Chapter 1

Introduction

1.1. Background

The rising incidence of colorectal cancer over the years has been a global concern. Colorectal Cancer (CRC) is currently the third most commonly diagnosed cancer worldwide, with a steady rate of increasing cases among developing countries. Also known as colorectal carcinoma, its most prevalent subtype is adenocarcinoma, which comprises over 90% of all diagnosed CRC cases (Fleming et al., 2012). Colorectal adenocarcinoma mostly arises from the epithelial lining of colorectal mucosa. This cellular abnormality rises due to the defect of multiple proto-oncogenes and tumor suppressor genes, including *RAS*, *BRAF*, *APC*, and *TP53* (Kasi et al., 2020), which leads to the transformation of normal epithelial lining into polyps, that if remained untreated will develop into adenocarcinoma. Despite the currently better-than-ever cancer management, CRC is still the second deadliest cancer worldwide in 2018, encouraging research to develop a better and more effective treatment for the disease (Bray et al., 2018).

Combinatorial treatment has been one of the solutions for better cancer therapies. By combining different drugs, a better cancer killing effect can be produced. By prescribing a more effective treatment, the drug dosage can be reduced, lowering the chance of side effects and possible toxicity. One of the possible sources of anti-cancer compounds come from natural-based products. Etiologically speaking, a large proportion of anti-cancer therapy were derived or made to mimic the activity of bioactive compounds derived from natural sources, including plants (Cragg & Pezzuto, 2016). The main reason for the outsourcing of natural products for cancer therapy is due to its persevered unique, and diverse bioactive compound, which have a higher possibility to hit multiple targets in cancer molecular signalling. Even so, the development process of a natural-based product into an actual usable drug remains an intricate process, requiring complex modifications in the synthesis and formulation of the pro-drug molecule. The result, however, is a drug that will have a

more functional pharmacokinetic and pharmacodynamic profile, and higher versatility, enabling it for future modifications including for combinatorial therapy (Cragg & Pezzuto, 2016).

The extract of the soursop plant (*Annona muricata*), a fruit bearing tree originating from Central America, has become an interesting subject in natural-based cancer drug research. The ethyl acetate extract of soursop leaves has shown selective cytotoxic effect in multiple cell lines, including CRC adenocarcinoma cell line HT-29 and HT-116, due to its rich biochemical content with the most exceptional one being acetogenins (Gavamukulya et al., 2017). There is even an already conducted phase I study using soursop leaf extract as a supplementation for CRC patients with positive results, although the study did not include efficacy parameters in their study (Indrawati et al., 2017). The previous clinical study includes patients that have received initial chemotherapy. In addition, the use of *Annona muricata* leaf extract in combination with other drugs for treating multiple ailments aside from cancer have been recorded (Yajid et al., 2018). Combined, this suggests a potential for *Annona muricata* leaf extract to be used with other anti-cancer drugs, in order to maximize the anti-cancer effect that is sought.

Another alternative source of anti-cancer drugs is through drug repurposing. Drug repurposing refers to the use of machine learning to effectively screen existing drugs against disease targets, including for cancer, making it a cost-effective drug-research due to the already-available pharmacological data (Pantziarka, 2017). One of the currently studied repurposed anti-cancer drugs are statin families (Longo et al., 2020). Simvastatin is a fungal derived statin that inhibits 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase. It has been reported to exert anti-cancer activity in multiple cancer types including CRC, though no consensus has been made due to contradictory findings between research groups (Hoffmeister et al., 2015; Y. Li et al., 2019). Several studies have claimed the ability of simvastatin to even rescue CRC patients with KRAS mutation that has become resistant towards first line cetuximab therapy through the modulation of BRAF pathway (J. Lee et al., 2011, 2014).

Even though the effects of both ethyl acetate extract of Annona muricata (EEAM) and simvastatin have each been individually assessed against CRC cell line, there are no available studies that investigates the combinatorial effect of both compounds. By combining both EEAM and simvastatin, a synergistic type of drug interaction may potentially be produced, therefore producing a better cancer-killing effect. Moreover, a synergistic or additive drug interaction can lower the dosage of each drug component, thus lowering possible toxicity that might come with the drug (Mokhtari et al., 2017). This dose-lowering effect of combinatorial treatment might benefit simvastatin due to its overall toxicity in high doses (Matusewicz et al., 2020).

The most accessible and relatively effective way of measuring the efficacy of an anti-cancer agent is through the cell viability assay. By comparing the viability of treated cells to a control, or other treatment, we can determine the best treatment formulation that works on that particular cell (Niepel et al., 2019). Aside from its viability, observing the type of cell death induced by the treatment is also an important aspect in cancer therapeutic study. Generally, apoptosis is a type of cell death preferably targeted with cancer treatment, since its pathways are regulated, well-studied, and commonly exploited. Therefore, targeting apoptosis will not cause additional inflammation and produce less systemic side effects (A. et al., 2012).

Recent advances in bioinformatics have brought biomedical research to a new height. One technology in particular is the affinity-based screening between biomolecules, including metabolite to protein interaction. Such technology allows for interaction analysis that might explain the actual laboratory data, leading to better understanding of the molecular mechanism of certain treatment. In pharmaceutical research, affinity-based screening can also be used to study drug-to-drug interaction, making it useful to advice or prevent the combination of certain therapies (Schneider et al., 2020). Therefore, utilizing affinity-based screening for combinatorial therapy would provide an invaluable insight into the mechanism of interaction, and subsequent effects in the cellular signalling of the affected cell.

In this research, we aim to observe the combinatorial activity of both EEAM and simvastatin against HT-29 colorectal adenocarcinoma cell line. The findings of this study will provide us with information regarding the benefit of combining both substances, compared to each individual substance in terms of their anti-proliferative capability, cell-death inducing ability. In addition, this study will also cover the phytochemical identification, and metabolite-protein docking analysis through molecular docking. Therefore, the bioinformatic analysis may provide an explanation for the study result and strengthen the finding of this research.

1.2. Aim and Objectives

The aim of this research is to assess the combinatorial activity of EEAM and simvastatin, against the HT-29 colorectal adenocarcinoma cell line. Initially, we will do the extraction of soursop leaf, using ethyl acetate as a solvent via maceration. The extract will be subjected towards several phytochemical tests to identify the composition of the extract and perhaps able to explain its anti-cancer activity. To assess the combinatorial activity, the IC_{50} of each treatment compound will be initially determined using cell viability assay. From the IC_{50}, the combinatorial ratio of EEAM and simvastatin will be formulated and each combination ratio will be assessed by calculating its combinatorial index (CI). The endpoint of the combinatorial cell viability assay will be its CI, which can be used to classify the drug interaction, whether it is synergistic (value <1), additive (value =1), or antagonistic (value >1). We are also targeting to investigate the type of cell death caused by both substances, using the DNA ladder assay. Finally, one of the bioactive compounds in the extract (i.e., Annomuricin E) will be subjected towards affinity-based screening against the various pathways related to its anti-cancer activity, which potentially explains its interaction with simvastatin and its individual anti-cancer activity.

1.3. Scope of Work (Activities)

The scope of this project will include all the activities that will be used to achieve the research objectives that includes:

- Sample extraction from *A. muricata* leaves powder using ethyl acetate via Phytochemical profiling of the EEAM including flavonoid, alkaloid, saponin, terpenoid, phenolic, and antioxidant (DPPH) tests.
- IC_{50} determination of both EEAM and simvastatin on HT-29 colorectal adenocarcinoma cell line by measuring its viability after treatment, using MTS assay.
- Assessing combinatorial activity of EEAM and simvastatin by combining both treatment and determine the combinatorial activity type of EEAM and simvastatin by assessing its combinatorial index.
- Conducting DNA ladder assay to determine the type of cell death caused by EEAM and simvastatin, both individually and in combination.
- Conducting the affinity-based screening of the EEAM bioactive component against potential binding partner.