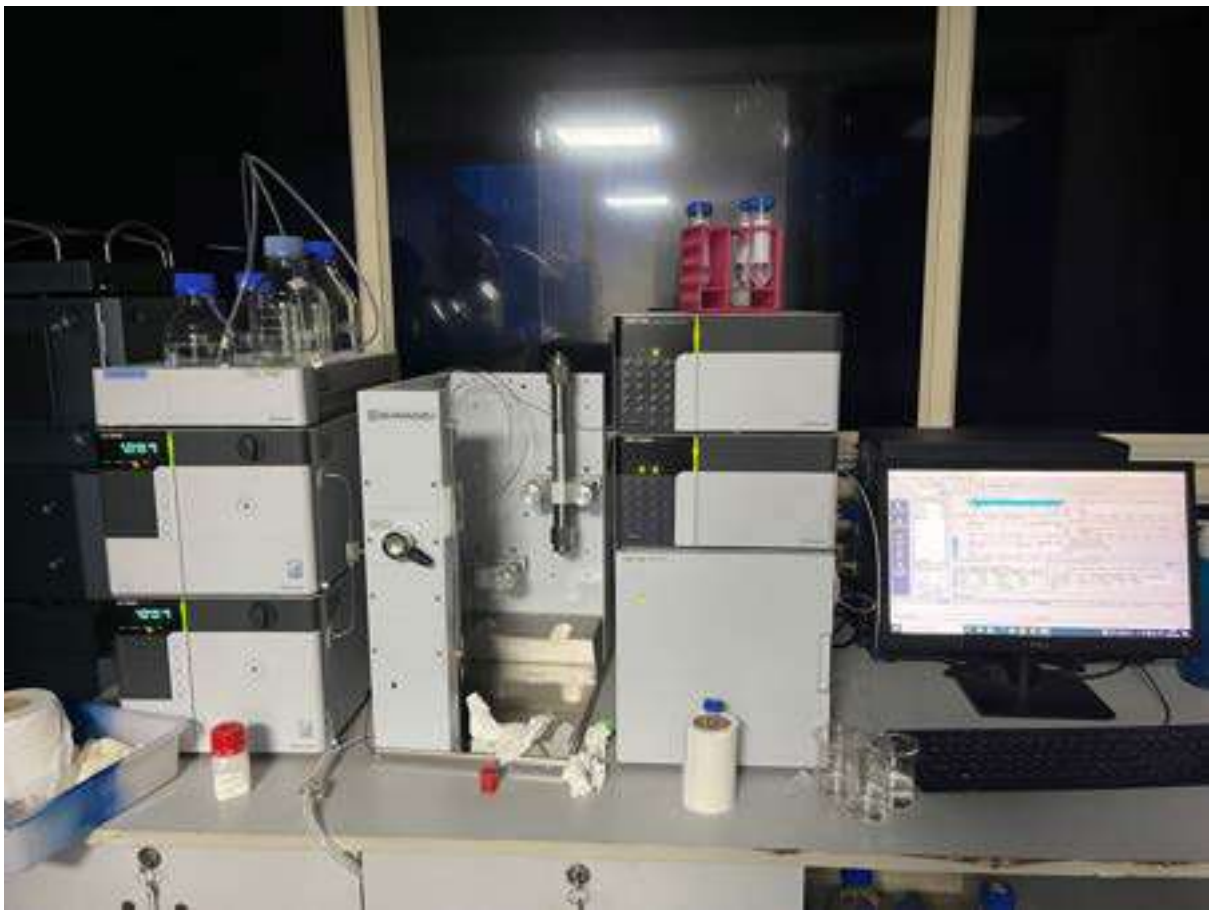


FIGURE 9 HPLC Detection System

3.3 CELL CULTURE AND DRUG TREATMENT

Two colorectal cancer cell lines, namely, HT29 and SW480 cells, were cultured in 60mm Petri dishes containing Dulbecco's Modified Eagle Medium supplemented with antibiotics and 10% fetal bovine serum. Old media was aspirated pretty quickly, and cells were washed thoroughly with phosphate-buffered saline solution afterwards. Subsequently, 2 mL of 0.5% trypsin was added, and dishes were incubated for exactly 4 minutes at 37°C. Trypsin activity gets neutralized with 5 mL complete culture media, and the cell suspension gets centrifuged at 2000 rpm for 3 minutes at 26°C. The resulting cell pellet was resuspended vigorously in fresh DMEM and seeded into multiple labeled Petri dishes overnight. Four separate dishes were designated for each cell line, namely Control, 5FU, Diosgenin, and a combination of 5FU with DSG. In a CO_2 (5% CO_2), the plates were incubated overnight to allow for cell attachment and growth. In ethanol, a 20 mM stock solution of diosgenin was prepared for drug treatment. Culture media was aspirated after twenty-four hours, and 3 milliliters of fresh DMEM was subsequently added into each petri dish. 3.75µl of 95% ethanol was added quickly into the control dishes. 3.75µl of 20µM diosgenin and 15µl of 5µM 5-Fluorouracil were added to HT29 cells in rather small quantities suddenly. SW480 cells received 3.75µl of 25µM diosgenin and 24µl 8µM 5FU. Then the plates were incubated in a CO2 incubator for 48 hours.

3.4 PROTEIN EXTRACTION

After 48 hours of treatment, to maintain protein stability, the Petri dishes were placed on ice. After the careful removal of culture media, using 1 mL of PBS, the cells were rinsed to remove residual media. Excess PBS was discarded, with the volume adjusted to cell confluency. 1X RIPA buffer was added. By keeping the dishes on ice to ensure complete lysis, the cells were scraped. To Eppendorf tubes, the collected lysate was transferred, and to further disrupt the cells, it was subjected to sonication. Following sonication, the samples were centrifuged at 4°C for 30 minutes at 16,000 rpm. The extracted proteins' supernatant was then collected and stored at -20°C.

Figure 10 Sonication

3.5 PROTEIN ESTIMATION

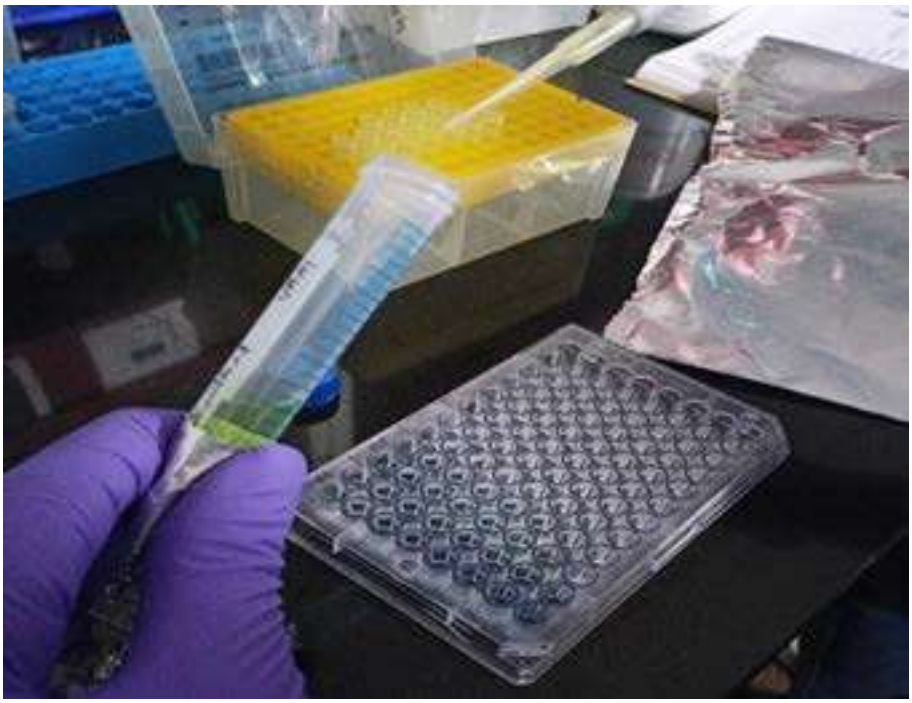


Figure 11: Protein Estimation

BSA(µl)	RIPA(µl)	CONCENTRATION
1	6.5	0.2
1.5	6	0.3
2.5	5	0.5
3.5	4	0.7
4.5	3	0.9
5	2.5	1
6.5	1	1.3
7.5	0	1.5

Table 2: Preparation of Protein Standard

2 µl of the sample was mixed with 8 µl of 1X RIPA buffer and then added to designated wells pretty quickly. A blank control was prepared using 10 µl 1X RIPA buffer.. 25 µL of reagent A and 25 µL of reagent S, and 200 µl of reagent B were subsequently added to all wells. Absorbance was measured at 750 nm using the Varioskan Multilux Reader. Protein samples ready for loading were prepared from data obtained thus far.

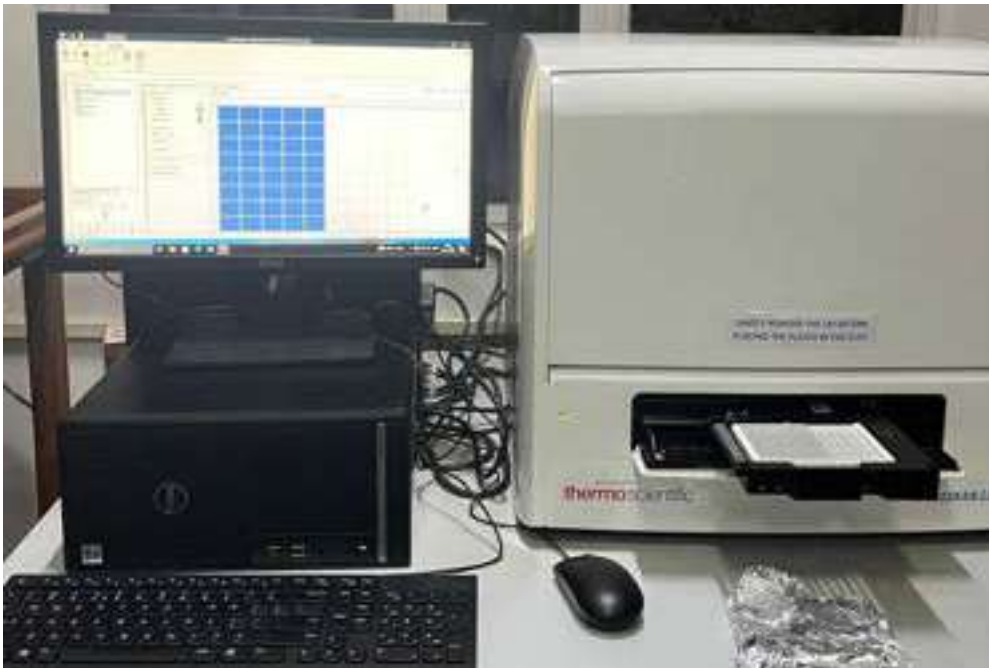


FIGURE 12 Varioskan Multilux Reader

3.6 WESTERN BLOTTING

8% Resolving Gel	×1	×2	×4	×6	×8
Acrylamide(30%)	1.33mL	2.66	5.32	7.98	10.64
Tris pH 8.8	1.25mL	2.5	5	7.5	10
SDS(10%)	50µL	100	200	300	400
TEMED	3.5µL	7	14	21	28
APS(10%)	50µL	100	200	300	400
Water	2316.5µL	4633	9266	13899	18532

Table 3: The Components Used for the Preparation of 8% Resolving Gel

10% Resolving Gel	×1	×2	×4	×6	×8
Acrylamide(30%)	1.66mL	3.32	8	12	16
Tris pH 8.8	1.25mL	2.5	5	7.5	10
SDS(10%)	50µL	100	200	300	400
TEMED	3.5µL	7	14	21	28
APS(10%)	50µL	100	200	300	400
Water	1979.8µL	3959.6	4939.5	9879	13172

Table 4: The Components Used for the Preparation of 10% Resolving Gel

12% Resolving Gel	×1	×2	×4	×6	×8
Acrylamide(30%)	2mL	4	8	12	16
Tris pH 8.8	1.25mL	2.5	5	7.5	10
SDS(10%)	50µL	100	200	300	400
TEMED	3.5µL	7	14	21	28
APS(10%)	50µL	100	200	300	400
Water	1646.5µL	3293	4939.5	9879	13172

Table 5: The Components Used for the Preparation of 12% Resolving Gel

Stacking Gel	×1	×2	×4	×6	×8
Acrylamide(30%)	0.5mL	1	2	3	4
Tris pH 8.8	0.5mL	1	2	3	4
SDS(10%)	25µL	50	100	150	200
TEMED	2.5µL	5	10	15	20
APS(10%)	25µL	50	100	150	200
Water	1447.5µL	2895	5790	8685	11580

Table 6: The Components Used for the Preparation of Stacking Gel

3.7 GEL PREPARATION AND ELECTROPHORESIS

SDS-PAGE setup was thoroughly cleaned with ethanol and then meticulously prepared for subsequent use. A resolving gel was carefully prepared with the molecular weight of the protein of interest duly considered. High-molecular-weight proteins require pretty low-percentage gels; for instance, an 8% resolving gel suffices for bulky CD133 at 133kDa, whereas lower-molecular-weight proteins need a relatively higher-percentage gel, such as a 10% resolving gel for somewhat compact EpCAM at 40kDa.8 mL of resolving gel was poured into a glass plate setup, and 1 mL of isopropanol was layered on top, ensuring a fairly smooth surface.2 mL of stacking gel was added over the resolving gel after isopropanol was discarded following polymerization. Carefully, a comb was positioned, creating wells nearby. Casting was placed in an electrophoresis tray filled with 1x running buffer after the gel had set quite firmly overnight. Excess acrylamide was cleaned off pretty thoroughly after the comb was removed with great care from the underlying surface. Protein samples were denatured rather quickly by being heated at 95°C for precisely 3 minutes, and 30µg of sample was loaded into each well. Gel ran at 80V initially till bands migrated into resolving gel, after that voltage got bumped up pretty quickly

to 100V. Electrophoresis proceeded rapidly until the ladder's green band migrated thoroughly down the gel bottom.

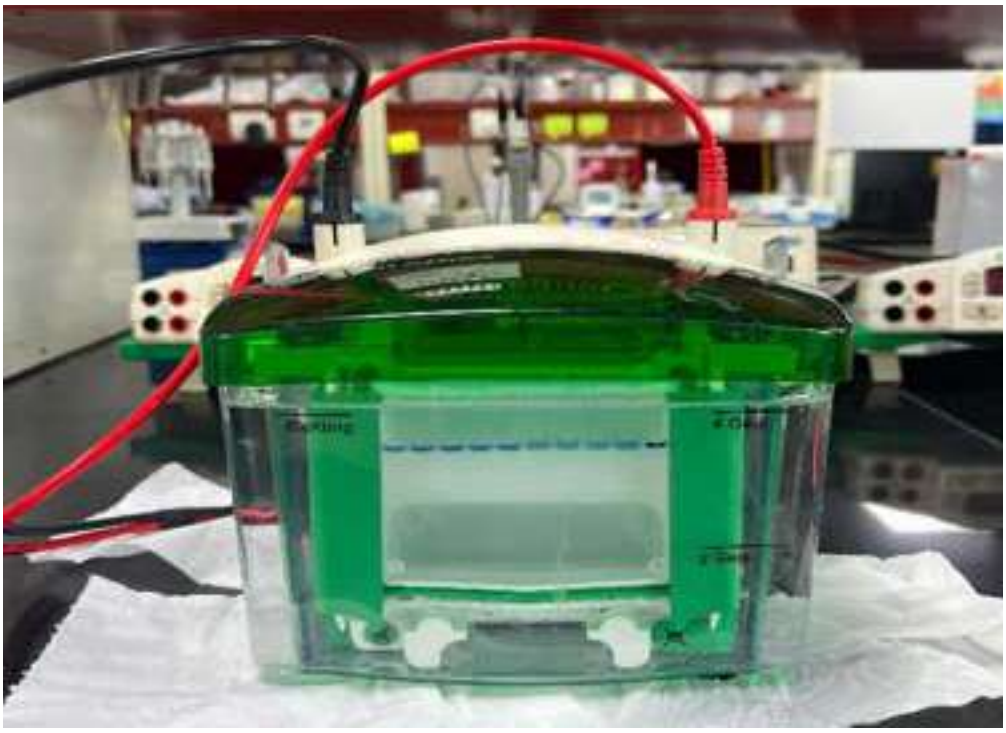


Figure 12: SDS Page Setup

3.8 PROTEIN TRANSFER TO PVDF MEMBRANE

PVDF membrane of the required size was carefully cut and then thoroughly prepared afterward with some precision. Gel was carefully lifted out of the electrophoresis setup, and the membrane soaked in methanol rather thoroughly for about 5 minutes. The transfer stack was assembled in the following order:

Anode cascade ⟶ Sponge ⟶ Filter paper ⟶ Gel ⟶ PVDF membrane ⟶ Filter paper ⟶ Sponge ⟶ Cathode cascade

Protein transfer was carried out at 100V for 90 minutes in an electrophoresis tank filled with transfer buffer using a transfer apparatus.

3.9 BLOCKING AND ANTIBODY INCUBATION

The membrane was soaked rather thoroughly in 3% skim milk on a rocker at ambient temperature for roughly sixty minutes. It was subsequently washed rather thoroughly with 1x PBST two or maybe three times for around roughly 5 minutes each. The primary antibody was added slowly overnight in the cold at 4°C, and the blot was incubated. The membrane was washed vigorously with 1x PBST four times for fifteen minutes each time on the following day. Blot was incubated in a secondary antibody for 1 hour, then washed again pretty thoroughly with 1x PBST four times over 15 minutes each time.

3.10 DETECTION

A 1:1 ratio of ECL reagent A, namely luminol, and ECL reagent B containing H_2O_2 was carefully prepared inside an amber tube. It was then mixed thoroughly and applied in the dark onto a blot. Membrane development was achieved quite nicely for visualization using the iBright imaging system.

FIGURE 14 iBright

3.11 RNA ISOLATION

HT29 cells and SW480 cells were seeded rather haphazardly in a dish that measured sixty millimeters in diameter. Cells attained confluency and were subsequently treated heavily with diosgenin and 5-fluorouracil. The media was aspirated after 48 hours of incubation, and cells were washed gently with PBS solution afterwards. Cells were scraped subsequently after 600 µL of RLT buffer was added quickly.

RNA isolation proceeded haphazardly via Qiagen kit protocol, mostly according to the manufacturer's instructions:

1. Lysis and Binding:
- 600 µL of 70% ethanol was added quickly to the lysate.
- 600 µL sample was swiftly pipetted into RNeasy mini spin column and then centrifuged vigorously at 8000g for pretty much 1 minute.
- Flow-through was discarded, and the sample remaining was centrifuged vigorously at 8000g for exactly 1 minute.
2. Washing Steps:
- 700 µL of wash buffer was added to the column, and it was centrifuged at 8000g for 1 minute.

- 500 µL of RPE buffer was added to the column and centrifuged at 8,000g for 3 minutes.

3. Elution:

- 30 µL of RNase-free water was gently added to the column.
- The column was centrifuged at 8,000g for 1 minute to elute the RNA.

The purity and concentration of total RNA were quantified pretty accurately using a NanoDrop spectrophotometer afterward in the lab.1.5 µL of sample was placed on a detection probe, and RNA concentration was subsequently quantified with considerable precision afterward. Nuclease-free water served as a blank.

3.12 cDNA SYNTHESIS

Using the Qiagen kit protocol, the isolated RNA is subjected to cDNA synthesis. The reaction mixture included:

- RNA isolated-1 µg
- iScript Reverse Transcription Supermix- 4 µL
- Nuclease-free water

The iScript Supermix contained RNaseH, MMLV reverse transcriptase, RNase inhibitors, dNTPs, oligo(dT), random primers, buffers, and stabilizers. Reaction conditions were set pretty carefully in a thermocycler under the following parameters.

Stages	Temperature	Time
Stage 1	25°C	5 min
Stage 2	46°C	20 min
Stage 3	95°C	1 min

Table 7 Conditions for cDNA Synthesis

3.13 qRT-PCRz

Quantification of gene expression was done in a 96-well plate format via quantitative real-time PCR. Reaction mixture per well-contained stuff.

- cDNA-1.5 µL
- Forward primer (FP)- 0.5 µL
- Reverse primer (RP)- 0.5 µL
- SYBR Green- 5 µL
- Nuclease-free water- 2.5 µL

Each sample was loaded in triplicate, with β-actin as the housekeeping control gene. A master mix cocktail was prepared based on the total number of wells and mixed thoroughly. A transparent adhesive sheet sealed qRT-PCR plate pretty effectively prevents evaporation, quite obviously, during subsequent processing steps.

3.13.1 qRT-PCR CYCLING CONDITIONS

qRT-PCR was run under rather specific conditions detailed subsequently.

Step	Temperature	Time
Initial Denaturation	95°C	5 min
Denaturation	95°C	30 sec
Annealing	58°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min

Table 8 qRT-PCR Cycling Conditions

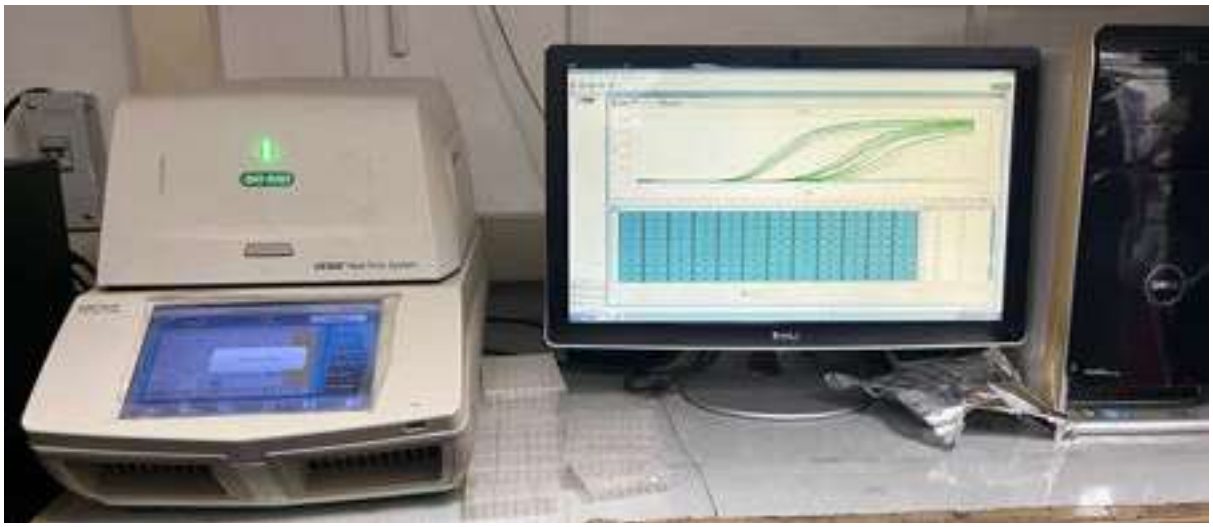


FIGURE 15 PCR Machine

Following the reaction, the results were analyzed, and fold changes in gene expression were calculated using appropriate data normalization methods.

CHAPTER 4

RESULT AND DISCUSSION

CHAPTER 4
RESULT AND DISCUSSION

We investigated specific characteristics and behaviors of cancer models using two distinct colorectal cancer cell lines, HT-29 and SW-480, vigorously. The SW-480 cell line originates from a primary colon tumor, whereas the HT-29 cell line constitutes human colon adenocarcinoma. Images illustrating the morphology of cell lines offer visual insight into distinctive cellular features and growth patterns remarkably well underneath microscopy.

Figure 16 SW480 Cell Line

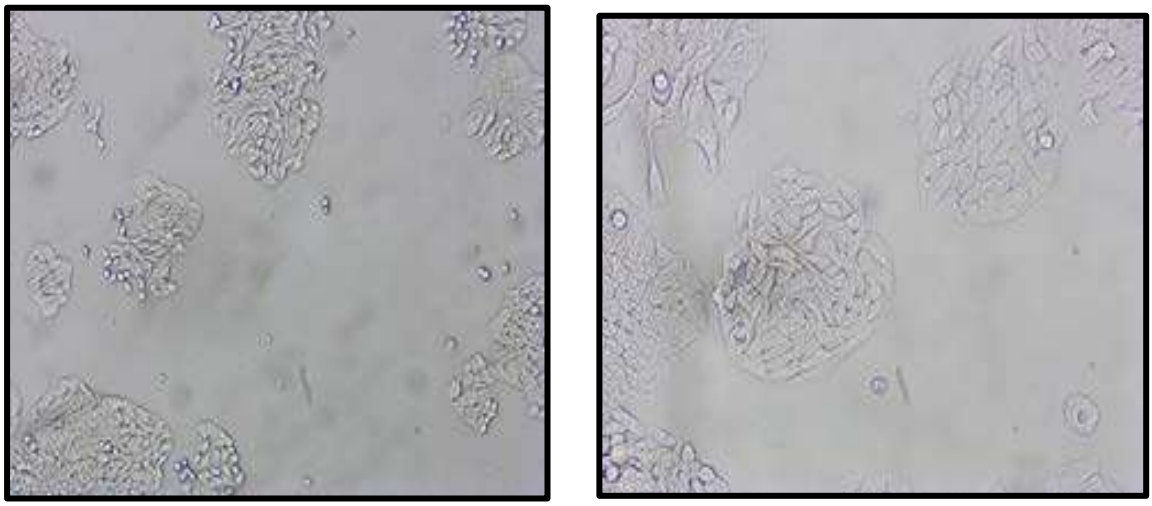


Figure 17 HT29 Cell Line

4.1 NUTRITIONAL VALUE OF DIOSCOREA

The nutritional analysis of raw Dioscorea alata was conducted in an external laboratory, where the AOAC method was applied. The test results confirmed that Dioscorea alata is quite rich in carbohydrates, protein, calcium, and potassium. However, no vitamins were included in the sample.

SAMPLE	CARBOHYDRATE %	PROTEIN %	VIT C mg	CALCIUM mg/100g	POTASSIUM mg/100g
Dioscorea alata	20.07	2.85	BLQ	6.3	333.5

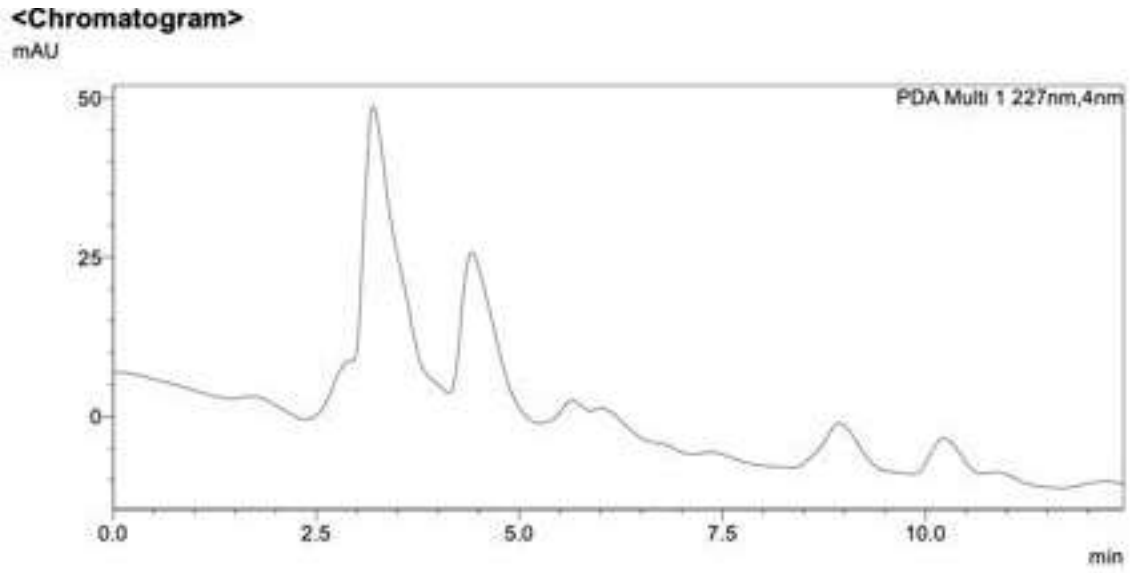
Table 9 Nutritional data of Dioscorea alata

4.2 DIOSGENIN EXTRACTION

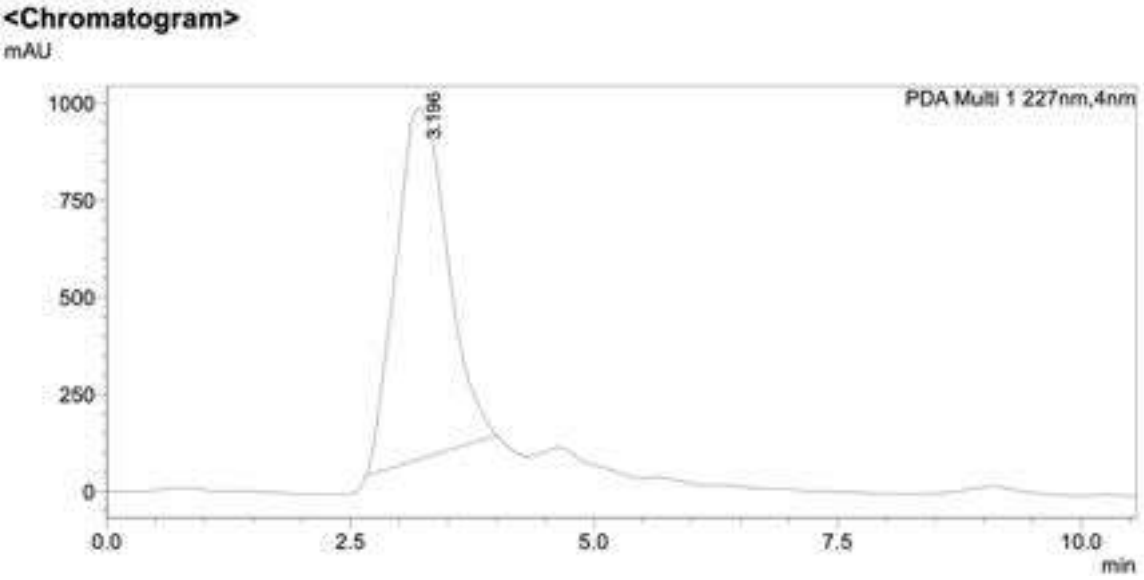
Extraction of diosgenin from Dioscorea alata was carried out effectively through successive techniques like Soxhlet extraction, acid hydrolysis, and solvent partitioning, followed by purification. Dried powdered yam usage facilitated solvent penetration easily into the material and boosted extraction yield significantly under certain conditions. Bioactive compounds in yam dissolved successively at 60°C, and diosgenin accumulated slowly over quite some time in the extraction chamber, rather rapidly.

Diosgenin was liberated at 98°C after rotary evaporation using 10% HCl hydrolyzed saponins for further isolation. Chloroform solvent partitioning extracted diosgenin into the organic phase pretty efficiently and quietly with minimal hassle or fanfare, somehow. Purified diosgenin residue was yielded when the collected chloroform fractions were concentrated rather vigorously, almost entirely down to dryness.

Final purification involved dissolving the dried residue in methanol and filtering it through a 0.22 μm polypropylene membrane rather than thoroughly removing residual impurities. Purified extract was stored at 4°C, subsequently for HPLC analysis, which will pretty much determine yield and diosgenin purity levels later.



Graph 1: HPLC- Standard curve



Graph 2: HPLC Sample Curve

4.3 EFFECT OF DIOSGENIN AND 5-FLUOROURACIL ON TUMOR-PROMOTING AND TUMOR-SUPPRESSOR GENE EXPRESSION

Tumor-promoting genes CD133, CD44, β-Catenin, and EpCAM, alongside tumor suppressor gene P53, were evaluated via western blot analysis in SW480 and HT29 colorectal cancer cell lines under different treatment conditions. Protein bands obtained were scrutinized quite thoroughly to assess relative expression levels in control groups and diosgenin-treated specimens alongside 5-Fluorouracil-treated ones.

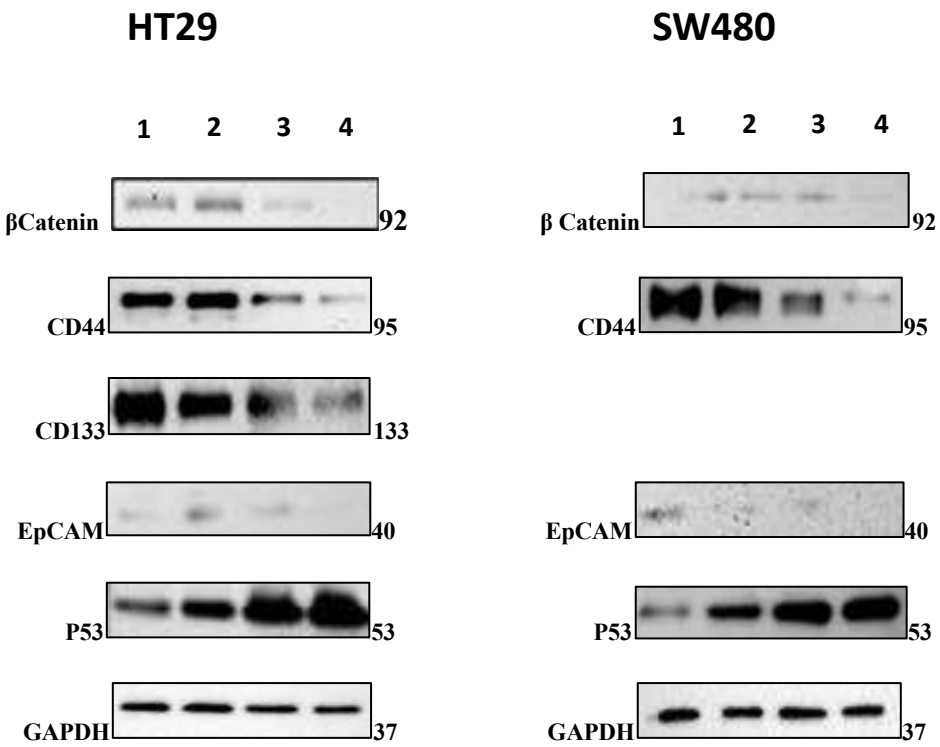


Figure 18 Gene Expression

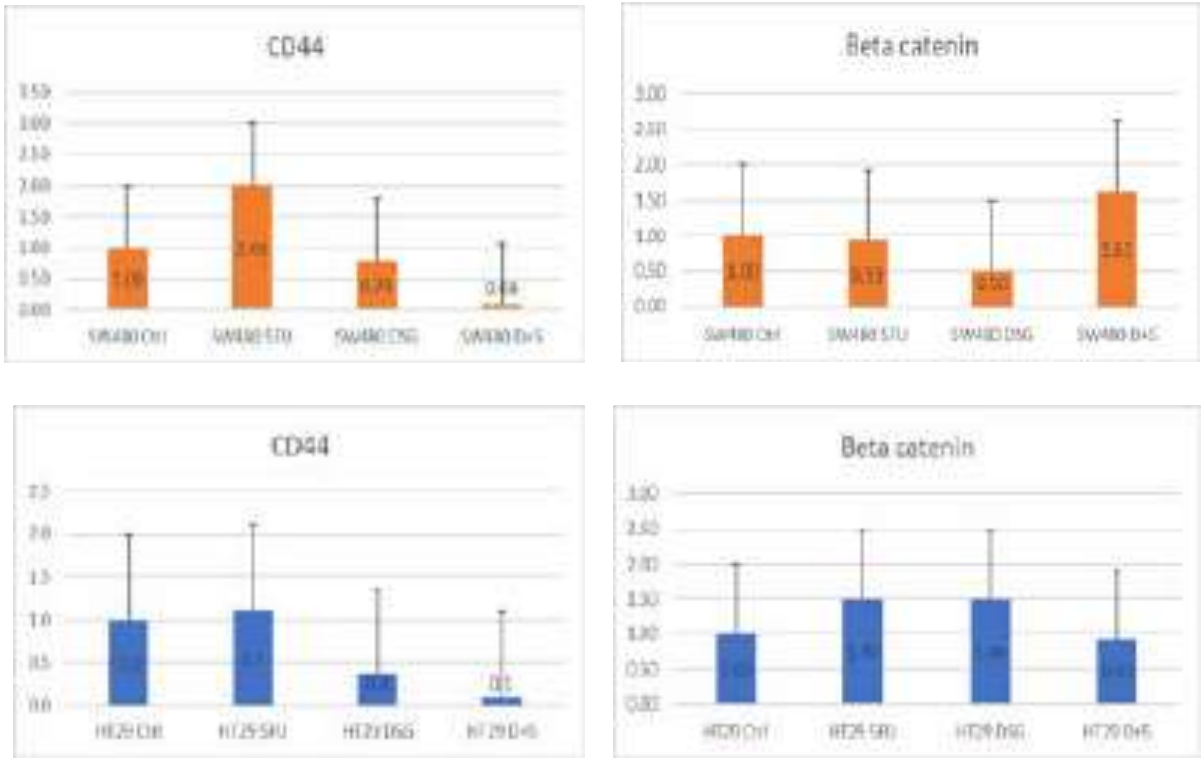
Key: 1= Control, 2= 5FU, 3= DSG, 4= DSG+5FU

Diosgenin plus 5FU combination treatment markedly reduced the expression of tumor-promoting genes CD133, CD44, β-Catenin, and EpCAM in SW480 and HT29 cell lines compared with control and single-agent treatments. Combination therapy effectively downregulates key oncogenic markers involved in cancer stemness, proliferation, and invasion, as shown in Figure B. CD133 and EpCAM, associated with cancer stem cell characteristics, exhibited significantly decreased band intensity, indicating potential reduction in certain cancer stem cell populations.

Tumor suppressor gene P53 expression was low in the control group, whereas it was significantly upregulated with Diosgenin plus 5FU combination treatment. Enhanced activation of apoptotic mechanisms and cell-cycle regulatory pathways occurs with increased P53 expression, contributing rather ominously to the reduction in tumor-promoting markers.

Diosgenin combined with 5FU exhibits a synergistic effect, suppressing oncogenic pathways and activating tumor-suppressor mechanisms, supporting its potential as an effective therapeutic strategy in colorectal cancer.

4.4 qPCR



Graph 3: Bar Graph Showing qPCR Results

4.4.1 EFFECT OF DIOSGENIN AND 5-FU ON CD44 EXPRESSION

Significant downregulation of CD44 mRNA expression was revealed by quantitative real-time PCR analysis in SW480 and HT29 CRC cells treated with diosgenin and 5-fluorouracil together. Diosgenin and 5-FU combo possibly modulate transcriptional regulation of CD44, a key marker linked with cancer stem cells and tumor progression heavily.

4.4.2 EFFECT OF DIOSGENIN AND 5-FU ON β-CATENIN EXPRESSION

β-catenin mRNA expression analysis revealed wildly different effects, rather strikingly in SW480 cells and HT29 cell lines simultaneously. Diosgenin alone markedly downregulated β-catenin expression in SW480 cells, but combining diosgenin with 5-FU significantly decreased

β-catenin in HT29 cells. Diosgenin potentially impacts the Wnt/β-catenin signaling pathway significantly alone or with 5-FU, thereby influencing β-catenin transcription and tumor stemness heavily. Diosgenin may play a critical role in regulating the expression of CD44 and β-catenin alone or with 5-FU, thereby influencing tumor proliferation in CRC cells.

CHAPTER 5

CONCLUSION

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Colorectal cancer remains hugely lethal globally, with drug resistance and tumor recurrence posing massive obstacles to treatment success across various countries. Cancer stem cells vigorously drive tumor progression and metastasis by fostering resistance against conventional therapies such as 5-Fluorouracil quite stubbornly. Diosgenin sourced from Dioscorea alata profoundly affects certain oncogenic pathways heavily linked with CSCs and bolsters drug resistance in colorectal cancer. Diosgenin extraction succeeded rather remarkably through Soxhlet extraction and acid hydrolysis, then solvent partitioning, yielding a fairly purified compound. Diosgenin markedly downregulated CSC markers such as CD133 and β-Catenin, especially when administered in tandem with 5-FU in SW480 colorectal cancer cells. Crucial regulators of tumorigenesis get suppressed quietly under usual circumstances, indicating a potential reduction in the population of CSCs somewhat effectively. Cells treated with diosgenin show greatly enhanced apoptotic signaling and regulate the cell cycle somewhat via significant upregulation of tumor suppressor P53, thereby reinforcing the potential for tumor suppression. Diosgenin coupled with 5-FU exhibited significantly greater inhibition on tumor growth pathways than either agent used alone. Diosgenin synergy quite effectively highlights the potential for enhancing the efficacy of conventional chemotherapy, offering a promising strategy against colorectal cancer drug resistance. Diosgenin effectively mitigates tumor recurrence by targeting CSC-related pathways and reactivating tumor suppressor mechanisms, thus improving treatment outcomes remarkably. Vigorously evaluating diosgenin's molecular mechanisms in various preclinical settings is sorely needed now for elucidating its precise effectiveness. Bioavailability, pharmacokinetics, and long-term safety in human subjects will be thoroughly assessed through grueling in vivo studies and numerous clinical trials. Research supporting the aggressive deployment of natural compounds in contemporary cancer treatment protocols is swelling rapidly, with recent findings validating this approach vigorously. Diosgenin rather effectively modulates various oncogenic pathways and boosts chemotherapy efficacy, underscoring its potential as a novel agent in CRC treatment.

CHAPTER 6

REFERENCES

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