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THE CYTOTOXIC COMPOUNDS OF FRACTIONATED EXTRACT OF TIBIG (*FICUS NOTA*) LEAVES AGAINST HCT116 HUMAN COLORECTAL CARCINOMA CANCER LINE

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ABSTRACT

Introduction: Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths, with early detection often hindered by the absence of initial symptoms. Natural sources like *Ficus* species offer potential alternatives for cancer treatment, yet studies on *Ficus nota* remain limited. This study aimed to identify the most cytotoxic fraction of *Ficus nota* extract and evaluate its cytotoxic activity. **Methodology:** The plant was authenticated, dried, macerated, and extracted, followed by phytochemical screening, solvent partitioning, and gravity column chromatography. Fractionation was guided by the Brine Shrimp Lethality Assay (BSLA) and MTT Assay, LC-MS/MS identified compounds in the most active fraction, and molecular docking was used to assess their potential binding interactions with cancer-related targets. **Results:** BSLA showed that the 1000 ppm hexane partition exhibited the highest cytotoxicity, while the MTT Assay revealed the 2:8 fraction as the most active. The extract exhibited moderate cytotoxicity against HCT 116, with an IC_{50} comparable to Vinblastine and a selectivity index above 1, suggesting cancer cell specificity. LC-MS/MS detected 18 potentially cytotoxic compounds, the most abundant being Phytolacca Cerebroside and Neoline. **Discussion:** Molecular docking revealed strong multi-target interactions with TNIK, PI3Kα, and EGFR, particularly from Phytolacca Cerebroside, which showed exceptional binding energies but poor drug-like properties due to high lipophilicity and low solubility. In contrast, Neoline showed consistently strong binding and favorable ADME characteristics, making it a more promising lead for drug development.

KEYWORDS: Cytotoxicity, *Ficus nota*, bioactive compounds.

CHAPTER 1

THE PROBLEM AND ITS BACKGROUND

INTRODUCTION

Cancer is a deep-rooted problem that humanity has been battling against. Cancer is defined as a disease involving uncontrolled cell growth and the risk of metastasizing or invading nearby tissue (National Cancer Institute, 2021). The American Cancer Society (2024) defines colorectal cancer as a cancer that starts in either the colon (colon cancer) or the rectum (rectal cancer), and is often grouped due to their many similarities. The CDC (2024) lists risk factors for colorectal cancer: age, physical activity, family history, smoking, alcohol intake, obesity, diet, and inherited genetic mutations. Globally, colorectal cancer is the third most common cancer and is the second leading cause of cancer-related deaths (WHO, 2023). In the Philippines, colorectal cancer is the third most common cancer with 20,736 new cases in 2022, and ranks fourth in terms of number of deaths with 10,692 in the same year (WHO - IARC GLOBOCAN, 2024).

Polyps, a growth in the lining of the colon, usually mark the beginning of colorectal cancer. However, the presence of polyps does not mean that a patient already has cancer, only the risk of the possibility that it may progress into cancer (American Cancer Society, 2024). This underscores the importance of early diagnosis and treatment. Similar to most cancers, colorectal cancer is most treatable while at the localized stage becomes more fatal as it progresses. This is made evident with the 5-year relative survival rates of colon and rectal cancer: 91% and 90% in the localized stage and 13% and 18% in the distal stage (American Cancer Society, 2024). However, early diagnosis is made difficult by the absence of symptoms in the early stages of colorectal cancer.

Therefore, it is essential for early and consistent screening for the disease. Fortunately, there exists a number of screening tests and techniques available today: guaiac-based fecal occult blood test (gFOBT), fecal immunochemical test (FIT), FIT-DNA test, flexible sigmoidoscopy, colonoscopy, and CT colonography (CDC, 2024). Unfortunately, the Philippines lacks a formal colorectal screening program; thus, most cases are diagnosed as symptoms appear, in the late stages (Fernandez et al., 2024). Fernandez et al. (2024) also state that the lack of formal screening, along with the cost and accessibility, further lowers patient participation in colorectal cancer (CRC) screening, leading to increases in incidence and mortality rates.

Moreover, CRC treatments can be costly or inaccessible to certain areas. This is especially true for low-middle-income countries like the Philippines, as the main treatment for CRC is surgical resection (Philippine Society of Gastroenterology, 2019). Although surgical resection serves as the cornerstone of CRC treatments, the PSG-CRC Handbook (2019) also suggests alternative treatments such as chemotherapy. The National Cancer Institute also provides alternative treatments for CRC, such as radiation therapy, targeted therapy, and immunotherapy. CRC treatments prove to be either costly, have many side effects, or both. Nearly all of the aforementioned treatment plans can be financially crippling (Baclig, 2023). Moreover, most cancer treatments, while effective, have many undesirable consequences.

Natural sources offer possibilities of less toxic, cheaper, faster, and more eco-friendly ways to combat cancer. The potential of bioactive plants is already often utilized—plant metabolites like vinblastine, vincristine, and taxol have already been brought to light for having antitumor properties. In particular, vinblastine and vincristine still serve as one of the many natural medications that are utilized today. Vincristine has even become a standard therapy for pediatric

acute leukemia (Taub et al., 2024). This underscores the potential of natural sources in providing anticancer medications that are cost-effective, safe, and efficacious.

The Philippines, which is rich in plant biodiversity (Aureo et al., 2020) and has a history of herbal use, offers many endemic plant sources. Tibig (*Ficus nota*) is an endemic tree in the Philippines and is easily accessible. The Ficus family contains numerous compounds (Cruz et al., 2022) that have shown cytotoxic properties and activity against cancer (Rajasekar et al., 2023).

Although many anticancer drugs are available, the search for new treatments continues due to the high costs and side effects of current medications. Tibig (Ficus nota) has shown promising cytotoxic and anticancer effects against certain cancer cell lines. This study focuses on identifying the compounds in Tibig leaves that exhibit the strongest cytotoxic activity against colorectal cancer cells (HCT116), aiming to uncover potential new natural therapies.

Background of the Study

More than 800 species make up the Ficus genus in the Moraceae family, which is primarily found in tropical and subtropical regions (Pimentel et al., 2023). Cruz and others. According to al. (2022), the entire genus Ficus has a wide range of substances with various functions and impacts, including alkaloids, flavonoids, glycosides, saponins, steroids, tannins, and terpenes. Ficus species have demonstrated noteworthy applications: Ficus polita and Ficus thonninggii showed cytotoxicity by increasing the death rate of brine shrimp, Ficus racemosa leaves showed cytotoxicity towards the Dalton Lymphoma Ascites (DLA) cell line (Khan et al., 2017), and Ficus crocata was found to exhibit selective toxicity to the cancer cell line MDA-MB-231 cells (Sánchez-Valdeolívar, 2020).

Colorectal cancer is the third most common cancer worldwide, making up about 10% of all cases, and it is the second leading cause of cancer-related deaths globally, according to the World Health Organization (2023). A key tool in colorectal cancer research is the HCT116 cell line, which comes from a colorectal tumor in an adult male and is widely used to study this disease. The G13D mutation in codon 13 of the KRAS gene renders it significant. The oncogenic transformation of these cells is a result of the mutation. Both can be ideal for in vitro and in vivo studies since they are also employed for studying mice.

Though there are other Ficus species which are well investigated for their cytotoxicity, few studies of the cytotoxicity of Ficus nota (Tibig) and the compounds found in the plant for its cytotoxic activity, and especially its selective cytotoxic activity against HCT116 cells, have been carried out. As per Wang et al., (2021) the selective cytotoxicity of the compounds towards HCT116 cells encompasses, but is not restricted to induction of apoptosis, oxidative stress mechanisms, and cycle arrest. Exploring the leaves of Ficus nota (Tibig) will provide further insight concerning the profile of the plant and learn more about its cytotoxic compounds. This is especially interesting for anticancer drug discovery against cancer, especially colorectal cancer. In addition, this research will enhance the understanding of cancer and medicine.

Operational Framework

The HCT116 cell line was used to assess the effects of the ethanolic extracts of Tibig (*Ficus nota)* leaves. A comprehensive approach was employed, commencing with the preparation of the plant extract in order to perform various tests and fractionations needed for testing cytotoxicity. The preliminary measure of cytotoxicity was

determined using the Brine Shrimp Lethality Assay, while the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay quantified cell metabolic activity as an indicator of cell health. Control experiments with untreated cells and the standard, Vinblastine and Fluorouracil, provided natural and synthetic benchmarks to validate and compare the effects of the plant-derived compounds. To identify the specific bioactive constituents responsible for the observed cytotoxicity, Liquid Chromatography–Mass Spectrometry (LC-MS/MS) was conducted on the most active fractions. Finally, in silico analysis was performed to computationally predict and model the interaction between the identified compounds and molecular targets relevant to colorectal cancer, as well as assessing the drug-likeness and pharmacokinetic properties of the identified compounds.

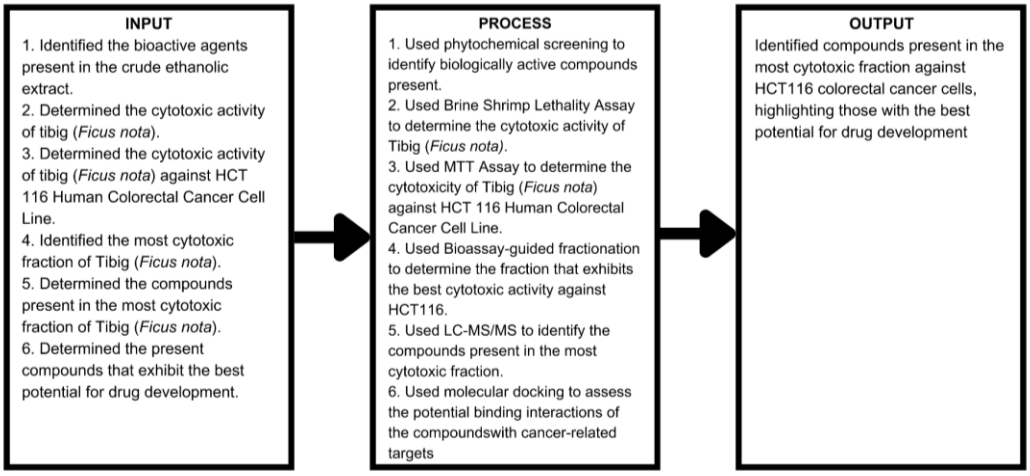

Figure 1: Operational Framework.

Research Objectives

General Objectives

This study aimed to identify and characterize the compounds of Tibig (*Ficus nota)* leaves exhibiting the most cytotoxic effects using In Vitro and In Silico analysis.

Specific Objectives

To evaluate the cytotoxic property of the identified fraction from the Bioassay-Fractionation from Tibig (*Ficus nota)* leaves ethanolic extract.

The specific objectives of this study were:

1. To obtain Tibig (*Ficus nota)* leaves ethanolic crude extract and compute the percentage yield.
2. To conduct phytochemical screening for the confirmatory test of the different constituents present in the ethanolic crude extract.
3. To determine the cytotoxic property of the biologically active compounds of Tibig *(Ficus nota)* leaves using Brine Shrimp Lethality Assay.
4. To determine the cytotoxic property of the biologically active compounds of Tibig *(Ficus nota)* leaves against HCT116 Human Colorectal Carcinoma Cancer Cell Line using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay.
5. To obtain and identify the fraction of Tibig *(Ficus nota)* leaves that exhibit the highest cytotoxic activity.

6. To identify the compounds present in the fraction that exhibit the highest cytotoxic activity.
7. To perform in silico analysis of the identified fractionated compounds, predict their binding affinity to colorectal cancer-related proteins, and assess their pharmacokinetic properties.

Hypotheses

Ho: The computed IC50 of the fractionated extract of Tibig (*Ficus nota)* leaves has no observable difference with the published IC50 of the standard drugs Fluorouracil and Vinblastine against HCT116 Human Colorectal Carcinoma Cell Line.

Ha: The computed IC50 of the compounds of the fractionated extracts of Tibig (*Ficus nota*) leaves has an observable difference with the published IC50 of the standard drugs Fluorouracil and Vinblastine against HCT116 Human Colorectal Carcinoma Cell Line.

Significance of the Study

This study investigated the medicinal potential of *Ficus nota*, an endemic plant in the Philippines, as an alternative for the treatment of colorectal cancer.

The findings of the study aim to benefit the population of this study, specifically cancer patients with colorectal cancer. Focusing on the medicinal potential of Ficus nota as a natural drug for human colorectal carcinoma cancer cells (HCT 116), this study seeks to provide medical practitioners with a natural therapy that can offer fewer side effects than traditional methods. The methodical process of isolating bioactive compounds may result in the identification of new anti-cancer agents, providing new drug development avenues that can potentially provide more effective and less toxic drugs. Moreover, this research points to herbal cancer therapies as a cost-effective alternative to the pricey drugs in the market. Research into the therapeutic activity of Ficus nota provides an avenue for investigation into its potential effectiveness against other forms of cancer.

This study not only sought to expand scientific knowledge of the therapeutic significance of Ficus nota but also provided solutions to health issues that highlight the advantages of Ficus nota in enhancing human health.

Scope and Delimitations of the Study

This study investigated the cytotoxic compounds in Ficus nota (Tibig) leaves against the human colorectal cancer cell line HCT116 using bioassay-guided fractionation to identify the most active components. The *Ficus nota* leaves used were harvested and collected from the same source to ensure consistency. The fractions obtained were subjected to the Brine Shrimp Lethality Assay for a preliminary assessment of the cytotoxic effect of Tibig, and the MTT assay to further determine cytotoxic activity was conducted. Liquid Chromatography-Mass Spectrometry was utilized to identify the compounds responsible for the cytotoxicity. The identified compounds were structurally modeled using ChemDraw to generate optimized 2D representations. Binding site analysis of the protein targets; TNIK, PI3Kα, and EGFR was conducted using CASTp, a surface topology tool for mapping potential binding pockets based on volume and accessibility. Molecular docking simulations were performed using AutoDock Vina to predict binding affinities and interaction profiles, with Chimera utilized for the visualization and analysis of docking results. Additionally, SwissADME was employed to evaluate the pharmacokinetic properties and drug-likeness of the identified compounds to support in silico findings.

The following delimitations were observed during experimentation. Leaves were the only plant part used. Biologically-assay guided isolation of other pharmacologic compounds was not conducted. The in vitro cytotoxic studies were exclusive to the HCT116 human colorectal carcinoma cancer cell line. The Brine Shrimp Lethality Assay and MTT assay were the only assays used to determine the cytotoxicity. Furthermore, Liquid Chromatography-Mass Spectrometry was the only primary method used for compound identification. For in silico analysis, ChemDraw was the only application used for structural modeling, CASTp was the sole tool for binding pocket mapping, AutoDock Vina was the only software used for molecular docking, Chimera was the only platform used for molecular visualization, and SwissADME was the only tool used for pharmacokinetic and drug-likeness prediction. No additional computational tools or experimental validations beyond those stated were conducted.

Definition of Terms

This part of the study provides readers with a thorough understanding of the terms used throughout the paper.

- **Bioassay- Guided Fractionation -** A technique to be used for profiling and screening of plant extracts for bioactive compounds with potential sources of new bio-based drugs (Mani et al., 2022)
- **Brine Shrimp Lethality Assay** - A tool to be used to assess the preliminary cytotoxicity assay of plant extract based on its ability to kill a laboratory cultured larvae (nauplii) (Sarah et al, 2017)
- **Cancer** - These are the diseases to be assessed, which are characterized by a large number of abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissue. (Cancer - Symptoms and Causes, n.d.)
- **Cell Culture** - This is an in vitro process involving the isolation of cells under extremely controlled conditions (Gilmore et. al., 2023)
- **Colorectal cancer -** This is the type of cancer that develops in the tissues of the colon or rectum. (National Library of Medicine, n.d.)
- **Cytotoxicity** - This is the degree to which an agent has specific destructive action on cells (Rock et.al, 2023)
- ***Ficus nota*** - A species of fig tree native to the Philippines (Reyes, et. al., 2020)
- **HCT116** - This is a human male colon cancer cell line that is commonly used to study colon cancer progression. (HCT116 Cell Line - a Comprehensive Guide to Colorectal Cancer Research, n.d.)
- **In Silico** - These are experiments or analyses carried out using computer simulations or models to explore and understand biological systems and processes.
- **MTT Assay**- A test that measures metabolic activity by detecting color changes (Nga et. al., n.d.)

CHAPTER 2

Review of Related Literature and Studies

This chapter takes a close look at a variety of source books, articles, journals, and other important references that are relevant to this research. It digs into several key areas about plants, like their physical characteristics, the chemicals they contain, how they might affect the body, and their potential to be toxic. On the medical side, it also reviews studies about colorectal cancer, focusing especially on the HCT116 cell line. It covers different lab techniques used in this study, such as breaking down plant extracts through bioassay fractionation, testing toxicity with the Brine Shrimp Lethality Assay, measuring cell viability using the MTT assay, analyzing compounds with LC-MS/MS, and running computer simulations through in silico analysis.

The Genus Ficus

Falistoco informs us that among the largest genera of angiosperms, the Ficus group has 800 species worldwide in the tropics and subtropics. Such diversity makes these plants very important as regulators of biophysical processes within ecosystems. Most surprisingly rich, as it offers the species, Ficus is the type for holding such big treasures of fundamental minerals and different natural compounds, conferring upon them strong antioxidant activities. With these characteristics, some are hepatoprotective, antibacterial, and cardioprotective, and they may also be antineoplastic. Such plants indeed constitute a very good source of natural health remedies.

Phytochemical Studies on the Genus *Ficus*

The Ficus genus is rich in a variety of compounds found throughout its bark, roots, and above-ground parts. These include alkaloids, flavonoids, glycosides, saponins, steroids, tannins, and terpenes, all of which contribute to its diverse biological properties.

Several bioactive compounds, such as phenols, flavonoids, tannins, saponins, coumarins, and steroids, have been identified through phytochemical analysis of Ficus nota. These compounds considerably contribute to the plant's possible health benefits (Cungihan, 2024). The following secondary metabolites have also been identified in extracts of Ficus nota leaves: glycosides, flavonoids, carbohydrates, saponins, and tannins. They all possess antihyperglycemic and alpha-glucosidase inhibition activities (Franco et al., 2019).

Pharmacological Studies on the Genus *Ficus*

Due to the diversified pharmacological activities that the genus Ficus displays, its species are greatly valued in both traditional and complementary medicine. Ficus species have proved useful in the management of several diseases, including diabetes, cystitis, and diseases of the central nervous system, heart, and lungs (Salehi et al., 2020). These plants' high phytochemical content, with anti-inflammatory, antibacterial, and antioxidant chemicals, is to blame for their numerous therapeutic applications. Cytotoxic activities of Ficus species are one of these effects. It has been a notably interesting area of study.

Based on recent research, a variety of extracts from various Ficus species exhibit excellent cytotoxic activities on various cell lines of cancer. Ficus carica and Ficus religiosa, for instance, have shown excellent inhibitory activities on the growth of cancer cells, which highlights their possible applications as natural anticancer drugs (Salehi et al., 2020). Ficus carica has also been proved by Soltana et al. (2019) to possess antitumor and antiproliferative activity against colorectal cancer, in this case, using the cell lines HCT 116 and HT-29. This indicates it is a potent chemotherapeutic or chemopreventive agent against the human colorectal carcinoma cell line. Ficus extracts are compelling prospects in the search for effective cancer therapies due to their ability to induce apoptosis and inhibit the progression of the cell cycle. The increase in demand for new, safe, and effective cancer therapies that can complement or enhance existing therapies is the particular reason we chose to examine cytotoxicity. Traditional chemotherapeutic agents often result in profound adverse reactions, and natural compounds obtained from Ficus spp. could give a more targeted approach with less harmful effects (Salehi et al., 2020).

Also, some bioactive compounds of *Ficus* species have been purified, and their potent cytotoxic activities have been shown. Dongnoside E from *Ficus glumosa*, for instance, has been shown to have activity comparable to that of standard

chemotherapeutic agents (Salehi et al., 2020). This aspect of *Ficus* species not only signifies their therapeutic use but also warrants further studies of their mechanisms of action in a bid to design new cancer therapy strategies.

Botanical Description of *Ficus nota*

Often called "tibig" or "sacking tree" in local terms, *Ficus nota (Blanco) Merr.* is a flower-bearing species and a member of the Moraceae family. This native species hails from the Philippines, though it's often seen growing wild in Luzon and the Visayas; however, it does grow in parts of Malaysia as well as in northern Borneo (Mancia et al., 2019). It has a height of up to nine meters and is often found near bodies of water at low elevations. Birds are responsible for its dissemination, as they eat the fruit and pass on the seeds. In the Philippines, Filipinos usually eat the tasteless but palatable fruits with sugar and cream. As a vegetable, its young leaves are also eaten.

Cytotoxic Activity of *Ficus nota*

Ficus nota is a plant known for its ethnic medicinal utilization in the treatment of various illnesses, including muscle aches, hypertension, hyperglycemia, and fever. The ethanolic extracts of *Ficus nota* exhibited active cytotoxic activity against the brine shrimp, proven through the Brine Shrimp Lethality Assay, indicating potential bioactive components of the plant (Arquion et al., 2015). Moreover, the results of the tests conducted proved that ethanol extract exhibited greater cytotoxic activity compared to the decoction extract which accounts for further phytochemical screening for the determination of its bioactive components.

Additionally, the evaluation of the cytotoxic properties of ethanolic extract of *Ficus nota* indicates that the plant possesses significant bioactive compounds that warrants further investigation for their other therapeutic applications. The potency of these extracts was significantly higher than the extracts derived from the stem of the plant, highlighting the superior efficacy of the leaf extracts based on its cytotoxic properties, which supports the traditional use of *Ficus nota* and aims to identify the bioactive components responsible for its cytotoxic effects (Latayada et al., 2016).

Colorectal Cancer

Colorectal cancer is among the leading cancers in both incidence and mortality. According to the World Health Organization (2023), colorectal cancer is the third most common cancer and is the second leading cause of cancer-related deaths. It is also the third most common cancer and the fourth leading cause of cancer-related death in the Philippines (International Agency for Research in Cancer, 2024).

Colorectal cancer is defined as any cancer originating from the colon, termed as colon cancer, or from the rectum, termed as rectal cancer, and the term colorectal cancer is used due to the similarities that these cancers share. Colorectal cancers usually start with the formation of polyps, growths on the inner lining of the colon or rectum. From there, the cancer may spread into the outer layers of the lining and eventually, to other parts of the body. (American Cancer Society, 2024).

In its early stages, colorectal cancer is the easiest to treat and has the highest survival rate, both for colon and rectal. However, diagnosis is often difficult due to there being no symptoms during these stages. Usually, symptoms appear when the cancer has already metastasized and is in its regional stage. Only in this stage will most people experience the usual symptoms: persistent changes in bowel habits, rectal bleeding, abdominal pain or cramping, fatigue, and unexplained weight loss. As is the case with most cancers, the survival rates drop as the disease advances. Thus, early

detection is crucial, as demonstrated by the fact that CRC survival rates also depend on the stage of the disease when it was diagnosed. (Cleveland Clinic, 2022 & American Cancer Society, 2024 & World Health Organization, 2023).

Regular screening is necessary in order to reduce the risk of developing CRC, especially for people fitting the risk factors of CRC: age (>50 years old), family history and genetic conditions, personal history of polyps or CRC, and lifestyle factors such as obesity, sedentary behavior, smoking, and excessive alcohol intake. This is also highlighted by the fact that even though modern developments in anticancer treatments have already improved survival rates drastically, patients who were diagnosed before the disease has metastasized still have more favorable survival rates (Dekker et al., 2019). There are numerous screening techniques available today: guaiac-based fecal occult test (gFOBT), fecal immunochemical test (FIT), FIT-DNA test, flexible sigmoidoscopy, colonoscopy, and CT colonography (CDC, 2024). Early detection along with effective modern treatments such as surgical expurgation, radiotherapy, chemotherapy, immunotherapy, and a combination of them (Kumar et al., 2023) vastly improve the survivability of patients battling CRC. However, it is still a necessity to explore potential anticancer medication that might offer safer and more effective treatment.

HCT116 Cell Line

The HCT116 cell line is widely recognized in the study of cancer, specifically in colon cancer proliferation. This cell line has limited differentiation potential and is characterized by their high oncogenic aggressiveness which is ideal for aggressive tumor phenotype studies. HCT116 cells mainly serve as a backbone for the continuing study of colorectal cancer and have brought massive leaps into the disease's pathogenesis and anticancer treatments. Further evidence of the efficacy of this cell line revealed and proved that HCT116 facilitates focal studies on tumor behavior and the overall potency of drugs (Kurasaka et al., 2021).

Kurasaka with his co-researchers further characterized HCT116 cells and provided valuable information regarding the significance and benefits of this cell line. Their research showed that HCT116 provided studies with clarification on the mechanisms of drug action, drug resistance, drug of choice in treating colorectal cancer, and a lot more discoveries which proved why this cell line stands out and is being widely-recognized in vitro model for colorectal cancer, the third most common cancer globally (WCRF International, 2024).

One remarkable feature of HCT116 cells is their high amenability to transfection, particularly those with viral vectors. This characteristic is extremely helpful in gene therapy research, as it allows efficient and precise introduction of genetic material, supporting advanced genetic manipulations and functional studies. (Kurasaka, C., Ogino, Y., & Sato, A. 2021). Another advantage of this cell line is its homogeneity. Their research showed that around 70% of HCT116 cells display consistent genetic profiles, the main determinant of its homogeneity. This characteristic is a crucial factor especially in studies which focus on gene expression, cellular signaling pathways, and studies which assess the efficacy of drug treatments. However, among its limitations are that it does not offer any information regarding the selective toxicity of samples being tested.

HEK293 Cell Line

The Human Embryonic Kidney cell line, also known as HEK293, was created in 1973 through transforming human embryonic kidney cells with altered adenovirus-5 DNA (Abaandou, L., Quan, D., & Shiloach, J. 2021). This cell line belongs to a class of kidney-derived non-cancerous models that has already been established in the field of biochemical

and toxicological studies. The origin of HEK293 remains debated, while some evidence suggests it may be derived from neuronal cells, others firmly believe it came from kidney tissues. (Chen, Y., et al., 2019).

Human cell lines provide a significant potential for the improvement of biotherapeutic production as these produce proteins with post-translational modifications that closely match those found in natural human proteins (Picanco-Castro, V., Biaggio, R., Cova, D., & Swiech, K., 2013). Among human cell lines, HEK293 stands out for its versatility. With its high transferability, efficient insertion of genetic material, allowing precise control over protein production, specifically viral vectors and making it an ideal choice for gene expression studies.

Specialized variations of HEK293 were established by scientists over time, leading to discoveries of the applications of this cell line. These variations include HEK294-T modified to create better DNA replication, HEK293-F adapted for serum-free suspension growth, HEK293-E used for enhanced plasmid replication, and HEK293-6E with improved growth and gene expression. These derivatives are widely used to provide contributions to therapeutic studies of proteins and vaccines as they grow easily, adapting to large-scale production and most importantly, mimicking human cell traits. While the HEK293 cell line proposes significantly strong traits, researchers must prudently interpret results for this cell line, especially in studies focused on kidney-specific effects due to its uncertain origin and genetic modifications (Tan, E., et al., 2021).

Bioassay-guided fractionation

Bioactives are compounds that exist in nature and have favorable effects on human health. These compounds, which include phytochemicals, can be extracted and purified from a variety of sources using various methods and utilized as medicines or health-promoting products (Khademi, 2023). Nothias et. al. (2018) state that nature has played a crucial role in the discovery of numerous therapeutic agents derived from organisms such as microbes, plants, and insects. The search for new bioactive natural products, which can serve as leads for therapeutic development, is often inspired by ethnopharmacological knowledge or accomplished through the screening of extract collections for bioactivity. This process can involve in vitro, in cellulo, and in vivo assays. When a natural extract is found to have bioactivity, bioassay-guided fractionation is typically conducted. This process involves several steps: (i) extracting metabolites from the biomass using solvents, (ii) fractionating the extract via chromatography, (iii) screening each fraction for bioactivity, (iv) isolating active molecules from the bioactive fractions, and (v) identifying the isolated molecules and evaluating their bioactivity. Although this method has been used by chemists, pharmacologists, and toxicologists since the early 1900s, it was formally established after the 1950s and continues to be employed today by researchers in academic, government, and industrial laboratories worldwide. However, challenges such as loss of activity or failure to isolate bioactive compounds during the fractionation process are common and can be costly. These setbacks are often unpublished or later reported as chemical characterization studies. Common reasons for these failures include: (1) degradation of bioactive compounds during purification, (2) the presence of bioactive compounds in concentrations too low for effective isolation, and (3) the bioactivity being due to synergistic effects between multiple compounds. Therefore, it is critical to identify candidate bioactive molecules early in the purification process to streamline the isolation efforts and be able to accurately identify compounds of interest.

Brine Shrimp Lethality Assay

The brine shrimp lethality bioassay is a widely utilized method for assessing the toxicity of various substances, including heavy metals, pesticides, pharmaceuticals, and natural plant extracts. It serves as a preliminary screening tool

to evaluate toxicity for plant extracts before conducting further experiments on mammalian animal models by assessing their ability to eliminate laboratory-cultured brine shrimp larvae (nauplii) (Wu, 2014). In this procedure, nauplii are exposed to various concentrations of the plant extracts for 24 hours, after which the number of motile nauplii, indicating survival, is counted to evaluate the extract's effectiveness. This method is recognized for its simplicity, cost-effectiveness, and low material requirements (Quazi et al., 2017).

Whether natural or synthetic, the Brine Shrimp Lethality Assay is valued for its rapid and comprehensive nature in testing bioactive compounds. It is affordable, simple, and does not require aseptic techniques. This assay allows for the use of a large number of organisms, which supports statistical validation and does not require specialized equipment. A small sample amount (2-20 mg or less) is sufficient for testing (Quazi et al., 2017).

Accurate and reliable assays are essential for cytotoxicity evaluation, and studies have noted limitations in the Brine Shrimp Lethality Assay. While valuable for initial toxicity screening, it primarily indicates general toxicity without identifying specific mechanisms of action. This assay is complemented with a cell line-based cytotoxicity assay, the MTT assay, to further confirm and explore the mechanisms of active compounds.

MTT Assay

MTT assay is an effective tool that measures the viability of cells to determine the cytotoxicity of drugs at different concentrations. It is a widely used method for quantifying mitochondrial activity of the cell indicated by conversion of MTT to formazan crystals (Van Meerloo et al., 2011). Researchers use this quantitative colorimetric assay to assess cell activation, proliferation, or cytotoxic activity of various samples. Using ELISA reader, a multiwell scanning spectrophotometer, the assay results are obtained, offering a rapid and precise approach for understanding cellular responses.

In the MTT assay, the central tetrazole ring is broken down, resulting in the formation of a violet-blue, water-insoluble molecule known as formazan, specifically (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). This mono-tetrazolium salt consists of a positively charged quaternary tetrazole ring core with four nitrogen atoms surrounded by three aromatic rings with two formazan. Due to its positive charge and lipophilic structure, the MTT reagent can pass through the cell membrane and penetrate the inner membrane of the mitochondria of living cells. Metabolically active cells can turn MTT into formazan, which can be quantified due to the chromogenic nature of this redox chemical reaction, enabling the measurement of the intracellular formazan through colorimetric analysis. Mosmann et al. used this to create the MTT test in 1983, leading to a very useful way to measure the metabolic activity of cells. Although it is useful, it does not directly determine cell survival or account for other confounding factors that may influence metabolic activity, which may result in inaccurate results. The MTT test is typically done after the cells have been incubated with MTT for a few hours. Then, a liquid like dimethyl sulfoxide (DMSO) is used to break up the water-insoluble formazan that was made. Then, the optical density (OD) at a wavelength that MTT-derived formazan absorbs the most light at (around 570 nm) is used to measure how much the blended MTT-formazan solution reduces light transmission through absorption and other means. The observed OD values are thought to show the quantity of formazan and, therefore, the MTT decrease inside the cell. This has been the basis of MTT assay application for nearly forty years as a tool to measure cytotoxicity, cell proliferation, and metabolic activity of cells.

Evaluation of cell viability and cytotoxicity requires an accurate and reliable assay. Limitations of the MTT assay, including limited sensitivity, dynamic range, and chemical interference, can affect percentage inhibition. Therefore, many scientists use a combination of complementary assays to gain a more comprehensive view of cell viability and cytotoxicity.

Fluorouracil

Fluorouracil (5-FU) belongs to the class of chemotherapy drug antineoplastics (Casale J, Patel P. 2024). Chemically, it is a heterocyclic aromatic organic compound that has a structure similar to the pyrimidine molecules of DNA and RNA. 5-FU is a uracil analogue with a fluorine atom at the C-5 position in place of hydrogen. This structural feature enables 5-FU to disrupt nucleoside metabolism, incorporating into RNA and DNA and ultimately inducing cytotoxicity and apoptosis (Zhang et al., 2008). Because of these findings, fluorouracil has been widely used in the treatment of cancer. Numerous studies of this drug, especially its mechanism and established toxicity made it a valuable reference compound in cytotoxic studies.

From a number of studies in relation to this drug from the past years, these has led to deeper and accurate understanding of the mechanism of action of Fluorouracil which helped scientists and researchers develop appropriate strategies that may increase Fluorouracil's anticancer activity (Longley, D. B., Harkin, D. P., & Johnston, P. G. 2003). Fluorouracil is a notable chemotherapy agent in combination with other therapeutic agents indicated for patients with colorectal cancer (Lee, J.J., Beumer, J.H. & Chu, E. 2016).

Despite all recorded efficacy, 5-FU exhibits limitations including a short biological half-life due to rapid metabolism, inconsistent oral absorption, variable bioavailability, and non-selective effects on healthy cells. Possible ways indicated to overcome these limitations is to modify the delivery of 5-FU to extend its circulation time and enhance its efficacy, thus becoming essential. Targeted delivery systems aim to sustain therapeutic concentrations of 5-FU while minimizing systemic levels, thereby improving its therapeutic index. (Entezar-Almahdi, E., Mohammadi-Samani, S., Tayebi, L., & Farjadian, F. 2020).

Vinblastine

Vinblastine, a vinca alkaloid naturally isolated from *Catharanthus roseus* leaves, is a natural drug known for its anticancer properties and for treating Hodgkin's disease (Banyal et al., 2023). According to the study by Dhyani et al. (2022), various plant parts of this plant (*Catharanthus roseus*) during the Ayurvedic era were used in folkloric herbal medicine to treat various ailments, and one of these is the treatment of cancer (Dhyani et al., 2022).

Vinblastine is widely known as the standard regimen in Hodgskin's lymphoma (Ozdemir et al., 2014). Further studies of this vinca alkaloid proved its efficacy in treating various malignancies, especially in combination therapies; thus it is indicated for diseases such as testicular cancer, ovarian cancer, breast cancer, head and neck cancer, and non-Hodgkin's lymphoma (Sears & Boger, 2015). One key factor that vinblastine has is its effectiveness in inhibiting tumor growth, which is essential in combination therapy regimens.

Vinblastine's mechanism of action involves cell death. It binds to microtubule proteins of the mitotic spindles, preventing cell division during the anaphase of mitosis. This activity eventually leads to mitotic arrest and apoptosis in rapidly dividing cancer cells (Knottenbelt et al., 2016). In relation to the antitumor effect of vinblastine, this happens

due to their ability to bind to intracellular tubulin, which inhibits DNA repair and RNA synthesis (Dhyani et al., 2022). Vinca alkaloids from *Catharanthus roseus*, in general, have a mechanism of action that alters the microtubular dynamics, resulting in cell growth regression and apoptosis (Dhyani et al., 2022).

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Liquid chromatography-mass spectrometry, an analytical chemistry method that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (Parasuraman et al., 2015). Due to its high sensitivity, selectivity, and ability to provide both qualitative and quantitative data on complex plant extracts, LC–MS is a powerful technique useful in research and clinical applications. (Briki et al., 2024)

LC-MS/MS functions by separating compounds in a sample using liquid chromatography first, followed by ionization and detection based on their mass-to-charge ratio (m/z). The tandem mass spectrometry (MS/MS) features further fragments of selected ions into smaller ions, which are then analyzed to provide detailed structural information. This method identifies the molecular structure, functional groups, and possible isomers of the compounds, thereby offering deeper insights into their chemical composition (Pitt, 2009). This technique provides significant advantages in phytochemical research, including high sensitivity, the ability to differentiate compounds with similar chromatographic behavior, detailed structural information through MS/MS fragmentation patterns, and broad applicability in analyzing diverse phytochemical constituents and the potential sources of its cytotoxic properties.

In Silico Analysis

In silico strategies significantly help in natural product drug discovery, offering predictive insights during hit discovery, hit-to-lead transition, and lead optimization. These tools enhance the identification of bioactive phytochemicals while rationally prioritizing plant sources for experimental validation, thereby directing synthetic efforts toward structurally optimized candidates (Chen and Kirchmair, 2020).

Docking methodologies serve as valuable tools for virtual screening campaigns, facilitating the efficient identification of potential bioactive compounds from large molecular libraries. According to Hasan et al., therapeutic potential of any compound can be assessed using molecular docking, significantly reducing time and cost in drug development. Given that compounds may possess diverse biological activities, computational approaches are utilized to systematically assess their pharmacologic effects against the diseases. Molecular docking represents a critical in silico methodology for screening and characterizing potential drug molecules. The technique's ability to predict ligand-protein binding interactions significantly enhances the efficiency of drug discovery and optimization processes (Azam et al., 2022).

Traf2 and Nck-interacting kinase (TNIK)

Traf2- and Nck-interacting kinase (TNIK) is a serine/threonine-protein kinase that plays a crucial role in the canonical Wnt/β-catenin signaling pathway, which is essential for regulating cell fate, proliferation, and stem cell maintenance. TNIK functions by phosphorylating and activating transcription factors such as TCF4 in complex with β-catenin, thereby promoting the transcription of Wnt target genes (UniProt Consortium, 2023).

This pathway is frequently dysregulated in various cancers, especially colorectal cancer (CRC), where over 90% of cases exhibit mutations in Wnt pathway components like APC or β-catenin. These mutations lead to constitutive Wnt signaling, promoting cancer cell proliferation, survival, and the maintenance of cancer stem cells (CSCs). TNIK is

particularly vital in this process, serving as a transcriptional co-activator that stabilizes the β-catenin/TCF4 complex, making it indispensable for sustaining tumor-initiating stem-like properties (Masuda et al., 2016).

In the landmark study by Masuda et al. (2016), TNIK was validated as a therapeutic target in CRC. The researchers developed NCB-0846, an oral small-molecule inhibitor that binds to TNIK in its inactive conformation. Inhibition of TNIK with NCB-0846 effectively suppressed Wnt signaling, diminished colorectal CSC properties, and reduced tumor formation in preclinical models. These findings underscore TNIK's central role in CRC pathogenesis and highlight it as a promising target for anti-cancer therapy.

Phosphoinositide 3-kinase (PI3K) isoform (PI3Kα)

Phosphoinositide 3-kinase alpha (PI3Kα), a lipid kinase, is composed of two main subunits, namely the catalytic p110α, encoded by PIK3CA, and a regulatory subunit, commonly p85α (PIK3R1). It plays a key role in the PI3K/AKT/mTOR signaling pathway, which is essential for certain cellular processes like growth, metabolism, proliferation, and survival (Zheng et al., 2023). Activation of PI3Kα occurs downstream of receptor tyrosine kinases, where it converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3), promoting the recruitment and activation of downstream effectors like AKT. This signaling cascade is tightly regulated in normal physiology but is frequently dysregulated in cancer (Voutsadakis, 2021).

Mutations in the PIK3CA gene in human cancers are one of the most frequent oncogenic changes, which are particularly common in breast (18%–40%), colon (32%), and endometrial (35%–53%) cancers (Zheng et al., 2023). Due to PI3Kα's key role in cancer, scientists have continuously developed drugs that may block its activity. An example of this drug is alpelisib, which is an already approved drug to treat certain patients with advanced breast cancer, specifically those who carry mutations in the PIK3CA gene (Narayan et al., 2020).

Epidermal Growth Factor Receptor Tyrosine Kinase Domain (EGFR-TKD)

Epidermal Growth Factor Receptor is a transmembrane protein that binds extracellular ligands, triggering dimerization and activating intracellular signaling pathways involved in cell growth, division, and survival (Mumphrey, B., et al., 2023).

EGFR is involved in activating several key pathways, including MAPK/ERK, PI3K/Akt, and PLCγ/PKC cascades, which collectively regulate cellular processes such as proliferation, survival, and differentiation. In colorectal cancer cells, these pathways contribute to tumor growth and progression.

Moreover, studies suggest that upon stimulation by the muscarinic acetylcholine receptors, EGFR works together with Protein Kinase C to activate ERK1/2 and P90 Ribosomal S6 Kinase (RSK), which leads to increased cell growth. The pharmacological inhibition of EGFR significantly reduces growth, highlighting the important role as a mediator of colorectal cancer cell proliferation. (Choi, J.H., et al., 2015).

Synthesis of Related Studies

The *Ficus* genus, recognized for its therapeutic versatility and cytotoxicity from *Ficus religiosa* and *Ficus carica*, remains largely unexplored. *Ficus carica* has also contributed to this, as it exhibits antitumor and antiproliferative activity against colorectal cancer, specifically with cell lines HCT 116 and HT-29. *Ficus nota*, widely common in the Philippines, is among the many species that remain uncharted. Therefore, this study investigated the cytotoxic activity

of *Ficus nota* against HCT116 Human Colorectal Carcinoma Cell Line and will also provide further insights into its constituents. Current studies and literature of *Ficus nota* lacks advanced methodologies for assessing its toxicity and potential therapeutic effects on humans or mammals. Thus, the need for a modern and robust approach in evaluating its uses (Braganza L., 2024, Latayada, F. S. and Uy, M.M., 2016 and Arquion, C., and Uy, N., 2015)

Colorectal cancer (CRC) is a leading cancer in incidence and mortality rates both globally and in the Philippines (WHO, 2023 & IARC, 2024). CRC can be cured even before the polyp becomes cancerous, lowering the incidence and mortality; however, early detection is difficult due to it being asymptomatic. Usually, CRC patients are diagnosed when they are already in the regional stages, or when the disease has spread to nearby tissues (Cleveland Clinic, 2022 & American Cancer Society, 2024 & World Health Organization, 2023). Lower-middle-income countries like the Philippines face many difficulties in the early detection of CRC: screening movements, cost, and accessibility (Fernandez et al., 2024). The main treatment for CRC is surgical removal and chemotherapy, which can be costly and pose side effects (MayoClinic, n.d., & Philippine Society of Gastroenterology, 2019). HCT116, a well-known CRC cell line, imitates the progression of cancer in the body and is widely used for treatment studies for CRC, thus offering a fitting cell line to be used in the study. The HEK293 cell line is widely recognized for its ability to produce therapeutic proteins with human-like post-translational modifications, which are essential for maintaining protein functionality and minimizing immunogenicity (Picanco-Castro, V., Biaggio, R., Cova, D., & Swiech, K., 2013). However, HEK293 cells are not often utilized for commercial production due to their unsuitability for gene amplification and lack of selectivity mechanisms for stable foreign protein production. This limitation justifies the exploration of other cell lines, specifically HCT116, to assess the cytotoxic effects of *Ficus nota* leaf extracts against colorectal cancer.

The Brine Shrimp Lethality Assay (BSLA) is widely used as a preliminary cytotoxicity screen to identify active cytotoxic and anti-tumor agents by testing plant extracts and other compounds for their ability to eliminate lab-cultured brine shrimp larvae (Meyer et al., 1982). However, it is limited only to general toxicity; it does not identify the mechanism of action. This is why BSLA will only serve as a starting point for the bioassay-guided fractionation for preliminary screening. To complement BSLA, the MTT assay—a cell-line-based cytotoxicity test—offers more precise insights into cellular responses by measuring mammalian cell survival and proliferation (Mosmann, 1983). Using *Ficus nota* (Tibig) as the plant of interest, these assays will guide the bioassay-guided fractionation process to separate and test fractions, identifying bioactive fractions with the highest cytotoxic potential (Nothias et. al., 2018). This approach, combining the two assays for the fractionation, refines the identification of the bioactive compounds while maintaining the feasibility, cost-effectiveness, and efficiency of the procedures.

Vinca alkaloids exhibit potential anti-cancer properties but significantly lack analysis of resistance mechanisms across various cancer types (Banyal, A., et al., 2023). The literature reviewed highlights the crucial applications of Vinca alkaloids, failing to provide comprehensive pharmacokinetic data, addressing the hindrances brought by drug resistance. Additionally, comparative efficacy between traditional and newer vinca analog scarcity paves the way for assessing the cytotoxicity of potential novel compounds with new and improved efficacy with reduced resistance through MTT assay by conducting direct comparison of vinca alkaloid analogs and the *Ficus nota* extract utilized in this study.

This study utilized LC-MS/MS in order to identify the cytotoxic compounds after bioassay-guided fractionation with MTT assay, determining the cytotoxic activity of the extracts of *Ficus nota*. While LC-MS/MS provides structured

information, its slower throughput compared to automated immunoassays could limit the number of fractions that can be comprehensively analyzed (Grebe, S.K., & Singh R.J., 2011). Therefore, combining MTT assay with LC-MS/MS for detailed analysis optimizes the efficiency of identifying the potential anti-cancer compounds present in the plant extract. Using computer-based methods such as molecular docking helps speed up the search for active compounds in natural products. These techniques predict which plant-derived molecules might work against disease targets, helping researchers decide which ones to test further. When combined with lab methods like LC-MS/MS for identifying compounds and MTT assays for testing their effects on cells, this approach makes the drug discovery process more efficient and focused, saving time and resources.

AUTHOR	TITLE	YEAR	RESEARCH GAP
Braganza, L.	Tibig: 8 Medicinal and Health Benefits of *Ficus Nota*, Description, and Side Effect	2024	This study provides insufficient details regarding the specific compounds responsible for the anticancer properties of *Ficus nota*. A thorough identification and characterization of these bioactive components are essential, as they would offer valuable insights into the plant's therapeutic potential and aid in the development of targeted treatments. Understanding which compounds exert the most significant effects could enhance the efficacy of future research and facilitate the formulation of more effective cancer therapies based on *Ficus nota*.
Salehi, B., Mishra, A. P., Nigam, M., Karazhan, N., Shukla, I., Kiełtyka-Dadasiewicz, A., Sawicka, B., Głowacka, A., Abu-Darwish, M. S., Tarawneh, A. H., Gadetskaya, A. V., Cabral, C., Salgueiro, L., Victoriano, M., Martorell, M., Docea, A. O., Abdolshahi, A., Calina, D., & Sharifi-Rad, J.	*Ficus* plants: State of the art from a phytochemical, pharmacological, and toxicological perspective.	2020	This study expands on research showcasing the anticancer potential of "*Ficus*" species, widely recognized for their therapeutic versatility. While *Ficus religiosa* and *Ficus carica* are well-studied and demonstrate significant cytotoxic effects—such as inhibiting cancer cell growth and inducing apoptosis—*Ficus nota* (Tibig) remains comparatively underexplored. Our research addresses this gap by isolating and identifying cytotoxic compounds from *Ficus nota* effective against HCT116 colorectal carcinoma cells, using bioassay-guided fractionation and cytotoxicity assays. Given the need for safer, targeted cancer therapies, *Ficus nota* presents a promising yet underutilized resource, potentially offering a natural alternative with fewer side effects than traditional chemotherapy. This work seeks to highlight the unique contributions *Ficus nota* can make in advancing natural cancer treatments.
Soltana, H., Pinon, A., Limami, Y., Zaid, Y., Khalki, L., Zaid, N., Salah, D., Sabitaliyevich, U. Y., Simon, A., Liagre, B., & Hammami, M.	Antitumoral activity of *Ficus carica* L. on colorectal cancer cell lines.	2019	Studies on *Ficus carica* L. have identified bioactive compounds with anticancer effects on colorectal cancer cells, inspiring further exploration within the *Ficus* genus. This research isolates compounds from *Ficus nota* to evaluate their cytotoxicity against HCT116 cells, aiming to uncover selective anticancer properties and expand therapeutic insights for colorectal cancer treatment.
Cungihan, L.L	Effect of *Ficus nota* (Blanco) Merr on the Hematobiochemical	2024	Various *Ficus* species, including *Ficus nota*, contain bioactive compounds like flavonoids, tannins, saponins, and steroids. Prior research

	Profile and Growth Performance in Broiler Chickens		focuses on identifying these compounds across *Ficus* plant parts generally. Our study, however, specifically examines the cytotoxic compounds in *Ficus nota* leaves against HCT116 colorectal cancer cells, aiming to identify therapeutic agents. A focus not covered in prior studies.
Franco, M.J.S., & Cruz, V.G	Screening of the Mammalian Alpha-Glucosidase Inhibitory Activity of Selected *Ficus* Species from Mount Makiling, Laguna, Philippines	2019	Extracts from *Ficus nota* leaves contain carbohydrates, flavonoids, saponins, tannins, and glycosides, which have demonstrated antihyperglycemic effects. However, existing research has not explored the potential cytotoxic effects of these compounds against colorectal cancer cells. Our study fills this gap by specifically targeting and identifying cytotoxic compounds in *Ficus nota* leaves.
Latayada, F. S. and Uy, M.M.	Antimicrobial Activities and Toxicities of the Leaf Extracts of *Ficus nota* (Blanco) Merr.	2020	This study lacks a comprehensive toxicity assessment that accurately reflects the potential effects on humans or other mammals. While the brine shrimp lethality assay provides some insights into toxicity, it may not fully represent how *Ficus nota* extracts could affect human health. Additional studies using mammalian cell lines or animal models are needed to offer a clearer understanding of the safety and possible adverse effects associated with these extracts.
Dekker, E., Tanis, P. J., Vleugels, J. L. A., Kasi, P. M., & Wallace, M. B.	Colorectal Cancer	2019	This review discusses the current trends in Colorectal cancer in terms of its incidence and mortality. This also reviewed the common risk factors for CRC. Discusses the usual treatments for colorectal cancer and how this has improved the survival rate.
Fernandez JKU, Borlongan MAB, Baliton MAA, Sacdalan DL, Sy FFA, Agoncillo AR, Arenos CLC, Tatoy VF, Uy TJS, Reveldez IAL, Lim SJL	Knowledge, Attitude, and Practices in Colorectal Cancer Screening in the Philippines	2024	This study delves unto the difficulties and obstacles faced by the Filipino masses in early diagnosis of colorectal cancer. This served as a reference in ascertaining the current trend in early diagnosis of CRC
Jafari, M., Laraqui, A., Baba, W., Benmokhtar, S., Zaitouni, S. E., Ali, A. A., Bounaim, A., Moujahid, M., Tanz, R., Mahfoud, T., Sbitti, Y., Annaz, H. E., Abi, R., Tagajdid, M. R., Kochri, S. E., Lahlou, I. A., Hsaini, H. E., Belayachi, L., Benjouad, A., . . . Ennibi, K.	Prevalence and patterns of mutations in RAS/RAF/MEK/ERK/ MAPK signaling pathway in colorectal cancer in North Africa. BMC Cancer	2022	This study highlights the prevalence of KRAS, NRAS, and BRAF mutations in the North African population but it has a limited understanding of the underlying factors that can impact mutation rates and cancer development. A number of external factors have also been stated, however, further research is needed to explore how these specific factors interact to influence cancer risks and progression. This study is also focused on a large scale population making it difficult to tailor preventions and treatment strategies suited for the population being studied.
Kumar, A., Guatam, V., Sandu, A., Rawat, K., Sharma, A., & Saha, L.	Current and emerging therapeutic approaches for colorectal cancer: A comprehensive review	2023	This study reviews the modern treatments and emerging CRC treatments, essential for understanding what methods of treatment are being explored and how.
Montemayor, M. T.	Cancer third leading cause of death in PH	2023	This reference highlights the alarming mortality and incidence rate of cancer in the Philippines. This article also tackled various factors why a

			lot of Filipinos don't get diagnosed early, like finances and lack of knowledge, as well as some risk factors that contribute to cancer diagnosis.
Buchler, T.	Microsatellite Instability and Metastatic Colorectal Cancer – A Clinical Perspective	2022	This study necessitates the need to improve immunotherapy treatments for the human subjects due to the low percentage of positive outcomes. The need for more reliable and more personalized approaches are of much need to find reliable determinants that may serve as standard for a positive outcome.
Khademi, F.	Beyond The Present: Future Research Directions On The Extraction And Fractionation Of Bioactives, With The Focus On Phytochemicals	2023	This article stated that extracting and fractionating bioactive compounds are useful for medicine and health-promoting products, as they are compounds that are available in nature and have desirable effects for the health of humans. Key challenges as well the changing dynamics of the procedure were also discussed in the article.
Ghasemi, M., Turnbull, T., Sebastian, S., & Kempson, I.	The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis	2021	This article stated that MTT assay has been used to infer secondary processes like cell viability that might not always be an acceptable indicator. Another gap identified is that the values of optical density in spectrophotometer correlates directly with formazan quantity and MTT reduction
Nothias, L., Nothias-Esposito, M., Da Silva, R., Wang, M., Protsyuk, I., Zhang, Z., Sarvepalli, A., Leyssen, P., Touboul, D., Costa, J., Paolini, J., Alexandrov, T., Litaudon, M., & Dorrestein, P. C.	Bioactivity-Based Molecular Networking for the Discovery of Drug Leads in Natural Product Bioassay-Guided Fractionation	2018	This article enumerated multiple organisms that serve as our source in nature for the discovery of therapeutic agents. It was also mentioned that bioassay-guided fractionation is typically conducted for natural extracts that have been found to have bioactivity. The steps of the said process were stated, as well as the history of the methods and their reasons for failures.
Kurasaka, C., Ogino, Y., & Sato, A.	Molecular mechanisms and tumor biological aspects of 5-Fluorouracil resistance in HCT116 human colorectal cancer cells.	2021	This study provided significant information regarding HCT116 cell line but it requires a deeper understanding of the specific mechanisms such as the KRAS mutation for example. The need to pinpoint the specific mechanisms and conduct a research study focusing in these fields is much needed to further explain and understand how to manage colorectal cancer in the aid of HCT116 cell lines.
Quazi S. S., Fatema C. A., & Mir M.	Brine Shrimp Lethality Assay	2017	The Brine Shrimp Lethality Assay (BSLA) is a widely used, cost-effective method for preliminary cytotoxicity screening of bioactive compounds, especially plant extracts. While BSLA's simplicity and effectiveness make it a valuable tool in early-stage research, this lacks specificity in the mechanism of action and variability due to differing environmental conditions like salt concentration and pH. Thus, these factors indicate that assay needs to be refined further to enhance the accuracy of the result and expand its range of applicability to different types of compounds.
Wu, D., Li, J., Hu, X., Ma, J., & Dong, W.	5-Fluorouracil: Mechanisms of resistance and reversal strategies	2018	This study provides basic information about the mechanisms involved in the drug Fluorouracil, however, several gaps can be identified. One of which is the limited evidence on research

			mechanisms, incomplete gene characterization, limited optimization of combination therapies and the need for a much more recent research study.
Ghafouri-Fard, S., Abak, A., Anamag, F. T., Shoorei, H., Fattahi, F., Javadinia, S. A., Basiri, A., & Taheri, M.	5-Fluorouracil: A narrative review on the role of regulatory mechanisms in driving resistance to this chemotherapeutic agent	2021	This narrative review examines the mechanisms of resistance to 5-Fluorouracil which served as a reference for further research studies, however, the need for a deeper understanding on how this resistance works is must to be able connect further affected factors which root back to these specific mechanisms.
Casale, J., & Patel, P.	Fluorouracil	2024	This review study has limited understanding and information regarding the mechanism of resistance of fluorouracil. Further research studies are of much need to be able to effectively enhance Fluorouracil's efficacy in cancer treatment.
Longley, D. B., Harkin, D. P., & Johnston, P. G.	Molecular Mechanisms and Tumor Biological Aspects of 5-Fluorouracil Resistance in HCT116 Human Colorectal Cancer Cells	2021	This research study highlighted the significance of understanding the mechanism of action of 5-Fluorouracil and the understanding of drug resistance, yet issues in comprehensive mechanism understanding should be resolved.
Dodevska, T., Hadzhiev, D., & Shterev, I.	Recent advances in electrochemical determination of anti cancer drug 5-fluorouracil	2023	This research study focuses only on the current status of the efficacy of fluorouracil in the treatment of cancer in correlation with past studies and this paper suggests to improve the efficacy of fluorouracil based on combination regimens and the need to reduce its toxicity.
Selvaraj, V., & Alagar, M.	Analytical detection and biological assay of antileukemic drug 5-fluorouracil using gold nanoparticles as probe	2021	This research study lacks the information of the mechanism of antibacterial and antifungal activity. In addition it does not address strategies of targeted drug delivery and controlled release of the drug fluorouracil.
Entezar-Almahdi, E., Mohammadi-Samani, S., Tayebi, L., & Farjadian, F.	Recent Advances in Designing 5-Fluorouracil Delivery Systems: A Stepping Stone in the Safe Treatment of Colorectal Cancer	2020	This study is in need of the development of combination therapies and the exploration of innovative, responsive drug delivery systems that are tailored to target specific tumor sites to achieve better results in treatment and management of the disease.
Banyal, A., Tiwari, S., Sharma, A., Chanana, I., Patel, S. K. S., Kulshrestha, S., & Kumar, P.	Vinca alkaloids as a potential cancer therapeutics: recent update and future challenges	2023	This research study is a great help in understanding vinca alkaloids potential in cancer treatment but it lacks the information about resistance mechanism in cancer treatment and the comparison of the efficacy of traditional and newer vinca alkaloid analogs.
Knottenbelt, D., Patterson-Kane, J., & Snalune, K.	Clinical Equine Oncology: Vinblastine/Vincristine	2016	This book mainly explained vinblastine/vincristine in equine oncology but it didn't specify their full pharmacokinetics. It also lacks important information that might be helpful in the study of these alkaloids as it focuses mainly on one type of veterinary oncology.
Dhyani, P., Quispe, C., Sharma, E., Bahukhandi, A., Sati, P., Attri, D. C., Szopa, A., Sharifi-Rad, J., Docea, A. O., Mardare, I., Calina, D., & Cho, W. C.	Anticancer potential of alkaloids: a key emphasis to colchicine, vinblastine, vincristine, vindesine, vinorelbine, and vincamine.	2022	This study highlights the different types of vinca alkaloid applications but it does not contain needed information about their analogs, resistance challenges in cancer therapies, etc.

Ozdemir, N., Dogan, M., Sendur, M. A., Yazici, O., Abali, H., Yazilitas, D., Akinci, M. B., Aksoy, S., & Zengin, N.	Efficacy and safety of first line vincristine with doxorubicin, bleomycin and dacarbazine (ABOD) for Hodgkin's lymphoma: a single institute experience. Asian Pacific journal of cancer prevention	2014	This study suggests the replacement of vinblastine with vincristine mainly in the treatment for Hodgkin's lymphoma but it still lacks necessary research and comparative studies with other standard treatments to prove its long-term benefits.
Sears, J. E., & Boger, D. L.	Total synthesis of vinblastine, related natural products, and key analogues and development of inspired methodology suitable for the systematic study of their Structure–Function properties	2015	Although this study helped in understanding vinblastine analogues, the need for the assessment of its long-term resistance development, pharmacokinetics, and clinical applicability of these analogues in diverse cancer types still remains crucial for their effective treatment in cancer therapies.
Yu, D.Y., Noh, S.M., & Lee G.M.	Limitations to the development of recombinant human embryonic kidney 293E cells using glutamine synthetase-mediated gene amplification: Methionine sulfoximine resistance	2016	Although the HEK293 cell line exhibits excellent post-translational modification properties, these are seldom utilized only for commercial production of therapeutic proteins. On the other hand, the HEK293 cell line lacks an effective gene amplification and selectivity for the stable production of foreign proteins unlike CHO, which restricts the suitability for industrial applications.
Gika, H. G., Wilson, I.D., & Theodoridis, G.A.	LC-MS-based holistic metabolic profiling problems, limitations, advantages, and future perspectives	2014	Despite significant advancements, LC-MS based metabolic profiling still notably faces hindrances, specifically, the incapability of comprehensively profiling a whole metabolome in a single analytical run. More importantly, developing unbiased analytical methods are complex due to the diverse nature of metabolites.
Grebe, S. K., & Singh, R. J.	LC-MS/MS in the clinical laboratory - where to from here?	2011	LC-MS/MS has several limitations related to its balance of sensitivity, specificity, and speed. Although it processes more samples than traditional HPLC or GC-MS, it is slower than automated immunoassays.
Masuda, M., Uno, Y., Ohbayashi, N., Ohata, H., Mimata, A., Kukimoto-Niino, M., Moriyama, H., Kashimoto, S., Inoue, T., Goto, N., Okamoto, K., Shirouzu, M., Sawa, M., & Yamada, T.	TNIK inhibition abrogates colorectal cancer stemness.	2016	The study validates TNIK inhibition as an effective strategy to reduce colorectal cancer stemness and tumorigenicity in preclinical models. Nonetheless, further investigation is required to evaluate the clinical efficacy, safety profile, and long-term impact of TNIK-targeted interventions.
UniProt Consortium	TRAF2 and NCK-interacting kinase (TNIK) [UniProt accession: Q9UKE5]	2023	The entry provides comprehensive molecular characterization of TNIK's kinase activity and its involvement in Wnt/β-catenin signaling pathways implicated in colorectal cancer. However, functional evidence linking these molecular properties to disease progression and therapeutic application remains limited.
Zheng, X.-M., Chen, Y.-S., Ban, Y.-J., Wang, Y.-J., Dong, Y.-X., Li Lei,	Design, synthesis and bioevaluation of PI3KA-selective	2023	The researchers developed and evaluated PI3KA-selective inhibitors as potential treatments for colorectal cancer. However, the

Big Guo, Wang, J.-T., Tang, L., Li, H.-L., & Zhang, J.-Q.	inhibitors as potential colorectal cancer drugs		study reveals gaps in clinical testing, understanding of resistance, and the need for predictive biomarkers.
Voutsadakis, I. (2021)	The Landscape of PIK3CA Mutations in Colorectal Cancer	2021	This article examines the landscape of PIK3CA mutations in colorectal cancer and their relevance to targeted therapies. It identifies gaps in understanding the functional roles of different mutations and their influence on treatment outcomes.
Wheeler, D. L., et al.	Mechanisms of tumor resistance to EGFR-targeted therapies.	2008	This article explains the inherent ability of the effects of EGFR inhibition that limits the clinical usefulness of drugs for a number of tumors. The genetic background of some tumors precludes a function for EGFR inhibition since cells are not dependent on this receptor for signaling

The studies reviewed highlight significant progress in understanding the medicinal potential of *Ficus nota* and its application in colorectal cancer treatment. However, there remain critical gaps in the research. These include the need for comprehensive identification and characterization of cytotoxic compounds specific to *Ficus nota*, as well as in vivo studies to evaluate its therapeutic efficacy and safety in complex biological systems. Furthermore, while some studies focus on related species or other therapeutic applications, Ficus nota remains underexplored compared to other Ficus species, presenting an untapped opportunity for advancing cancer treatments.

Additionally, research on drug resistance mechanisms and delivery systems for 5-Fluorouracil underscores the need for innovative approaches to enhance efficacy and reduce toxicity in colorectal cancer therapies. The development of combination therapies, targeted drug delivery systems, and optimization of bioactive compound extraction processes are essential next steps. Addressing these gaps will contribute to a more comprehensive understanding of both Ficus nota and 5-Fluorouracil, potentially leading to novel, safer, and more effective cancer treatment strategies.

The reviewed literature provides significant insights into the therapeutic potential of vinca alkaloids in cancer treatment technology, but still exhibits gaps that require thorough research. According to Banyal et al. (2023), the cytotoxic potential of vinca alkaloids lacked discussions of resistance mechanisms, particularly in the context of comparison between traditional and modern models, warranting developments and applications. Lastly, while the LC-MS/MS offers high specificity and sensitivity, it fails to provide thorough and time-efficient compared to automated immunoassays, limiting its capability in high-volume clinical settings (Grebe, S.K., & Singh, R.J., 2011).

In colorectal cancer, TNIK's role in Wnt/β-catenin signaling is well characterized at the molecular level, yet its functional impact in disease and therapy remains underexplored (UniProt Consortium, 2023). Preclinical studies show that TNIK inhibition reduces cancer stemness and tumor growth, but clinical safety and long-term efficacy require further investigation (Masuda et al., 2016). This reflects similar gaps identified in the development of PI3KA-selective inhibitors, where clinical testing, mechanisms of resistance, and predictive biomarkers require further investigation to improve treatment outcomes for colorectal cancer (Zheng, X. M., et al., 2023).

This article highlights an important limitation of EGFR-targeted therapies, showing that some solid tumors have genetic changes that make them naturally resistant because they do not rely on EGFR signaling to grow and survive

(Wheeler et al., 2008). This idea of inherent resistance fits with the larger challenges in cancer treatment, where a lack of understanding about how tumors become resistant slows the progress of effective therapies.

CHAPTER 3

Methods and Procedures

This chapter outlines the methodologies applied in the tests, including the steps for collecting and verifying the plant samples, extracting and preparing the plant extracts, performing phytochemical screening, conducting the cytotoxicity investigation, identifying compounds, and predicting interactions of identified compounds with relevant molecular targets.

Research Methods and Design

The researchers followed an experimental design in determining the compounds of *Ficus nota* with cytotoxic effect against HCT116 Human Colorectal Carcinoma Cell Line.

Figure 2: Paradigm of the Study.

Setting of the Study

The plant samples used in this study were sourced from a single specimen at the LCSBC Camp & Training Center in Alfonso, Cavite (Latitude: 14.1380464, Longitude: 120.8554205), to ensure uniformity and purity. These samples underwent authentication at the Jose Vera Santos Memorial Herbarium at the University of the Philippines-Diliman, Quezon City. Following authentication, the study was conducted at CEU Laboratories, where the planning, preparation, initial testing, Phytochemical Screening, Fractionation, Brine Shrimp Lethality Assay, and Gravity Column Chromatography took place. Additional specialized tests, including the MTT Assay and LC-MS/MS, were outsourced to external facilities like UP-Manila. Further analysis of the identified fractionated compounds and assessment of their potential cytotoxic properties was carried out through in silico testing at Centro Escolar University.

Research Procedures

Preparation of Plant Material

The leaves of *Ficus Nota* were obtained from LCSBC Camp & Training Center, Alfonso, Cavite, Philippines. The authentication of this species was conducted by Edwino S. Fernando, a curator at the Jose Vera Santos Memorial Herbarium, located at the University of the Philippines in Diliman, Quezon City.

Preparation of Extract

The leaves of *Ficus nota* went through a process of removing extraneous objects, then subjected to air-drying in a well-ventilated room for 4 days. It was then followed by cutting the leaves into small pieces and was subjected to grinding before being placed in an Erlenmeyer flask.

The weight of the ground leaves that was used in the experiment was 138.5 grams alongside 1.385 L of 70% ethanol. The ground leaves were subjected to a five-day soaking period at room temperature before promptly undergoing filtration, resulting in the collection of the filtrate.

The acquired filtrate was subjected to evaporation to obtain a more concentrated crude extract. Through a water bath, with the use of a hot plate that was set at a temperature of 300°C, a dark green syrupy extract was produced, resulting in the formation of the crude ethanol extract.

The percentage yield of the crude extract, was obtained through the use of the formula;

$$Percentage\ yield = \frac{weight\ of\ the\ extract}{weight\ of\ the\ plant\ sample} x\ 100$$

1. Phytochemical Screening

Further tests for the identification and screening of the different plant constituents of Tibig (*Ficus nota*) leaf extract was conducted by the researchers. Starting with the preliminary and confirmatory test while following standard methods previously reported for their identification and confirmation. The researchers conducted confirmatory tests and Quaternary Bases/Amine Oxide tests to determine the presence of Alkaloids (1) Mayer's Test, (2) Dragendorff's Test, (3) Wagner's Test. Moreover, confirmatory tests was conducted to determine the presence of Steroids specifically Cardenolides and Bufadienolides (1) Keller-killiani test, (2) Liebermann-Burchard test, (3) Salkowiski's test, Anthraquinone Glycosides, (1) Borntrager's test, (2) Modified Borntrager's test, Flavonoids, (1) Bate-Smith and Metcalf Test Method, (2) Wilstatter "Cyanidin" test, Tannins, (1) Gelatin test, (2) Ferric chloride test, and Carbohydrates (1) Fehling's test, (2) Molisch's test, (3) Benedict's test in the extract.

Test for Alkaloids

Preliminary Test

Using 2.6 g of plant sample, 5 mL of 2M hydrochloric acid (HCl) was added to the evaporated sample, then heated for approximately 5 minutes in a water bath. Sodium chloride (0.5 g) was then added. The sample was washed with enough 2M HCl until the filtrate reached a volume of 6 mL. Three 1 mL portions of the filtrate were taken, and each portion was tested with 2 to 3 drops of Mayer's reagent, Dragendorff's reagent, and Wagner's reagent.

Confirmatory Test

From the remaining 3 mL of the filtrate from the preliminary test, enough 28% ammonia was added until the solution became alkaline to litmus. The alkaline solution was extracted three times with small portions of 10 mL of chloroform. The lower chloroform extracts from each extraction were collected, while the upper aqueous layer was retained and used later in the test for quaternary bases. The collected chloroform extracts were evaporated to dryness, and 5 mL of 2M hydrochloric acid was added.

Test for Quaternary bases and/or amine oxide

From the reserved upper aqueous layer obtained in the previous test, it was acidified with 2M HCl then filtered. The filtrate was divided into three equal portions: Dragendorff's reagent was added to one portion, Mayer's reagent to another, and Wagner's reagent to the last portion. Expected results for Mayer's and Dragendorff's tests were (++) and (+++), indicating the presence of quaternary and/or amine oxide bases, while a score of (+) was considered negative.

Test for Steroids: Cardenolides and Bufadienolides

Using 1.3 g of plant extract that was previously evaporated to incipient dryness, the residue was defatted with 6 mL of hexane and water (2:1 v/v). This process was repeated to fully remove any colored pigments. The collected defatted aqueous layer was then heated in a water bath to remove residual hexane. This solution was divided into three equal portions for the Keller-Killiani test, Liebermann-Burchard test, and Salkowski's test.

Keller-Killiani test

From the divided portions, one portion of the defatted aqueous layer free from hexane was added with three mL of Ferric chloride. After letting it stand for a few minutes, 1 mL of concentrated sulfuric acid and a reddish-brown solution should be visible.

Liebermann-Burchard test

One portion of the defatted aqueous layer free from hexane was treated with 10 mL dichloromethane. The dichloromethane (CH_2Cl_2) extract was dried by passing the extract through about 100 mg anhydrous sodium sulfate (Na_2SO_4). The filtrate was filtered and then divided into two portions, one serving as the control. The other portion was treated with 3 drops of acetic anhydride and 1 drop of concentrated sulfuric acid. A noticeable color change should be visible when compared to the control.

Salkowiski's test

From the second portion previously used, an equal volume of sulfuric acid was added to obtain a red color appearance that will serve as an indicator for the presence of unsaturated sterol and/or triterpenes.

Test for Anthraquinone Glycosides

Borntrager's Test

Using 0.134 g of sample, 10 mL of water was added, and then filtered. The filtrate was extracted with 5 mL portions of Benzene twice. Both portions of the Benzene extracts were combined, then divided into two equal portions. One portion served as the control group, and the other portion was treated with 5 mL of ammonia. The results were then compared with the control group after shaking the solution. A red coloration in the lower ammoniacal layer indicated the presence of anthraquinones.

Modified Borntrager's Test

Using 0.134 g of sample, 10 mL of 5M Potassium Hydroxide and 1 mL of 5% Hydrogen Peroxide was added. The solution was stirred and the resulting mixture was heated over a steam bath for 10 minutes. It was filtered, and the residue was discarded. The filtrate was acidified with glacial acetic acid, collected, and extracted with two 5 mL portions of benzene. The filtrate was divided into portions: one portion served as the control group, while the other portion was alkalinized with ammonia.

Test for Flavonoids

Bate-Smith and Metcalf Test

Using 2 mL of extract, it was added to 0.5 mL concentrated hydrochloric acid (12 M) and observed for any color change. The solution was warmed in a water bath for 15 minutes. Further color change was observed within an hour

then compared with the control. A strong red or violet color indicated the presence of leucoanthocyanins, an example of flavonoids.

Wilstatter "Cyanidin" Test

Using 2 mL of extract, 0.5 mL of concentrated hydrochloric acid (12 M) and 3 to 4 pieces of magnesium turnings was added. Any color change was observed within 10 minutes. If there is a change in color or formation of colored layers, it indicates a positive result.

Froth Test

Using 0.126 g of extract, it was diluted with 10 mL of distilled water, and then shaken vigorously for 30 seconds. The mixture was allowed to stand for 10 minutes and observed. The presence of a 2 cm honeycomb froth indicated a positive result for the presence of saponin.

Test for Tannins

Using 1.3 g of sample, the extract was evaporated to incipient dryness, then the residue was extracted with 20 mL of hot distilled water. 5 drops of 10% sodium chloride solution were added before filtering the prepared sample. The filtrate was then divided into three portions, with one as the control and the other two for gelatin and ferric chloride test.

Gelatin test

From the prepared filtrate above, one portion was treated with three drops of gelatin salt solution, which served as the reference standard. Comparison with the control was then implemented. Formation of a jelly-precipitate indicated the presence of tannins.

Ferric chloride test

The last portion of the sample extract was treated with three drops of ferric chloride, and likewise to the tannic acid solution. The presence of a blue-black color afterwards indicated the presence of hydrolyzable tannins, while a brownish-green color may indicate the presence of nonhydrolyzable/condensed tannins.

Screening for Carbohydrates

Using 0.26 g equivalent of 80% alcoholic extract, it was evaporated to incipient dryness. The residue was then collected and combined to 10 mL of distilled water, which was later divided into three equal parts.

Fehling's Test

Fehling's solution was prepared by boiling the mixture of 2.5 mL of Fehling's A and 2.5 mL of Fehling's B in a test tube. An equal quantity of plant extract was then added before boiling again. The presence of a brick red precipitate indicated the presence of reducing sugars.

Molisch's test

Using 2 mL of the prepared filtrate, it will be mixed with 0.2 mL alcoholic solution of a-naphthol 10% in addition to 2 mL of sulfuric acid. A positive result will be expected if a red precipitate appears in the solution, which means that there is a presence of carbohydrates and/or glycosides.

Benedict's test

Using 1 mL of the filtrate, it was combined with 5 mL of Benedict's reagent. The mixture was then heated and observed for the appearance of a brick-red precipitate, which is an indicator for the presence of reducing sugars.

2. Bioassay-guided Fractionation

Solvent partitioning

Approximately 30 g of crude extract was exhaustively partitioned using three solvents in order of increasing polarity: hexane, ethyl acetate, and water. The crude extract was dissolved in a 1:1 ratio of water and hexane, then agitated mechanically to create a consistent mixture. The solution was then allowed to settle in a separatory funnel until the layers have fully separated. After separation, the upper hexane layer was collected and concentrated using a rotary evaporator. This process was repeated until all hexane-soluble constituents are extracted. The same procedure was applied to ethyl acetate and water. The final aqueous portion was refrigerated to slow down potential degradation. After all that, the concentrated partitions were tested for cytotoxicity using the Brine Shrimp Lethality Assay. The most active partition was then selected to undergo the next purification step.

Gravity Column Chromatography (GCC)

A silica gel column was prepared by packing 100 g of silica gel using the slurry method. The amount of silica gel used was 100 times the weight of the sample to be separated. 1000 mg of the hexane partition was dissolved in the appropriate solvent system and was carefully loaded onto the top of the column.

Elution was done using a step-by-step process of gradient solvents with increasing polarity, starting with a 6:4 ratio of hexane-ethyl acetate. After collecting the fractions of the first mixture, the polarity of the eluent was gradually increased through adjusting the hexane-ethyl acetate ratio and finishing with 100% absolute ethanol. Each fraction was collected individually according to the corresponding solvent system composition. The collected fractions were then concentrated using a rotary evaporator to remove excess moisture, and then subjected to respective cytotoxicity testing.

3. Brine Shrimp Lethality Assay

This assay served as the preliminary screening for cytotoxicity. The partitions (hexane, ethyl acetate, and aqueous) were tested in concentrations of 10 ppm, 100 ppm, and 1000 ppm. The fluorouracil served as the positive control and sea water as the negative control.

This assay follows the protocol by Quazi et al (2017). The extracts were tested by placing them in different test tubes, each containing ten brine shrimp, with three replicates each setup for the Brine Shrimp Lethality Assay. After 24 hours, the number of surviving *nauplii* is counted, and the percentage of deaths at each concentration is recorded. This data is then used to calculate the percent mortality allowing preliminary cytotoxic comparisons among samples using the formula below. The extract with the highest mortality rate was selected for further purification through gravity column chromatography.

$$\%Death = \frac{Number\ of\ dead\ nauplii}{Number\ of\ dead\ nauplii\ + Number\ of\ live\ nauplii}\ x\ 100$$

4. Cell Viability Assay

The chosen GCC fraction (2:8) was subjected to MTT assay to assess its cytotoxic activity. Human colorectal cancer cell line HCT-116 and human embryonic kidney HEK 293 cells were cultured separately.

The cells were seeded into 96-well microtiter plates at a density of 30,000 cells per mL and incubated at 37°C in a CO_2 humidified incubator to allow cell attachment. After 24 hours, the cells were treated with the ethanol extract and further incubated for 24, 48, and 72 hours. After the treatment period, the MTT reagent was prepared by dissolving it in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL. The solution was filter-sterilized and protected from light. The culture medium was decanted, and the cells were washed once with 1× PBS. Another 100 µL of PBS was added to each well, followed by 10 µL of MTT reagent. The plates were then incubated for 3 hours at 37°C in a CO_2 incubator to allow the formation of formazan crystals. Following incubation, 85 µL of the solution was carefully removed from each well. Then, 100 µL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, and the plate was homogenized. The plate was further incubated for 10 minutes at room temperature before measuring the absorbance at 550–600 nm using a microplate reader.

The amount of viable cells, which is measured through the homogenized MTT-formazan solution, was determined through plate reading spectroscopy at 570 nm. Colorimetrically, the amount of viable cells was indicated by the intensity of the blue or purple coloration and the lack of change from the yellow color, indicating that the cells are no longer viable. Similarly, the viability of the cells is indicated by a higher absorbance at 570 nm, and the opposite is indicated by a lower absorbance. The results were representative of the cytotoxic activities of the ethanolic extract of Tibig (*Ficus nota*) leaves against human colorectal cancer cell line HCT-116 and human embryonic kidney HEK 293.

5. Data Analysis

The data gathered from the MTT assay were assessed qualitatively. Cell viability and the cytotoxic activity of the samples were compared through analysis of observable trends and simple descriptives. Data visualization techniques such as bar graphs, were employed to help with data comparison and observation of trends. Computed IC50 values were also contrasted to those of published IC50 values through the same means to gauge the activity of the sample. No statistical measures were employed as the study focuses on the identification of fractions exhibiting notable cytotoxic activity and thus, warranting further investigation.

6. LC-MS/MS

A single sample of the GCC extract (DBMB-LCMS-2025-006), characterized as a sticky, viscous, dark green material, was prepared for liquid chromatography–mass spectrometry (LC-MS) analysis. A stock solution was created by dissolving the sample in LC-MS-grade methanol to a concentration of 10 mg/mL. The solution was sonicated for five minutes at room temperature to facilitate dissolution and then centrifuged at 10,000 rpm for five minutes to remove particulates. A 100 µL aliquot of the resulting supernatant was diluted with methanol to a final concentration of 1 mg/mL, producing the working solution, which was transferred to an LC-MS-certified vial for analysis.

Chromatographic separation was performed using the Waters ACQUITY® I-Class Plus Core UPLC system, which offers enhanced resolution, sensitivity, and speed due to its low dispersion design, enabling sharp peak shapes and precise compound quantitation (Nascimento et al., 2021). The system was equipped with a Waters ACQUITY HSS T3 C18 column (1.8 µm, 2.1 × 100 mm), known for its ability to retain polar compounds and improve separation

efficiency through a trifunctionally bonded stationary phase (Thurman et al., 2020). The column was maintained at 30°C. The mobile phase consisted of solvent A (LC-MS-grade water with 0.1% formic acid) and solvent B (LC-MS-grade acetonitrile with 0.1% formic acid). A gradient elution was programmed as follows: 0–2 minutes, 95% A; 2–6 minutes, linear gradient to 100% B; 6–9 minutes, held at 100% B; 9–12 minutes, returned to 95% A; and 12–15 minutes, held at 95% A to re-equilibrate the system. The flow rate was set at 0.3 mL/min, with an injection volume of 5 µL.

Mass spectrometric detection was carried out using a Waters Xevo® G2-XS Quadrupole Time-of-Flight (QToF) mass spectrometer, which offers high sensitivity, excellent dynamic range, and precise mass accuracy, making it ideal for structural elucidation and complex mixture profiling (Wang Alelyunas et al., 2017). The instrument operated in positive electrospray ionization (ESI^+) mode, a soft ionization technique that generates intact protonated molecules suitable for analysis of thermally labile or high molecular weight compounds (Alder et al., 2017). Key settings included a capillary voltage of 2.7 kV, cone voltage of 40 V, source temperature of 110°C, and desolvation temperature of 250°C. Nitrogen gas was employed for both cone (60 L/h) and desolvation (800 L/h) flows. Data was acquired in full scan mode over an m/z range of 50–1,200 with a scan time of 0.5 seconds. Collision energy was set at 6 eV for low-energy scans and ramped from 15 to 45 eV for high-energy scans. Leucine enkephalin was used as a reference lock mass to ensure real-time mass calibration.

7. In Silico Analysis

The compounds identified from the LC-MS/MS were initially modeled using ChemDraw to extract optimized 2D structures suitable for further computational analysis. These structures were converted to 3D and energy-minimized prior to docking. To evaluate the multi-target anticancer potential of these derived compounds, three key signaling proteins were selected for molecular docking: TRAF2 and NCK-interacting kinase (TNIK) (PDB ID: 5D7A), PI3Kα (PDB ID: 5DXT), and EGFR-TKD (PDB ID: 4HJO). These targets represent crucial nodes in cancer-associated pathways. TNIK is a serine/threonine kinase essential for the activation of the Wnt/β-catenin signaling pathway, a key driver of colorectal and other cancers due to its role in stem cell renewal and tumor proliferation (Masuda et al., 2016). PI3Kα, a catalytic subunit of the PI3K-Akt-mTOR pathway, regulates cell growth, survival, and metabolism; its mutation or overexpression is frequently implicated in breast, ovarian, and endometrial cancers (Heffron et al., 2016). EGFR-TKD plays a central role in the MAPK and PI3K signaling cascades, influencing cellular proliferation and survival. Dysregulation of EGFR via mutations or overactivation is a hallmark of lung, colorectal, and head and neck cancers (Park et al., 2012). The selection of these proteins aimed to assess the potential of these phytochemicals to interfere with multiple oncogenic mechanisms simultaneously.

The identification of the binding sites for the three target proteins was conducted using Computed Atlas of Surface Topography of proteins, a computational tool designed to analyze the geometric and topological features of protein surfaces, enabling the detection and characterization of pockets based on volume and accessibility. Each target protein was subjected to CASTp to systematically map the potential ligand-binding pockets, which were then employed in further docking analysis through evaluation of the spatial configuration and overlap with the residues available in the literature to determine functional activity or ligand interaction.

Following binding site identification via CASTp, molecular docking simulations were conducted using AutoDock Vina to predict the optimal orientation and binding affinity of selected ligands within the active sites of target proteins.

Protein structures—TNIK (PDB ID: 5AX9), EGFR (PDB ID: 4HJO), and alpha-beta tubulin (PDB ID: 1Z2B)—co-crystallized with known inhibitors were retrieved from the RCSB Protein Data Bank. Preprocessing of these structures was performed using UCSF Chimera's Dock Prep tool, which included removal of water molecules, addition of polar hydrogens, assignment of Gasteiger charges, and geometry optimization. Ligand structures, obtained from PubChem and selected based on previous cytotoxicity screening, were prepared through energy minimization and file conversion. To validate the docking protocol, native ligands were initially redocked into their respective binding sites using the same grid coordinates derived from CASTp, and results were compared to ensure reliability. The validated coordinates were then used to dock 18 test compounds, with binding affinities calculated in kcal/mol. Docking results were ranked based on affinity values to identify the most promising candidates.

SwissADME was utilized to evaluate the drug-likeness and ADME (absorption, distribution, metabolism, and excretion) profiles of selected compounds. Simplified Molecular Input Line Entry System (SMILES) notations were retrieved from the PubChem database and input into the SwissADME web tool. The evaluation included drug-likeness screening based on established pharmaceutical rules, including Lipinski, Ghose, Veber, Egan, and Muegge filters, along with bioavailability score assessment. Compounds presenting zero to two violations across these criteria and a bioavailability score of 0.55 or higher were classified as drug-like. Additional properties such as solubility, gastrointestinal absorption, blood-brain barrier permeability, P-glycoprotein substrate prediction, structural alerts (PAINS and Brenk filters), lipophilicity (LogP), topological polar surface area (TPSA), and synthetic accessibility scores were also computed to inform compound suitability for further development.

CHAPTER 4

Presentation, Analysis, and Interpretation of Data

This chapter outlines the qualitative and quantitative findings of the study, along with detailed analyses of each experimental method. The results include the outcomes of the phytochemical screening, fractionation, the brine shrimp lethality assay (BSLA), MTT assay, LC-MS, and In-Silico analysis, all performed on the plant extract.

1. Extraction of the crude from Tibig (*Ficus nota*) Leaves

To extract the bioactive components of the Tibig (*Ficus nota*) leaves, it was macerated and extracted, and the percentage yield of the extract was obtained.

Table 1: Percentage Yield of Crude Extract.

Weight of Plant Sample	Weight of Extract	Percentage Yield
130.182 grams	10.730 grams	8.24%

Table 1 shows that the *Ficus nota* (Tibig) leaves yielded a percentage yield of 8.24%. Banawe et al (n.d.) show that this is a comparable yield for the species. Their study yielded 7.19% using ethanol as a solvent. Their study also compares different ficus species: *F. elastica, F. septica, & F. ulmifolia*, which had lower yields. On the other hand, studies by Kurniawan & Yusuf (2021). Farnev, Tijjani, & Shamaki (2021) showed that *F. carica* and *F. sycomorus* had a significantly higher yield of 14.74% and 17.83% respectively.

2. Phytochemical Screening of the Crude Extract

Phytochemical screening was conducted to identify the presence of secondary metabolites in the extracts, including glycosides, flavonoids, tannins, and carbohydrates. Various tests were performed to detect these compounds, providing an initial profile of the chemical constituents of the extracts.

Table 2: Results for Phytochemical Screening.

SCREENING FOR GLYCOSIDES				
TEST FOR STEROIDS: CARDENOLIDES AND BUFADIENOLIDES				
TEST	**EXPECTED RESULT**		**ACTUAL RESULT**	**REMARKS**
SALKOWISKI'S TEST: Test for Unsaturated Sterol and or Terpenes	Appearance of Red color		Appearance of Red color	POSITIVE
TEST FOR FLAVONOIDS				
BATE-SMITH AND METCALF METHOD: Test for leucoanthocyanins	Strong Red or Violet Color		Formation of Strong Red Color	POSITIVE
WILSTATTER "CYANIDIN" TEST: Test for y-benzopyrone	Colors ranging from Orange to Red, Crimson, Magenta, and occasionally Green or blue		Formation of Orange Color	POSITIVE
TEST FOR TANNINS				
TEST	**POSITIVE RESULT**		**ACTUAL RESULT**	**REMARKS**
GELATIN TEST	Formation of Jelly- Precipitate		Formation of Jelly-Precipitate	POSITIVE
FERRIC CHLORIDE TEST	Blue Black color	Hydrolyzable tannins	Formation of Blue Black color	POSITIVE
SCREENING FOR CARBOHYDRATES				
TEST	**POSITIVE RESULT**		**ACTUAL RESULT**	**REMARKS**
FEHLING'S TEST	Brick Red Precipitate	Reducing Sugars	Formation of Brick Red Precipitate	POSITIVE
MOLISCH'S TEST	Bluish Violet Zone	Carbohydrates &/ Glycosides	Formation of Bluish Violet Zone	POSITIVE
BENEDICT'S TEST	Red Precipitate	Reducing Sugars	Formation of Red Precipitate	POSITIVE

Table 2 shows the specific tests conducted with visible positive results. According to the study of Cungihan (2024), focusing on the effect of *Ficus nota (Blanco) Merr* on the Hematobiochemical Profile and Growth Performance in chickens, a part of the methodology highlighted the result of the phytochemical screening of *Ficus nota* fruits which revealed the presence of various bioactive compounds including phenols, flavonoids, tannins, saponins, coumarins, and steroids. The table presented above portrays the results we obtained after undergoing phytochemical screening of the *Ficus nota* leaves for our research study. The constituents that yielded positive results consist of the unsaturated sterol and or terpenes, leucoanthocyanins, y-benzopyrone, carbohydrates, and hydrolyzable tannins.

From the list of positive constituents, the possible constituents with cytotoxic effects include terpenes, as research studies have proven that plant-derived terpenes have proven their effects against different cancer cells, especially in both in vivo and in vitro models (Camara et al., 2024).

Additionally, hydrolyzable tannins, which also tested positive, have shown significant cytotoxic activity against human oral squamous cell carcinoma and salivary gland tumor cell lines, with minimal effects on normal human gingival fibroblasts (Sakagami et al. 2000).

Moreover, steroids also exhibit potential cytotoxic effects. According to Jameborzogil et al. (2019), *Axinella cf. bidderi* revealed that the steroids extracted from this sponge exhibit potent cytotoxic effects against several human cancer cell lines, including ovarian (IGROV-ET), pancreatic (PANC1), and lung (NSCLC N6-L16) cancer cells.

Flavonoids also show anticancer properties. According to Sak (2014), flavonoids are important in human nutrition and health, with chemoprevention emerging as one of the most promising strategies for preventing malignant disorders. Combining flavonoids with conventional chemotherapeutic drugs has been shown to reduce dosage requirements and associated toxicity while overcoming resistance mechanisms, thereby minimizing genotoxic damage to normal cells and lowering the risk of secondary cancers.

3. Brine Shrimp Lethality Assay (BSLA)

The brine shrimp lethality assay was performed as a preliminary screen to assess the cytotoxicity of extracts derived from the fractions obtained through solvent partitioning. Various concentrations of the extract were used to expose *Artemia salina*, and the survival rates were recorded to evaluate the potential toxic effects based on mortality.

Table 3: Brine Shrimp Lethality Assay (BSLA) Result.

Partition	Concentration (ppm)	Number of surviving Nauplii after 24 hours			Accumulation of surviving Nauplii/ total Nauplii	Percent mortality (%)
		Trial 1	Trial 2	Trial 3		
Hexane	10	10	10	10	30/30	0
	100	10	10	10	30/30	0
	1000	0	0	0	0/30	100
Ethyl Acetate	10	10	10	10	30/30	0
	100	10	10	10	30/30	0
	1000	8	8	10	26/30	13.33
Aqueous	10	10	10	10	30/30	0
	100	10	9	10	29/30	3.33
	1000	10	7	9	26/30	13.33
Crude	10	10	9	10	29/30	3.33
	100	10	10	10	30/30	0
	1000	10	10	10	30/30	0
Positive control (Fluorouracil)	10	10	10	10	30/30	0
	100	9	9	10	28/30	6.67
	1000	1	3	1	5/30	83.33
Negative control (Sea water)	1000	10	10	10	30/30	0

Table 3 shows that the hexane partition exhibited the highest percent mortality, with 100% at 1000 ppm, indicating its potential cytotoxic properties. Ethyl acetate and aqueous partitions showed less activity, with 13.33% mortality at 1000 ppm. The crude extract displayed similar but lower cytotoxic activity, with 3.33% mortality at 10 ppm. The positive control, fluorouracil, exhibited cytotoxicity with 6.67% mortality at 100 ppm and 83.33% mortality at 1000 ppm, confirming the assay's reliability. The negative control (seawater) caused no mortality, further confirming the assay's validity. These findings suggest that the hexane partition is a suitable candidate for further cytotoxicity study.

4. Data Analysis

MTT Assay Results

MTT was employed to test the cytotoxic activity of the Tibig extract and to determine the fraction in which the highest cytotoxicity was exhibited. It was also done to obtain the Tibig extract's IC50.

Table 4: MTT Result of Different Fractions.

Extract	Concentration	Absorbance			Average Abs
Ethanolic Fraction	100	0.8094	0.7970	0.7028	0.7697
	10	0.7377	0.7277	0.9944	0.8199
6:4	100	0.2554	0.2687	0.2858	0.2700
	10	0.2534	0.5556	0.2287	0.3459
4:6	100	0.0485	0.0933	0.0496	**0.0638**
	10	0.2489	0.2148	0.1866	0.2168
2:8	100	0.1303	0.1536	0.1055	**0.1298**
	10	0.2974	0.2235	0.2538	0.2582

Results from the MTT assay reveal that the 2:8 fraction and the 4:6 plant extract had the highest cytotoxic activity with average absorbance readings of 0.1298 and 0.0638, respectively. However, due to limited sample volume, the 2:8 fraction was chosen for subsequent testing. The two fractions do not have a statistically significant difference, and the 2:8 fraction had a more polar solvent. Furthermore, because cytotoxic secondary metabolites such as flavonoids are better extracted with polar solvents, it was a better candidate for subsequent testing.

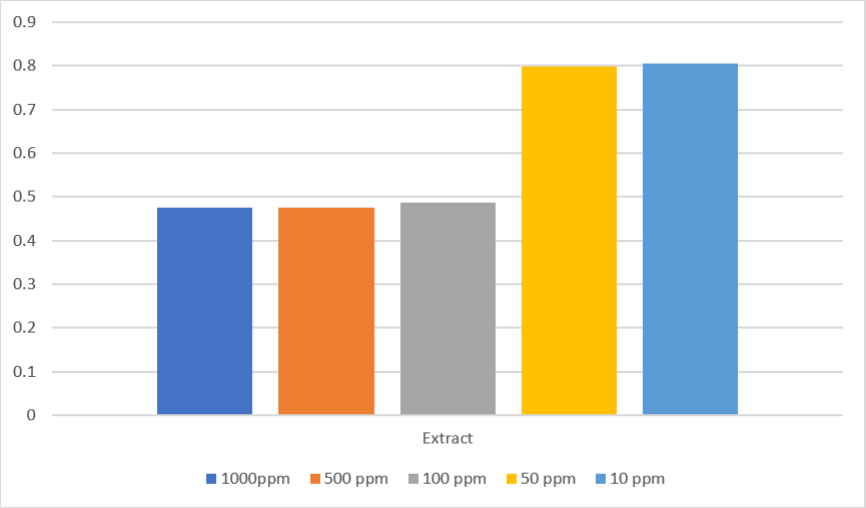

Figure 3: Average Absorbance of Plant Extract.

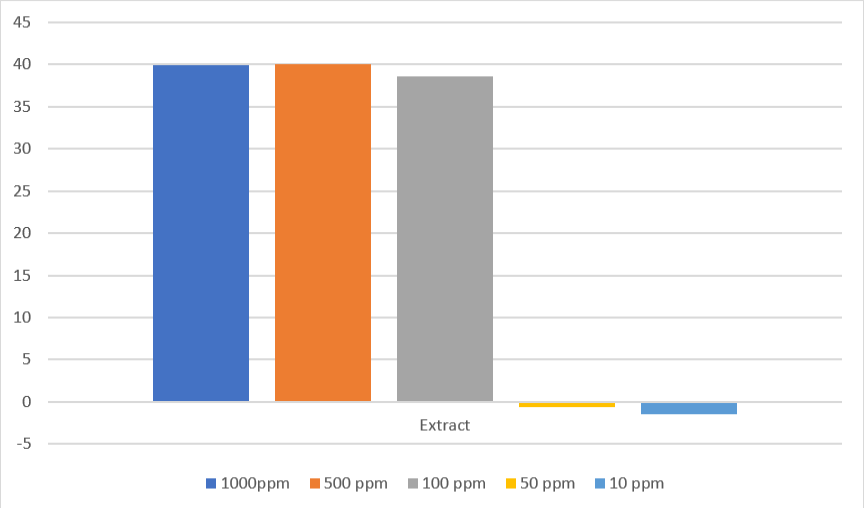

Figure 4: Average Percent Cell Inhibition.

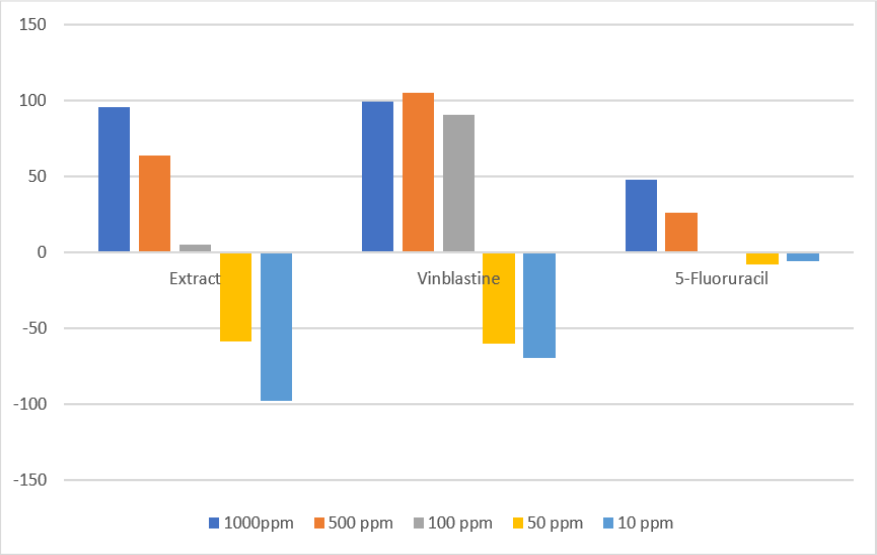

Figure 5: Cell inhibition on HEK293.

Table 5: Selectivity Index.

HCT116 IC50	HEK293 IC50	Computed Selectivity Index
59.7	107.6	1.80

The 2:8 fraction exhibited moderate activity according to the classification from Nordin et al. (2018). They classify IC50s of ≤ 20 ppm as very active, > 20 ppm to 100 ppm as moderately active, > 100 to 1000 ppm as weakly active, and > 1000 ppm as inactive. The plant extract's computed IC50 of 59.7 is similar to Vinblastine's published IC50 of 47.86 ppm. Moreover, they are under the same classification of moderate activity. However, both Tibig extract and Vinblastine exhibit a lower IC50 than fluorouracil at 7.93 ppm (Babazadeh et al., 2022), considered to be very active. Furthermore, Vinblastine and the Tibig extract exhibit a similar activity on HEK-293. In comparison to fluorouracil, however, it exhibits less toxicity against HEK-293. Similarities between Vinblastine and Tibig extract may suggest a comparable activity and safety profile. In addition, this may also suggest that their mechanisms of action are both as microtubule inhibitors or something similar. Upon computation of the drug's selectivity index using the computed IC50 for non-cancerous and cancerous cell lines, it was found that the Tibig extract's selectivity index is 1.80. A selectivity index of more than 1 suggests an efficacy against tumor cells greater than the toxicity against normal cells (Krzywik et al., 2020).

Moreover, at concentrations of 10 ppm and 50 ppm, the Tibig extract seems to exhibit a possible stimulatory effect, as shown with below below-zero cell inhibition. This may be due to the hormetic effects exhibited by all anticancer agents (Yoshimasu et al., 2015).

4. Liquid Chromatography - Mass Spectrometry

LC-MS/MS was conducted to identify the compounds present in the most cytotoxic fraction. The compounds were identified to examine the speculated cause of the extract's cytotoxic activity.

Table 6: Compounds Identified Through Liquid Chromatography - Mass Spectrometry with Possible Cytotoxicity.

Compound Name	Chemical Formula	Detector Counts	Alternative Assignments
(10E)1,10-Heptadeca-diene-4,6-diyne-3,8,9-triol	C17H24O3	4757.483887	Aurapten, Ciryneone F, Ostruthin, Shogaol, Mitiodiol
(E,E)-9-Oxooctadeca-10,12-dienoic acid	C18H30O3	26934.61914	
1-Nonene	C9H18	3273.7854	1,2,4-Trimethyl-cyclohexane
2-Monopalmitin	C19H38O4	8978.866211	1-Monopalmitin
Baicalein	C15H10O5	8654.013672	Genistein, APigenol, Galangin (Norizalpinin), 3'4'7-Trihydroxyflavone, Resokaempferol, 1,3-Dihydroxy-2- methoxyanthraquinone, Emodin, Aloe-emodin, Rubilactone, Sulfuretin, 1,3,6-Trihydroxy-2-methylanthraquinone. 1,6-Dihydroxy-2-methoxyanthraquinone, Digitopurpone
Bowdichione	C16H10O6	3937.38623	Irilone
Butyl isobutyl phthalate	C16H22O4	24995.03418	Dibutyl phthalate, Diisobutyl phthalate (DIBP), Isobutyl phthalate
Campesterol	C28H48O	8784.678711	Ergost-5-en-3-ol, 24-Methylcholest7-en-3β-ol, Pollinastanol
Hentriacontane	C31H64	2335.170166	
Kuwanon C	C25H26O6	5457.139648	Kadsurenin H, Kadsurenin J, Shanciol B, Kadsurenin L, Kuwanon C, Kuwanon T, Sanggenon I, Flavenochromane B, Kuwanon D, Schisandrin B, Shanciol B
Lupenone	C30H48O	4065.24585	Arborinone. δ-Amyrenone, β-Amyrenone, Taraxerone, Alnusenone
Neoline	C24H39NO6	36268.39453	Foresticine
Paeonisothujone	C10H14O3	8612.344727	Deoxypaeonisuffrone
Phytolacca Cerebroside	C48H93NO10	59326.64648	Momot-cerebroside I
Puerarol	C25H24O5	3351.459229	Ethylnotopterol
Salvianolic acid A	C26H22O10	2926.970459	
7Talatisamine	C24H39NO5	6326.072266	
Tonkinensisol	C25H24O6	8227.776367	Shanciol, Schanciol F, Teracrylshikonin, Cyclomulberrin, Kuwanon A. Kuwanon B, Mulberrochromene

A total of 39 compounds were identified through LC-MS/MS analysis. Table 5 highlights 18 compounds that may be responsible for the extract's cytotoxic effects. However, other biological activities and potential interactions of these compounds were not assessed in this study, and further investigation of the fraction is recommended. The three most abundant compounds were Phytolacca Cerebroside, (E, E)-9-Oxooctadeca-10,12-dienoic acid, and Neoline.

Of the four secondary metabolite classes previously identified through phytochemical screening as potential contributors to cytotoxicity, only flavonoids (Baicalein, Bowdichione, Kuwanon C, Tonkinensisol), steroids (Campesterol), and terpenes (Lupenone, Paeonisothujone, Talatisamine) were detected in the LC-MS/MS profile. Hydrolyzable tannins were not detected, likely due to their high polarity, as the analyzed fractions were mostly non-polar.

These findings help identify possible compounds responsible for the extract's cytotoxic effect and point to areas that need further study.

5. In Silico Analysis

Molecular docking analysis was performed to predict the potential interactions between the identified compounds from *Ficus nota* and key protein targets associated with colorectal cancer. Binding affinities and interaction profiles were examined to assess the likelihood of inhibitory activity, providing insight into the possible mechanisms behind the observed cytotoxic effects. These computational findings offer a complementary perspective to the experimental data.

Table 7: CASTp-Derived Area and Volume of the Chosen Active Site Pockets of TNIK, PI3Kα, and EGFR-TKD.

Protein	Active Site ID	Area ($Å^2$)	Volume ($Å^3$)
TNIK	1	3640.386	4065.512
	9	110.286	109.953
	10	104.321	101.975
	11	63.744	60.019
PI3Kα	5	424.418	323.175
EGFR-TKD	1	1094.184	963.437

Table 6 shows the geometric characteristics of the prioritized active sites in TNIK, PI3Kα, and EGFR-TKD as identified by CASTp. Protein binding sites for all three targets were identified using CASTp, which maps surface pockets based on geometric volume and accessibility.

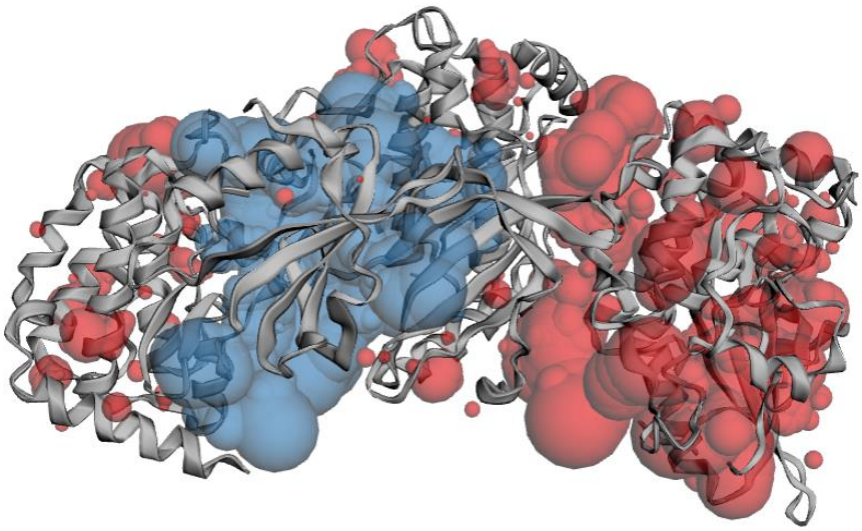

Figure 6: Highlighted Active Binding Site of TNIK Identified via CASTp.

For TNIK, out of 99 identified pockets, sites 1, 9, 10, and 11 were prioritized due to the inclusion of residues VAL31–VAL39 (ATP-binding loop), LYS54 (ATP coordination), and ASP153 (proton acceptor) (UniProt Consortium, 2023). These residues are reported as functionally relevant, corresponding to the known binding region of the TNIK inhibitor NCB-0846 (Masuda et al., 2016).

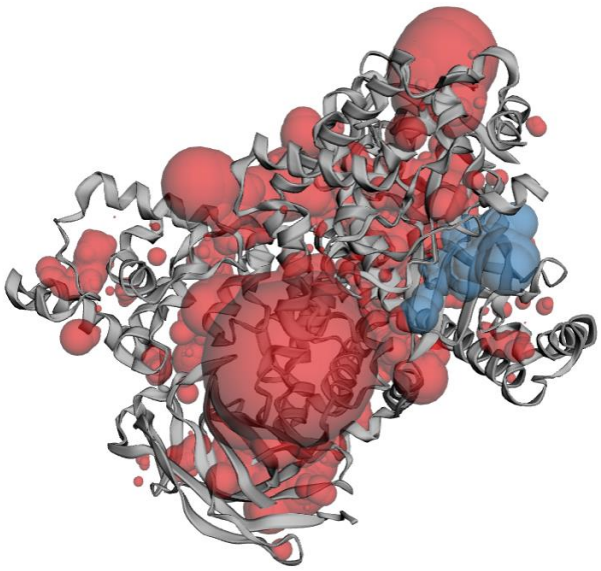

Figure 7: Highlighted Active Binding Site of PI3Kα Identified via CASTp.

Similarly, 118 predicted pockets were examined for PI3Kα, with Site 5 selected based on the presence of HIS855, GLN859, THR856, and SER854. These residues are known to confer isoform-specific inhibition, particularly structure-activity relationship (SAR) studies involving PI3Kα-selective inhibitors such as GDC-0326 and compound 24 (Heffron et al., 2016). Docking simulations confirmed that these residues formed consistent hydrogen bond networks and spatial configurations favorable to ligand engagement.

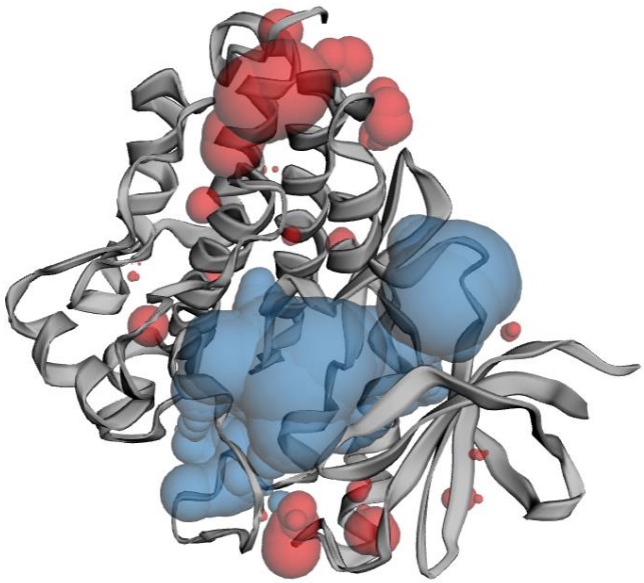

Figure 8: Highlighted Active Binding Site of EGFR-TKD Identified via CASTp.

In the case of EGFR-TKD, 32 potential pockets were identified, and Site 1 was selected due to its overlap with the ATP-binding cleft and catalytic core. This pocket includes LYS721 and ASP831 (DFG motif), essential for kinase function, alongside THR766, GLN767, and MET769, which stabilize inhibitor binding (Park et al., 2012). All binding site selections were cross-validated with structural and functional literature to ensure biological relevance and strengthen the interpretation of docking results.

Table 8: Docking Scores of the LC-MS/MS-Identified Compounds Against TNIK, PI3Kα, and EGFR-TKD.

Compound Number	Proteins		
	TNIK	PI3Kα	EGFR-TKD
1	-6.38	-6.649	-7.119
2	-4.742	-4.491	-5.522
3	-6.284	-9.285	-9.019
4	-8.159	-7.854	-8.014
5	-8.169	-8.709	-9.297
6	-5.216	-5.647	-5.601
7	-5.049	-5.469	-5.549
8	-6.487	-6.71	-7.384
9	-10.27	-8.639	-11.6
10	-8.568	-9.519	-9.449
11	-5.41	-5.924	-6.717
12	-8.619	-7.258	-8.457
13	-8.753	-9.31	-11.6
14	-8.554	-9.991	-10.68
15	-9	-10.18	-10.01
16	-10.34	-11.8	-11.38
17	-8.862	-8.94	-10.33
18	-9.879	-10.68	-11.66
19	-6.983	-7.128	-7.365
20	-9.792	-12.35	-10.7
21	-12.06	-12.16	-12.34
22	-10.98	-11.85	-13.65
23	-7.304	-6.674	-8.096
24	-6.283	-6.124	-7.11
25	-6.231	-6.195	-6.791
26	-13.5	-12.65	-15.34
27	-9.711	-10.9	-10.6
28	-9.949	-12.21	-10.82
29	-12.51	-11.63	-12.15
30	-12.81	-13.21	-13.1
31	-9.589	-10.99	-11.65
32	-6.007	-5.033	-5.709
33	-20.08	-19.48	-24.15
34	-12.68	-12.85	-12.98
35	-13.74	-14.99	-18.25
36	-6.566	-6.726	-7.23
37	-12.03	-13.35	-10.23
38	-11.37	-12.5	-12.42
39	-7.284	-7.574	-8.43

The docking results revealed several compounds with exceptionally strong binding affinities, suggesting their potential role in the extract's anticancer activity. Compound 33 (Phytolacca cerebroside) emerged as the most potent binder across all three protein targets with remarkable binding energies of -20.08 kcal/mol for TNIK, -19.48 kcal/mol for PI3Kα, and -24.15 kcal/mol for EGFR. These exceptionally low energy values indicate highly stable interactions with the active sites of these proteins, which are critically involved in cancer signaling pathways.

Compounds 21 (Ergot-7,22-diene-3β,5α,6β-triol) and 22 (Hentriacontane) also demonstrated consistently high affinities, particularly against EGFR, a key therapeutic target in colorectal cancer. The strong correlation between the docking scores of these compounds and the extract's in vitro cytotoxicity strongly suggests that they are the primary bioactive agents responsible for the observed anticancer effects. Interestingly, Compound 26 (Lignoceryl ferulate)

showed selective but potent binding against TNIK and PI3Kα, indicating its potential as a dual-pathway inhibitor that could simultaneously target multiple oncogenic signaling cascades.

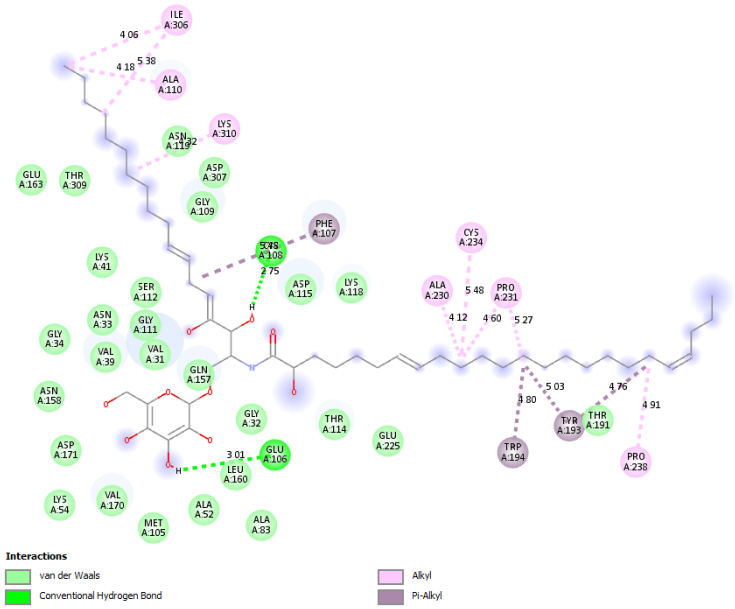

Figure 9: 2D Interaction map of Compound 33 with TNIK.

The molecular interactions of Compound 33 with TNIK, PI3Kα, and EGFR-TKD reveal a consistent pattern of high-affinity binding mediated by both polar and hydrophobic contacts. In TNIK, Compound 33 engages the ATP-binding loop (VAL31–VAL39) through van der Waals forces and LYS54, a residue essential for ATP coordination. This interaction mimics the binding mode of the known TNIK inhibitor NCB-0846, suggesting competitive ATP-site inhibition. The compound further stabilizes its position via alkyl interactions with hydrophobic residues (e.g., ALA52) in the pocket, explaining its exceptional binding energy (−20.08 kcal/mol).

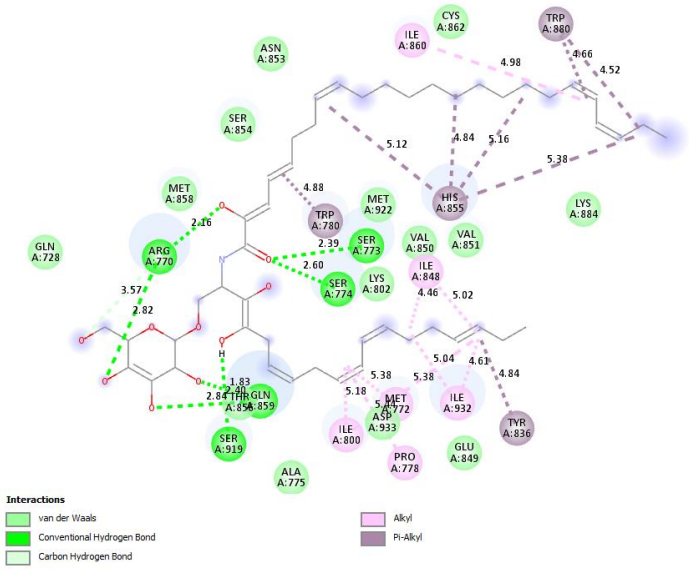

Figure 10: 2D Interaction map of Compound 33 with PI3Kα.

For PI3Kα, Compound 33 targets the isoform-specific site involving HIS855, SER854, and GLN859. A hydrogen bond with GLN859 (distance: 2.4 Å) anchors the ligand, while π-alkyl interactions with HIS855 and carbon-hydrogen bonds with THR856 enhance stability. These interactions align with the binding features of PI3Kα-selective inhibitors like GDC-0326, corroborating the compound's potential to disrupt PI3Kα signaling. The presence of multiple interaction types (van der Waals, hydrogen bonds, and hydrophobic contacts) accounts for its strong affinity (−19.48 kcal/mol).

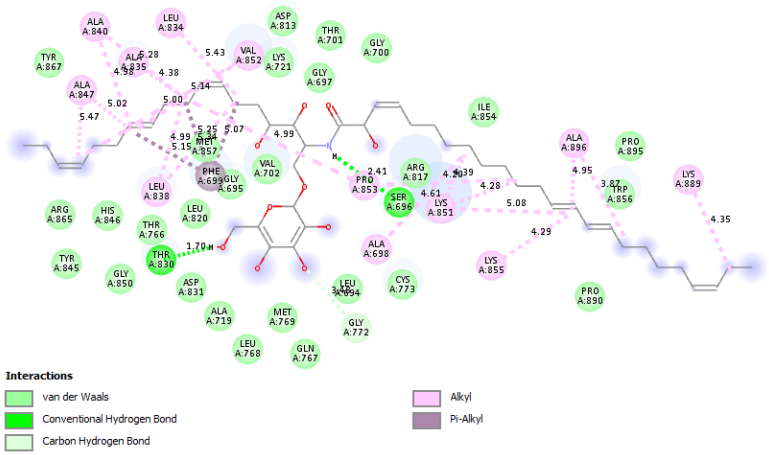

Figure 11: 2D Interaction map of Compound 33 with EGFR-TKD.

In EGFR-TKD, Compound 33 binds the catalytic cleft, forming hydrogen bonds with key residues SER696 and THR830. The compound's pi-alkyl interactions with PHE699 and additional van der Waals contacts with LYS721 and ASP831 (DFG motif), alongside GLN767, and MET769 mirror the binding mode of clinical EGFR inhibitors. This multi-pronged engagement, including a low-energy hydrogen bond (2.41 Å with SER696), underpins its ultra-high affinity (−24.15 kcal/mol). Compound 33's ability to target three distinct oncogenic kinases with high potency suggests broad-spectrum anticancer potential.

Table 9: ADME Results of the LC-MS/MS-Identified Compounds.

Properties	Parameters	Compound	
		33	**30**
Physicochemical Properties	MW	437.57	844.25
	#Heavy atoms	31	59
	#Aromatic heavy atoms	0	0
	Fraction Csp3	1	0.94
	#Rotatable bonds	5	42
	#H-bond acceptors	7	10
	#H-bond donors	3	8
	MR	117.87	243.57
	TPSA	91.62	189.17
Lipophilicity	iLOGP	3.25	8.72
	XLOGP3	-0.06	13.29
	WLOGP	0.12	8.45
	MLOGP	0.77	2.55
	Silicos-IT Log P	0.65	11.52
	Consensus Log P	0.95	8.9
Water Solubility	ESOL Log S	-2.19	-10.68
	ESOL Solubility (mg/ml)	2.86E+00	1.78E-08
	ESOL Solubility (mol/l)	6.53E-03	2.11E-11
	ESOL Class	Soluble	Insoluble

	Ali Log S	-1.41	-17.31
	Ali Solubility (mg/ml)	1.69E+01	4.09E-15
	Ali Solubility (mol/l)	3.86E-02	4.84E-18
	Ali Class	Very soluble	Insoluble
	Silicos-IT LogSw	-1.6	-9.72
	Silicos-IT Solubility (mg/ml)	1.10E+01	1.60E-07
	Silicos-IT Solubility (mol/l)	2.51E-02	1.90E-10
	Silicos-IT class	Soluble	Poorly soluble
Pharmacokinetics	GI absorption	High	Low
	BBB permeant	No	No
	Pgp substrate	Yes	Yes
	CYP1A2 inhibitor	No	No
	CYP2C19 inhibitor	No	No
	CYP2C9 inhibitor	No	No
	CYP2D6 inhibitor	No	No
	CYP3A4 inhibitor	No	No
	log Kp (cm/s)	-9.01	-2.01
Druglikeness	Lipinski #violations	0	3
	Ghose #violations	0	4
	Veber #violations	0	2
	Egan #violations	0	2
	Muegge #violations	0	5
	Bioavailability Score	0.55	0.17
Medicinal Chemistry	PAINS #alerts	0	0
	Brenk #alerts	0	1
	Leadlikeness #violations	1	3
	Synthetic Accessibility	6.4	9.37

The ADME profile of Compound 33 reveals several significant challenges that would need to be addressed for further development as a drug candidate. With a molecular weight of 844.25 Da and 59 heavy atoms, the compound already exceeds the typical limits for drug-like molecules, as evidenced by its three Lipinski rule violations. The extremely high lipophilicity is particularly concerning, with consensus log P values around 8.9 and all solubility predictions classifying the compound as either "insoluble" or "poorly soluble" across different models (ESOL, Ali, and Silicos-IT). This poor solubility is further reflected in the very low predicted aqueous solubility values, ranging from 1.78 × 10-8 mg/mL to 4.09 × 10-15 mg/mL, which would likely lead to formulation challenges and poor bioavailability. The compounds' large size and flexibility, indicated by 42 rotatable bonds and a molar refractivity of 243.57, contribute to these unfavorable properties. While Compound 33 shows some potentially favorable characteristics like moderate topological polar surface area (189.17 Å²) and no PAINS alerts that would indicate problematic structural motifs, its pharmacokinetic prediction raises multiple red flags. The compound is predicted to have low gastrointestinal absorption and is unlikely to cross the blood-brain barrier, though this may be desirable for avoiding CNS side effects in cancer treatment.

More concerning is the prediction that it would be a P-glycoprotein substrate, which could lead to efflux and reduced intracellular concentrations in cancer cells. The synthetic accessibility score of 9.37 suggests this would be a challenging molecule to synthesize, which could complicate structure-activity relationship studies. The combination of extremely high lipophilicity, poor solubility, and large molecular size presents substantial hurdles for drug development. However, the compound's remarkable binding affinities to multiple cancer targets may justify efforts to optimize its properties through medicinal chemistry. Potential strategies could include introducing ionizable groups to improve solubility, reducing molecular weight by removing non-essential moieties, or rigidifying the structure to

decrease rotatable bond count. Prodrug approaches might also be considered to address the solubility limitations. While the current ADME profile makes Compound 33 a challenging lead, its potent multi-target activity against TNIK, PI3Kα, and EGFR suggests it could serve as a valuable starting point for the development of more drug-like analogs with improved pharmacokinetic properties.

ADME analysis revealed that Compounds 16, 20, and 30 possessed both strong binding affinities and favorable drug-like properties. Among these, Compound 30 stood out due to its consistently high docking scores (-12.81 kcal/mol for TNIK, -13.21 kcal/mol for PI3Kα, and -13.1 kcal/mol for EGFR) alongside good ADME characteristics, making it the most promising candidate for further development. Compound 30 demonstrates favorable binding interactions across all three target proteins (TNIK, PI3Kα, and EGFR-TKD) while exhibiting significantly improved drug-like properties compared to Compound 33.

The ADME profile of Compound 30 presents a stark contrast to Compound 33, with significantly more favorable drug-like properties. With a molecular weight of 437.57 Da and only 31 heavy atoms, it comfortably adheres to Lipinski's rule of five (zero violations). The compound shows excellent solubility predictions across all models, classified as "soluble" or "very soluble" with values around 2.86 mg/mL (ESOL) and 16.9 mg/mL (Ali), addressing one of the major limitations of Compound 33. The moderate lipophilicity (consensus LogP 0.95) and topological polar surface area (91.62 Å²) suggest good membrane permeability while maintaining solubility. Compound 30 is predicted to have high gastrointestinal absorption and is not expected to cross the blood-brain barrier, which may be advantageous for minimizing CNS-related side effects in cancer treatment.

While the compound is predicted to be a P-glycoprotein substrate (which could potentially limit intracellular accumulation), its overall balanced properties make it a much more promising lead candidate than Compound 33. The synthetic accessibility score of 6.4 indicates it should be reasonably straightforward to synthesize and modify, facilitating further structure-activity relationship studies. The absence of any structural alerts (PAINS, Brenk) and good bioavailability score (0.55) further support its potential as a developable lead compound.

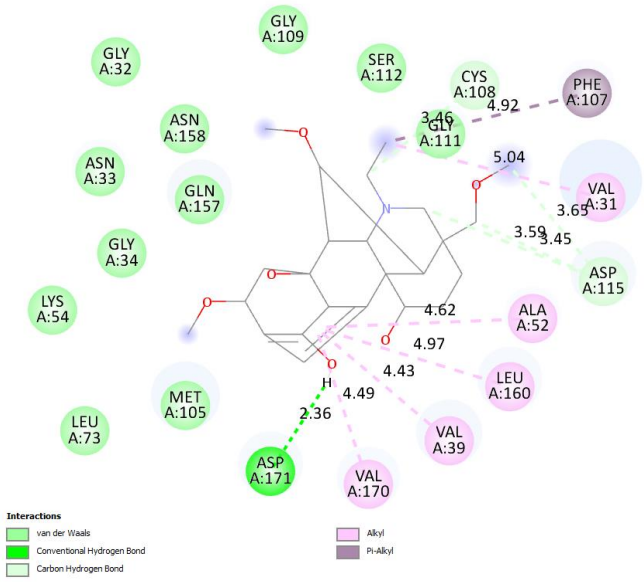

Figure 12: 2D Interaction map of Compound 30 with TNIK.

In TNIK, the compound 30 forms conventional hydrogen bonds with residues like ASP171, engages in multiple van der Waals interactions with LYS54 (critical for ATP coordination), GLY32-GLY34 (ATP-binding loop), and LEU73, and forms an alkyl interaction with VAL39 (ATP-binding loop). These interactions are complemented by carbon-hydrogen bonds with ASP115 and CYS108, creating a balanced mix of polar and hydrophobic contacts that contribute to its strong binding affinity (-12.81 kcal/mol). The presence of these interactions in the ATP-binding site suggests potential competitive inhibition similar to known TNIK inhibitors.

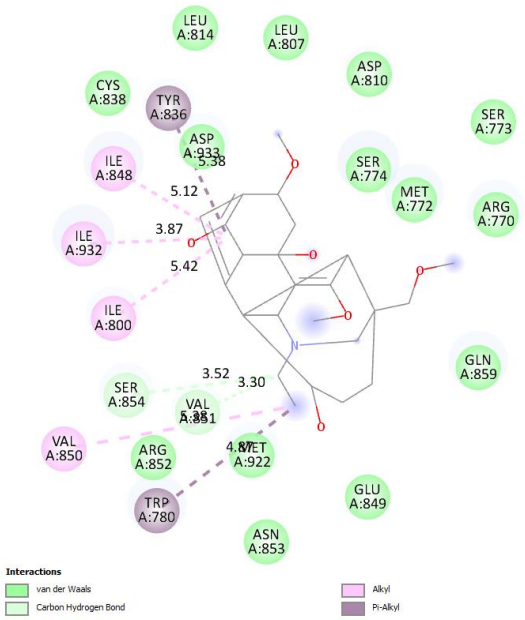

Figure 13: 2D Interaction map of Compound 30 with PI3Kα.

For PI3Kα, Compound 30 shows particularly interesting interactions with the isoform-specific pocket. It forms carbon-hydrogen bonds with SER854 (3.52 Å), VAL851 (3.30 Å), while maintaining van der Waals contacts with ASN853, GLU849, and GLN859. These interactions mirror those observed with selective PI3Kα inhibitors, explaining its notable binding energy (-13.21 kcal/mol). The compound's ability to interact with both polar and hydrophobic residues in this binding pocket suggests it could achieve good selectivity while maintaining potency.

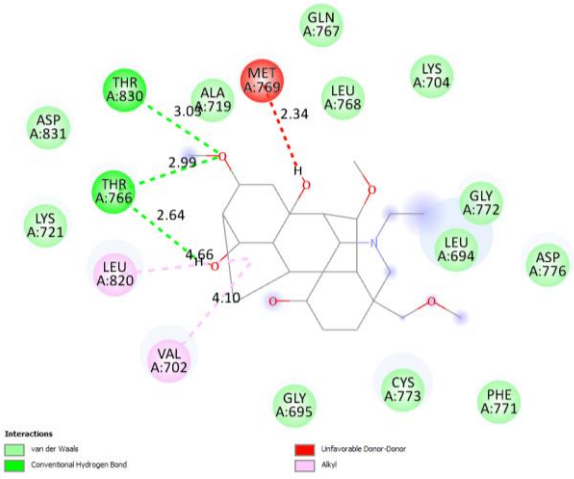

Figure 14: 2D Interaction map of Compound 30 with EGFR-TKD.

In EGFR-TKD, Compound 30 demonstrates strong interactions with key catalytic residues. It forms conventional hydrogen bonds with THR830 and THR766, while maintaining van der Waals contacts with LYS721, ASP831 (DFG motif), and GLN767. These interactions are particularly significant as they involve residues known to be critical for EGFR inhibitor binding, suggesting a mechanism of action similar to clinically approved EGFR inhibitors. The compound's ability to engage both the hinge region (through LYS721) and the hydrophobic back pocket contributes to its excellent binding affinity (-13.1 kcal/mol).

The combination of strong, multi-target binding interactions and favorable ADME properties positions Compound 30 as an excellent candidate for furher development. Its ability to potently inhibit three key cancer targets while maintaining drug-like characteristics suggests it could serve as either a single-target optimized lead or potentially as a multi-targeted agent.

The implications of these findings for cancer drug discovery are significant. The identification of multiple compounds with strong binding to cancer-related targets from a single plant extract demonstrates the potential of natural products as sources of novel anticancer agents. The fact that different compounds showed preferential binding to different targets suggests the possibility of developing combination therapies that could simultaneously inhibit multiple oncogenic pathways, potentially reducing the likelihood of drug resistance. For instance, a combination of Compound 30 (with its balanced potency and ADME properties) and Compound 33 (with its ultra-high affinity) might offer synergistic effects if formulation strategies can overcome the latter's pharmacokinetic limitations. Moreover, the structural diversity of these bioactive compounds provides multiple starting points for medicinal chemistry optimization to improve potency, selectivity, and drug-like properties.

In summary, this integrated approach combining phytochemical analysis, in vitro cytotoxicity testing, and computational molecular docking has successfully identified several promising bioactive compounds from Tibig leaves with potential anticancer applications. While Compounds 21, 22, and 33 represent highly potent inhibitors of cancer-related proteins, their development would require significant optimization to address pharmacokinetic challenges. Compound 30 emerges as the most immediately viable lead compound due to its combination of strong binding affinity and favorable ADME properties. Future research should focus on experimental validation of these computational predictions, specific isolation and synthesis, including in vitro target inhibition assays and in vivo efficacy studies, to fully realize the therapeutic potential of these *Tibig*-derived compounds. The findings underscore the value of traditional medicinal plants as sources of novel bioactive compounds and demonstrate how modern computational and experimental techniques can bridge the gap between traditional knowledge and contemporary drug discovery.

CHAPTER 5

Summary, Conclusion, and Recommendation

This chapter provides the researchers' summary, conclusions, and recommendations derived from the findings of the study.

Summary of Findings

The researchers were able to summarize the results with the list below:

1. The total percentage yield of 70% Ethanol extract was 8.24%.

2. The phytochemical screening showed that the following constituents were present in the plant sample: Unsaturated Sterol and/or Terpenes, Leucoanthocyanidins, y-benzopyrone, Hydrolyzable tannins, and Carbohydrates.
3. The Brine Shrimp Mortality Assay showed that the 1000-ppm hexane partition had the highest mortality rate of 100%, higher than that of 1000-ppm fluorouracil, the positive drug, which had a mortality rate of 83.33%. Thus, it serves as a basis for the selection of partitions for secondary cytotoxic screening.
4. The MTT assay showed that the Tibig extract has moderate cytotoxic activity against HCT 116, which is also comparable to the published IC50 of Vinblastine. With simple descriptives, the extracts and Vinblastine could have a comparable activity and selectivity profile. These similarities could be indicative of a similar mechanism of action as microtubule inhibitors. Moreover, the selectivity index computed of more than 1 is indicative of an activity on cancer cells greater than the activity on normal cells.
5. The LC-MS/MS identified that the compound/s responsible for the cytotoxic activity are: 10E)1,10-Heptadeca-diene-4,6-diyne-3,8,9-triol, (E,E)-9-Oxooctadeca-10,12-dienoic acid, 1-Nonene, 2-Monopalmitin, Baicalein, Bowdichione, Butyl isobutyl phthalate, Campesterol, Hentriacontane, Kuwanon C, Lupenone, Neoline, Paeonisothujone, Phytolacca Cerebroside, Puerarol, Salvianolic acid A, Talatisamine, and Tonkinensisol.
6. The in silico screening of *Tibig*-derived compounds revealed Compound 30 as the most promising lead, showing strong binding affinities alongside favorable ADME properties. Though Compounds 21, 22, and 33 also displayed potent target interactions, Compound 33, in particular, faces major pharmacokinetic limitations requiring further optimization. These findings, rooted in computational docking and ADME profiling, support Compound 30 as an ideal candidate for further development.

CONCLUSION

Based on the observable IC_{50}, the *Tibig* extract has no observable difference with Vinblastine but has an observable difference with Fluorouracil. On this account, the null hypothesis would be accepted on the comparison with Vinblastine and rejected on the comparison with Fluorouracil. In silico screening further supported the extract's therapeutic potential, with Compound 30 emerging as the most promising lead due to its strong binding affinities and favorable ADME profile.

Recommendation

The following recommendations are suggested for further research:

1. It is recommended that further studies should explore the extract's activity against a broader range of cancer cell lines representing different cancer types.
2. Further investigate the active compounds by identifying and isolating specific components of the plant that exhibit cytotoxic activity. This allows the researchers to investigate the specific mechanisms of action of each constituent.
3. Evaluate the anticancer efficacy in vivo to ensure its potential as a therapeutic agent. An in vivo study will provide the effectiveness of the plant extract and its therapeutic activity in living systems.
4. Utilize other parts of the plant to further identify different bioactive compounds present to maximize the therapeutic potential of the plant by discovering additional compounds that will exhibit pharmacological benefits.

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