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Evaluation And Determination Of The Phytotherapeutic Properties Of Selected Plants And Their Bioactive Metabolites On Targeted Genes In Colorectal Cancer Management

Ph.D. Thesis

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LIST OF ABBREVIATIONS/ ACRONYMS

AL: Aloin

APC: Adenomatous polyposis coli

AS: *Aloe secundiflora*

ATCC: American Type Culture Collection

ATP: Adenine triphosphate

BCL: B-cell lymphoma

CASP: Caspase

COX: Cyclooxygenase

CRC: Colorectal cancer

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ERK: Extracellular signal-regulated kinase

GI: Gastrointestinal

HNPCC: Hereditary nonpolyposis colorectal cancer

IG: Immunoglobulin

IL: Interleukin

LOX: Lipoxygenase

LPS: Lipopolysaccharides

MIC: Minimum inhibition concentration

MLH1: MutL homolog 1

MMP: Matrix metallopeptidase

NADH: Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

NF-kB: Nuclear factor-kappa B

PBS: Phosphate-buffered saline

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-PCR: Real-Time Quantitative Reverse Transcription PCR)

STAT3: Signal transducer and activator of transcription 3

TLR: Toll-like receptor

TME: Tumor microenvironment

VEGF: Vascular endothelial growth factor

WA: *Warbugia ugandensis*

WS: *Withania somnifera*

DEDICATION

First and foremost, I thank God for the grace He extended towards me throughout the course of my work. Second, I dedicate this dissertation to my close family, friends, and colleagues whose unfailing support and prayers helped me emerge strong and victorious. With special mention and gratitude, I also cordially dedicate my work to the Tempus Public Foundation (TPF) for fully funding my Ph. D. studies under the Stipendium Hungaricum Scholarship program in Hungary.

ABSTRACT

Colorectal cancer is among the most common malignancies and the third prime cause of cancer-associated mortalities. Research into tumorigenic pathways can aid in the development of more efficient cancer therapies and provide insight into the physiological regulatory mechanisms employed by rapidly proliferating cancer cells. Due to the severe side effects of cancer chemotherapeutic medications, plant metabolites, and their analogs are now explored more frequently for the treatment and prevention of colorectal cancer (CRC), opening the stage for new phytotherapeutic strategies that are considered effective and safe substitutes. This extensive research aimed to evaluate and determine the phytotherapeutic properties of selected plants and their bioactive metabolites on targeted genes in colorectal cancer management. The plants evaluated were *Withania somnifera* L*, Warbugia ugandensis,* and *Aloe secundiflora,* while the targeted genes were *COX-2, CASP9, Bcl-xL, Bcl2* and *5-LOX* in CRC cell lines (Caco-2). To establish critical gaps in this important topic, extensive and well-thought systematic reviews were conducted at the initial stage of the study. Results obtained from the reviews informed the choice of target genes for selective consideration. Plant extracts were obtained using serial exhaustive extraction using three different extraction solvents with varying polarities, for maximum metabolites extraction (Hexane, Ethyl acetate, and Methanol). They were then dissolved in Dimethyl sulfoxide (DMSO) appropriately for bioassay. Caco-2 cell lines were passaged, and treated with plant extracts at varying concentrations, and their RNA's isolated for evaluation. Our unique study reports on these important plants as efficient natural inhibitors of CRC growth, by directly linking their phytoconstituents to; the downregulation of *COX-2, 5-LOX, Bcl-xL*, *Bcl2,* and upregulation of *CASP9* genes dose-dependently. I strongly present *Withania somnifera, Warbugia ugandensis,* and *Aloe secundiflora* plant extracts as promising natural inhibitors for CRC carcinogenesis and recommend *in vivo* and subsequent clinical trials, with substantial clinical effects postulated. In addition, methanol and ethyl acetate can extract metabolites exhibiting higher regulatory effects on the targeted genes and are thus highly recommended for application in yielding maximum benefits. Finally, I suggest studies on the identification and characterization of the specific metabolites in these plants, that are involved in the modulatory mechanisms, resulting in the inhibition of CRC growth and possible metastases.

Keywords: Regulatory pathways; Angiogenesis; Phytotherapeutic inhibitors; *in vitro;* Apoptosis; Cytotoxicity; Colon carcinogenesis; Pharmacological activity

CHAPTER ONE

1. INTRODUCTION

1.1 Background information

1.1.1 Colorectal cancer malignancy

Colorectal cancer is among the most common malignancies but the third prime cause of cancer-associated mortalities in both men and women especially in developed countries [1]. Significant advances have been achieved in the knowledge of molecular activities leading to the formation of adenomatous polyps (cancer precursors) and cancer. Numerous colorectal tumors are sporadic but a substantial percentage (5–6%) have a distinct genetic association [1]. Epigenetic changes via aberrant promoter methylation and exertion of histone modifications play a key role in the origin and proliferation of colon cancers. Reversal of epigenetic marks using compounds targeting aberrant transcription factors, co-activator, co-repressor interactions, and histone-modifying activities, gives insightful possibilities where the epigenome of cancerous cells can be manipulated with probable therapeutic advantages [2].

Even though the pathophysiology of colorectal cancer (CRC) is complicated and poorly understood, interactions between risk factors such as genetics, lifestyle, and environment appear to be key in the development and progression of the disease occurring in developing countries [3]. Surgery and chemotherapeutic interventions are the most used forms of treatment for colon cancer due to the lack of scientifically explored alternatives. However, the development and identification of molecular compounds capable of killing or inhibiting transformed cells promoting carcinogenesis without inducing toxic effects or being toxic to normal cells are of utmost significance [4].

1.1.2 Phytotherapeutic intervention strategies

The development of different CRC therapies has failed to curb the mortality of patients suffering from CRC, due to the high incidence of metastasis. In light of this, management with dietary supplements derived from plants is beginning to receive due recognition as the most potent approach to lessen the burden of colorectal cancer-associated mortality [5]. Plants have significant bioactive compounds essential for growth and development in almost all living organisms. They are widely consumed as food and for their medicinal value in virtually all cultures. However, their pharmacological properties and efficacy are poorly understood. Most phytochemicals with determined bioactive potential have been associated with plants [6]. Nearly all phytochemicals established in plants are classified into four biochemical classes: terpenes, alkaloids, glycosides, and polyphenols [6].

Different plant organs have demonstrated substantial medicinal properties such as hypoglycaemic antioxidant and antitumor potentials [7]. It has been cited that 50% of cancer management options are derived from biologically active agents common in plants [8]. Ethnopharmacological studies should be encouraged to investigate the more appropriate technique of plant selection, extraction, chemical formulation, and dose calculation to ascertain and attain extensive benefits from these medicinal plant species [8]. A study performed using *Cassia fistula* demonstrated that the herb’s seeds and fruits exhibited active repression of cancerous cells derived from the colon, breast, and liver [7]. Plants' natural constituents in our study provide a new source of anticancer treatment with a sufficient novel mode of action. Compared to synthetic agents, phytoconstituents of plant origin are rarely seen to correlate with numerous side effects and have been demonstrated to present overwhelming therapeutic activities to heal numerous infectious diseases [9]. Natural products from plants have been a prolific

source of new anti-colon cancer medications, accounting for around half of all currently used anticancer treatments, either directly or indirectly. Our research sought to extract, analyze, and test the potency of phytochemical compounds from three important unexplored plant species natively occurring in Kenya, Africa, namely, *Withania somnifera* L. (WS)*, Warbugia ugandensis* (WU)*, and Aloe secundiflora* (AS).

1.2 Problem statement

Colon cancer is the third most diagnosed malignancy among men and women and the second leading cause of cancer death globally. The burden of colorectal cancer is largely attributed to industrialization and westernization. There is a notable shift in this global burden towards countries of lower economies as they continually become Westernized due to trade, tourism, professionalism, and other exchange programs across different continents. Genetic factors such as germline MutL homolog 1 (MLH1) and adenomatous polyposis coli (APC) mutations have an etiologic role, predisposing individuals to colon cancer. Synthetic treatments of CRC are largely associated with the development of cancer drug resistance and associated adverse side effects, provoking the exploration of natural alternatives as viable strategic options. However, even with this natural phytotherapeutic potent approach, knowledge of the existing beneficial medicinal plants remains not well researched and explored. Limited knowledge of the existing plant metabolites, their biological functions, and extraction methods slows down pharmacotherapeutic advances being made. This significant study, therefore, attempted to fill these critical gaps and to further advance phytotherapeutic approaches in CRC management.

1.3 Justification of the study

Since the existing chemotherapeutic treatment options are often associated with adverse reactions, medicinal plants in this research provide a safe treatment option for CRC, eliminating the chances of adverse reactions. Even at high concentrations, many natural phytoconstituents are quite well tolerated by humans and do not cause harmful effects. The interaction of conventional chemotherapeutics with natural phytoconstituents adds a new approach to CRC research and treatment. It could be a viable strategy for achieving gains while reducing the negative effects of conventional chemotherapy. Surgery and chemotherapeutic interventions are the most used forms of treatment for CRC due to the lack of scientifically explored natural alternatives. This study was therefore critical in filling these glaring gaps by providing substantive natural solutions through experimental research.

1.4 Research questions

1. Is extraction of hexanoic, ethanolic, and methanolic crude extracts from WS, WU, and AS possible?
2. Are active phytochemicals present in plant extracts derived using different extraction solvents?
3. Will active ingredients from plant extracts have any phytotherapeutic properties in the expression *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines?
4. Will it be possible to determine the modulatory expressions of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines, after treatment with different plant extracts?
5. Which extraction solvent can extract metabolites exhibiting the highest regulatory effects on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines?

1.5 Null hypotheses

1. Extraction of hexanoic, ethanolic, and methanolic extracts from WS*,* WU*,* and AS is not possible.
2. Active phytochemical compounds are not present in extracts derived from different extraction solvents.

1.6 Alternate hypotheses

1. Active ingredients from plant extracts have phytotherapeutic properties on the expression of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines.
2. It is possible to determine the modulatory expressions of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* after treatment with different plant extracts.
3. It is possible to determine the extraction solvents that can extract metabolites exhibiting the highest regulatory effects on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines.

1.7 General Objective

To evaluate and determine the phytotherapeutic properties of plants and their active phytoconstituents in the growth and proliferation of colorectal cancer using Caco-2 cell lines.

1.8 Specific Objectives

1. To obtain hexanoic, ethanolic, and methanolic crude extracts from three suspected medicinal plant species: WS, WU, and AS for CRC evaluation.
2. To determine and identify the presence of active phytochemical compounds in plant extracts obtained using different extraction solvents.
3. To determine the modulatory gene expressions of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* after treatment with different plant extracts.

4. To determine the most efficient extraction solvent in obtaining bioactive metabolites exhibiting the highest inhibitory potential on the growth of CRC cell lines.

1.9 Significance of the study

The purpose of this study was to highlight and present three selected medicinal plants with active metabolites, significant to induce beneficial effects in suppression of growth and proliferation of CRC. *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes associated with cancer development were treated with ethanolic, hexanoic, and methanolic extracts derived from the selected plants, with relevant history in medicinal application. The observed and tested plants are significant game-changers in the control of CRC if adopted for further medical scrutiny in both *in vivo* and subsequent clinical trials. Further, the novelty of this research is an addition of knowledge and insightful information to the existing literature on pharmacotherapeutic treatments derived from phytochemical compounds and their associated derivatives.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Incidence and Risk factors for carcinogenesis of colorectal cancer (CRC)

Globally, colorectal cancer (CRC) is the third most diagnosed malignancy and the second leading cause of cancer death. Approximately 70% of cancer fatalities occur in less developed countries [10]. About 9.96 million fatalities were reported in 2020, and by 2040, 16.3 million deaths are projected [11]. Arising through three major pathways, including adenoma–carcinoma sequence, serrated pathway, and inflammatory pathway, CRC represents an aetiologically heterogeneous disease according to subtyping by tumor anatomical location or global molecular alterations [12]. Long-term inflammation of the large intestine (colon) can promote carcinogenesis by facilitating oxidative stress which enhances DNA damage and therefore results in the tumor initiation step. Factors contributing to the high incidence of colon cancers include dietary and behavioral risks such as lack of physical exercise, smoking, and excessive consumption of alcohol as well as unhealthy diets lacking whole grains, vegetables, and fruits, high-fat diets, gender, race, age, family history, and geographical location. In addition, other factors include exposure to infectious agents, heavy metals, and certain chemicals [13]. The prevalence of these factors is reflected in the substantial variation in CRC incidence worldwide, which is highest in westernized countries like Europe and North America and lowest in sub-Saharan Africa. The relationship between CRC and a Western lifestyle is so strong that increasing rates of the disease are considered a marker of economic transition. People living in high-income countries who have a healthy lifestyle have lower CRC risk than the general population. A recent study found that maintaining a healthy weight, being physically active, limiting alcohol consumption, and eating a healthy diet reduces the risk of CRC by more than one-third (37%) [14].

Approximately 60–65% of CRC cases arise sporadically (it occurs in individuals without a family history of CRC or inherited genetic mutations that increase CRC risk), through acquired somatic genetic and epigenetic aberrations largely attributable to potentially modifiable risk factors [15]. CRC has a hereditary component, with studies having estimated the heritability of CRC to be 35–40% [12]. Approximately 25% of CRC cases have a family history of CRC without any obvious genetic cancer syndrome [15]. Only 5% are attributed to hereditary cancer syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch syndrome) or familial adenomatous polyposis (FAP), caused by inherited germline mutations in rare but high-penetrance susceptibility genes (for example, *MLH1* and *APC*, respectively) [15].

Some bacteria excrete polymers that can form the basis for biofilms. These biofilms create microenvironments for microbes that provide benefits including protection from external threats and access to resources/nutrients [13]. Biofilms are sometimes found in the colon, and they are often associated with colorectal cancer, particularly on the right side of the colon, which is the first portion of the colon that early-stage feces travel through [16]. Tissues with biofilms on them have been associated with greater permeability of the intestinal barrier and greater immune activation [14]. Exposure to pathogenic organisms, heavy metals, and certain chemicals are other factors [17,18]. There are several indications that diets rich in sucrose, compared with equicaloric diets containing the same amount of digestible complex carbohydrates such as starch, increase mucosal proliferation rate in the colon of rodents [14]

2.2 Survival rates for CRC

Survival rates are also available for colon cancer and rectal cancer separately. For colon cancer, the overall 5-year survival rate for people is 63%. If the cancer is

diagnosed at a localized stage, the survival rate is 90%. If the cancer has spread to surrounding tissues or organs and/or the regional lymph nodes, the 5-year survival rate is 71%. If colon cancer has spread to distant parts of the body, the 5-year survival rate is 14% [19]. For rectal cancer, the overall 5-year survival rate for people is 67%. If the cancer is diagnosed at a localized stage, the survival rate is 89%. If the cancer has spread to surrounding tissues or organs and/or the regional lymph nodes, the 5-year survival rate is 71%. If the cancer has spread to distant parts of the body, the 5-year survival rate is 15% [19]. By 2018, the incidence rate of colorectal cancer had gone up to 51.2%, per 100,000 [20], compared to the year 2010, where the incidence rate was reported at 49.9%, per 100, 000 [21].

2.3 Treatment approaches currently employed in the management of CRC

The therapeutic management and standard treatments for CRC include the use of cytotoxic drugs, immunotherapy, chemotherapy, surgery, targeted therapy, and radiotherapy [22]. In addition to these treatments, antiangiogenic agents are potentially used to treat and control the progression of cancerous cells. The development of different CRC therapies has failed to curb the mortality of patients suffering from CRC, due to the high incidence of metastasis. Methods used to prevent CRC are often dependent on approaches for diagnosing adenomatous polyps that are pre-CRC abrasions for colon cancer [23]. CRC progresses through various stages viz. 0, I, II, III, and IV. Stages 0-III are managed and treated using surgery; however, stage IV, as well as recurrent CRC, involves chemotherapy and surgery as treatment options [24]. Along with the individual characteristics of the patient, various drugs for chemotherapy as well as diets are currently in use to manage CRC. The first-line chemotherapeutic treatment involves folfiri (levoleucovirin, 5-FU, and irinotecan) and folfox (oxaliplatin and fluorouracil plus formyltetrahydrofolate) [25], combined with

cetuximab panitumumab or bevacizumab [23]. Of them, antibody-based medicines panitumumab and bevacizumab) have been found to improve the efficacy and safety of CRC treatment [25]. Mucositis, nausea, vomiting, and gastrointestinal toxicity are the most common side effects of folfiri chemotherapy [25]. When patients are intolerant to 5-FU, irox is favored, showing that it is a viable second-line treatment option for these advanced CRC patients. However, it causes more negative side effects in patients than when folfiri is administered [26]. Due to the sheer negative effects of these chemotherapeutic drugs, phytochemicals and their derivatives are becoming more prominent in CRC treatment and prevention [27]. The side effects associated with chemotherapy arise once healthy normal cells suffer damage [28]. All over the globe, diagnosing and managing cancer at the early stages generally increases the survival chances of individuals (Table 2.1). However, in third-world nations, access to modern facilities and effective diagnostic methods are frequently inadequate for the low-income population, particularly those residing in rural regions [29]. The WHO has informed that nearly 80% of the low-income population in the world uses traditional medicine. Among them, phytomedicines are used either alone or in combination to manage ailments and conditions.

Table 2.1 Anticancer management agents and associated cancer they treat [30]

S/N	Anticancer Agents	Treated Cancer Type
1	Alkylating	Breast, multiple cancers, myeloma sarcoma, lung and ovarian
2	Antibiotics	Colorectal, lung cancers, prostate, breast, and ovarian
3	Antimetabolites	Leukemia, intestinal tract cancers, breast, pancreatic and ovarian
4	Mitotic inhibitor	Breast, myeloma, leukemia, ovarian, lymphoma, and lung
5	Platinum compound	Testicular, lung, head, neck, ovarian, bladder, and colon cancers
6	Biological response modifier	Malignant melanoma, breast (trastuzumab), and non-Hodgkin lymphoma
7	Hormone therapies	Prostate, endometrial (uterine), and breast cancers

Research into tumorigenic pathways can aid in the development of more efficient cancer therapies and provide insight into the metabolic regulatory mechanisms employed by rapidly proliferating cancer cells [31]. The evaluation of natural biomass sources is a promising strategy for accelerating the development of novel anti-cancer medications to address the incidences of rising cancer problems. In the past, natural products frequently had a significant impact on the quest for novel medications and lead structures for active substances [32].

2.4 Tumour microenvironment and its associated tumorigenesis

The extracellular matrix, surrounding stromal and immunological cells, as well as metabolites and signaling chemicals in the intercellular space, constitute the tumor microenvironment (TME) [33]. The establishment of a TME is necessary for the upregulation of cell proliferation, migration, invasion, and metastasis from the initial site of the tumor [34]. Today, cancer ranks as the fourth leading cause of death worldwide, with a larger proportion of cancer fatalities. Even though the pathophysiology of colorectal cancer (CRC) is complicated and poorly understood, interactions between risk factors such as genetics, lifestyle, and environment appear to be key in the development and progression of the disease occurring in developing countries [3].

The understanding of the molecular processes that cause adenomatous polyps and cancer has made significant strides [35]. Oxidative stress increases deoxyribonucleic acid (DNA) damage leading to the tumor initiation stage. This results in chronic inflammation of the colon which can induce carcinogenesis [36]. Although many CRCs are sporadic, a sizable portion (5–6%) have a clear genetic connection. Among all gastrointestinal (GI) tract tumors, CRC has the greatest incidence [3]. The tumor microenvironment plays a pivotal role in tumorigenesis.

2.5 Targeted medicinal plants with suspected anti-CRC potency

2.5.1 Botanical description and the biodiversity of WS plant species

WS belongs to the kingdom Plantae (plants), the sub-kingdom Tracheophytes (vascular plants), division Angiospermae, class Eudicots, clade Asterids, order Solanales, family Solanaceae, sub-family Solanoideae, tribe Physaleae, genus *Withania*, and species *somnifera,* according to the biological classification system [37–39]. WS is a Solanaceae plant commonly called 'ashwagandha' in Hindi/ Sanskrit and is a small shrub growing in abundance in the subtropics. The leaves and roots are used in Ayurveda, the Indian traditional medicine [40]. The aerial part, particularly the stem, leaves, and calyx is sparsely covered with fine hairy tomentum [41]. It is an evergreen shrub growing to a height of about 1.5 m. They have simple, glabrous, ovate, petiolated, entire, shiny, smooth, and opposite leaves extending to a length of 10 cm. The branches are erect and are about 60-120 cm in length [41]. Flowers appear bright yellow or greenish and are approximately 1cm in length. They have small fruits growing to a size of approximately 6 mm in diameter. The fruits grow like berries and turn orange-red when they are mature. Their seeds are yellow and about 2.5 mm in diameter [42], and are small, flat, reniform, and very light. The crop is generally grown in Kharif season, and the plant has a tap root system 15-25 cm in length and light yellow (Figure 2.1). It requires dry weather conditions for the development of better root quality and alkaloid content [41].

Additionally, WS Dunal and WS Kaul have been identified as two subspecies [43]. In India, *Withania* species WS and *W. coagulans* are both widely farmed [38]. There are two WS cultivars utilized in Sri Lanka, although the Indian cultivar is better suited for drug research due to its starchy character, whereas the local species has fibrous roots that are challenging to powder [43]. The leaves and roots of this medicinal plant

species are used in Ayurveda, the Indian traditional medicine. In India, *Withania* species; WS and *W. coagulans* are both widely farmed [38]. In Ayurvedic, Allopathic, Unani, homeopathic, and other medical systems, its roots, seeds, and leaves have been utilized for more than 3000 years for a variety of health-related purposes. It is often referred to as the Queen of Ayurveda or as a Rasayana plant because of its exceptional medicinal powers [38]. It is grown throughout the arid tropical regions of Afghanistan, Baluchistan, the Canary Islands, China, Congo, Egypt, Israel, Jordan, Madagascar, Morocco, Nepal, Pakistan, South and East Africa, Spain, Sri Lanka, Sudan, and Yemen [44]. The traditional medical system of Ayurveda has a long history and is widely regarded. To achieve its therapeutic goals, this method makes use of numerous natural compounds in diverse forms [45]. The Ayurvedic system identifies thousands of plants that help prevent ailments and maintain health, including WS [45].

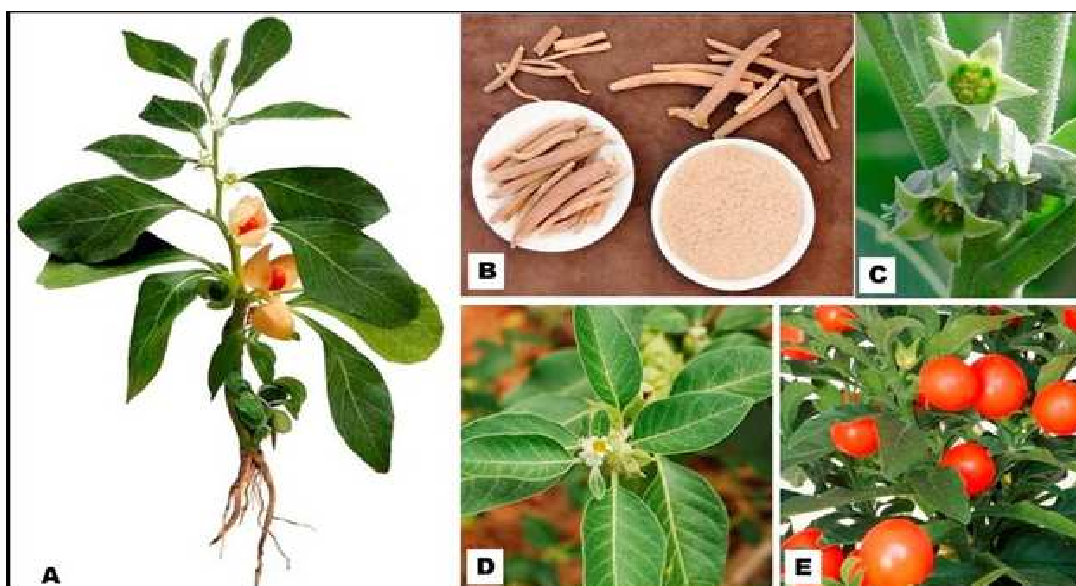


Figure 2.1 WS plant and its specific organs: (A) The main plant (B) roots and their ground root powder (C) the flowers (D) the leaves and (E) and the fruits [46]

2.5.2 Bioactive compounds present in WS

Withania's biochemistry has been thoroughly investigated, and several distinct metabolites have been identified and their biological activities described (Table 2.1).

Whereas withanolides (steroidal lactones that are highly oxygenated C-28 phytochemicals) are also isolated from other families, the *Withania* genus is a significant source of steroidal lactones in nature (Figure 2.2). Taccaceae, Solanaceae, and Fabaceae are among these families [47]. From various portions of WS steroidal lactones and phenolic acids are two important chemical families that have been discovered [48].

Withanolide A, Withaferin A, (Figure 2.3 & Figure 2.4) isopelletierine, anferine, sitoindosides VII and VIII, sitoindoside XI and X, withanoside V, withanolide B, and withanoside IV are the most physiologically active botanical constituents of several species of the *Withania* genus [49]. Vanillic acid, p-coumaric acid, syringic acid, benzoic acid, gallic acid, physagulin, and trigonelline are only found in the leaves, whereas palmitic acid, oleic acid, linoleic acid, linolenic acid, withanone, 5,6-epoxy withaferin-A, 27-deoxywithanone, 27-hydroxywithanone, withanolide withanolide B, withanoside IV, withanoside VI, (+)-catechin, and 12-deoxywithastromonolide are isolated from both leaves and roots [48], (Table 2.2).

Table 2.2 Active metabolites present in WS. and their pharmacological activities

Plant part/organ	Bioactive compounds present in WS	Pharmacological activity	Reference
Roots	Withanolide A, Withaferin A, Withanolide sulfoxide, Withanoside IV & VI, Withacoagin, dihydrowithanolide D, ixocarpalactone A, glucopyranosyl moieties, glycosides, Withasomidienone, 5,7 α-Epoxy-6α, 20α-dihydroxy-1-oxowitha-2, 24-dienolide, ß-sitosterol, stigmasterol, ß-sitosterol glucoside, stigmasterol glucoside, Viscosa lactone B, 16ß-acetoxy-17(20)-ene, 6a-hydroxy-5,7a-epoxy, 27 Hydroxy withanone, Hydroxy, 17-deoxy withaferin A, Deoxy withastromonolide, Physagulin, Benzyl alcohol, 2-Phenyl ethanol, benzoic acid, p-Hydroxy, phenyl acetic acid, Asparagine, Choline, Palmitic acid, Oleic acid, Linoleic acid, Porphyrine,	Anti-inflammatory, memory enhancement, cerebellar ataxia, anti-peroxidative, cardiotonic and antioxidative abilities. Fertility enhancement	[50–52], [53], [54], [55], [56], [57], [58] [59], [47], [60] [38,61,62] [48], [49].
Leaves	Withanolides, Withaferin, Polyphenols, saponins, alkaloids, steroids, steroidal lactones, flavonoids, glycosides, 27 Hydroxy withanone, Hydroxy, 17-deoxy withaferin A, Deoxy	Anti-stress, antianxiety, anti-carcinogenic activity, antimicrobial, antioxidative	[50–52], [53], [54], [55],

	withastromonolide, Physagulin, ß-sitosterol, ß-sitosterol glucoside, 2-Hydroxy propanol, 2-Hydroxy propanoic acid, 1-Octanol, Benzoic acid, Butandioic acid, Phenyl acetic acid, p-Hydroxy, phenyl ethano, p-Hydroxy benzoic acid, Alanine, Aspartate, Asparagine, Choline, Palmitic acid, Oleic acid, Linoleic acid, Porphyrine, Pheophytin, Sterol, TAG, Vanillic acid, p-coumaric acid, syringic acid, gallic acid, physagulin, and trigonelline	ability, Anti-helmantic and anti-inflammatory potency	[56] [57], [58] [59], [47], [48], [49], [60] [38,61,62]
Fruits and Fowers	Chamase, condensed tannins, peroxidases, proteolytic enzyme, cystine, favonoids, glutamic acid, amino acids, aspartic acid, alanine glycine, hydroxyproline, isopsoralen, Psoralen, proline, tyrosine, and valine.	Antimicrobial activity, management of respiratory illness	[38,61,62]
Stems	Alkaloids and its derivatives (Ashwagandhine, Isopelletierine, Pseudotropine, [3]-Tigloyloxtropine, Tropeltigloate, Dlisopelletierine, Hygrine, Mesoanaferine, Choline, Somniferine, Withanine, Withananine, Hentriacontane, Visamine, Withasomnine, Somniferinine, Somninine, Nicotine, Cuscohygrine, Pseudotropin, Anahygrine, Anaferine, Tropine), Glycosides and its derivatives (Withanosides I–VII, Withanamide), Flavonoids (Quercetin, 7-hydroxyfavone Kaempferol), and Phenolics and their derivatives (Coumaric acid, caffeic acid, chlorogenic acid, gallic acid, ferulic acid, and catechin)	Used to treat tumors, nocturnal leg cramps, coronary heart diseases, diarrhea, and psychiatric palpitation, improves blood cholesterol levels, antimicrobial, relaxant, anti-spasmodic, Sedative, muscle relaxant, diuretic, strengthen capillary walls, osteoporosis.	[38,61,62], [63], [62–64]

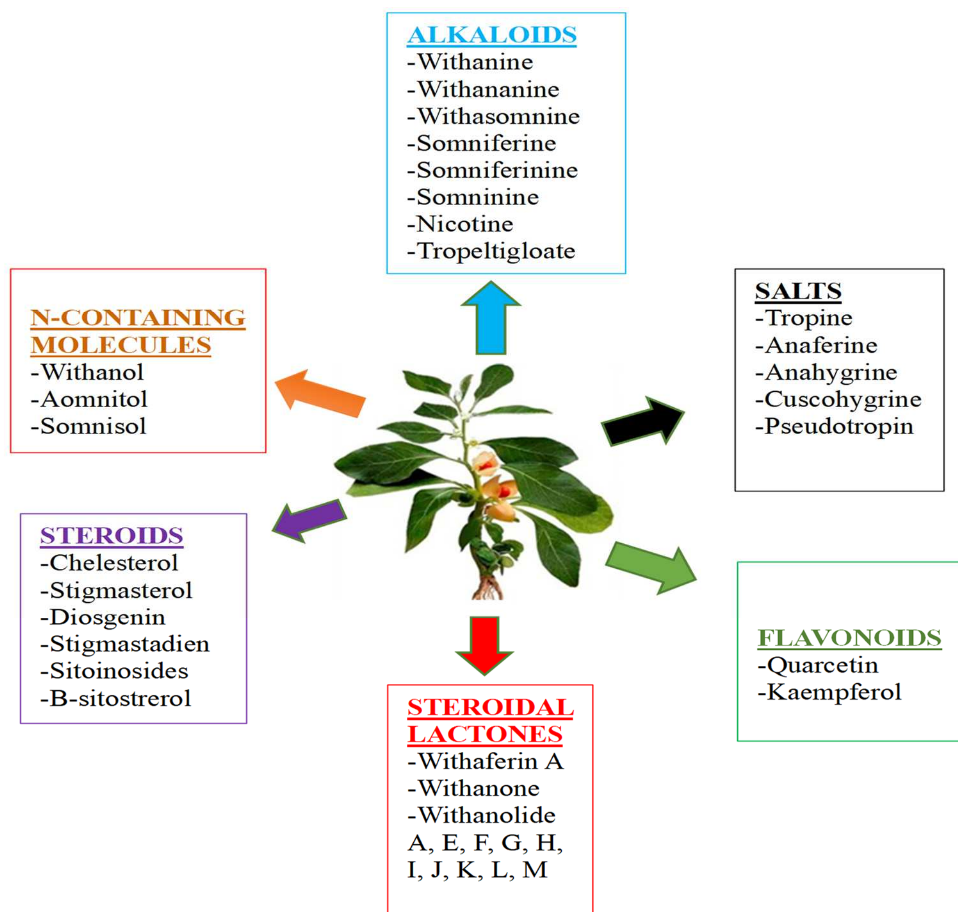


Figure 2.2. Classification of the various metabolites present in WS

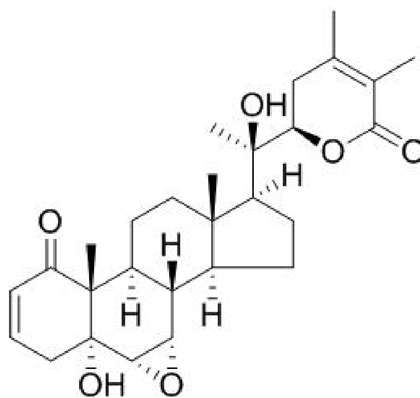


Figure 2.3. The chemical structure of Withanolide A

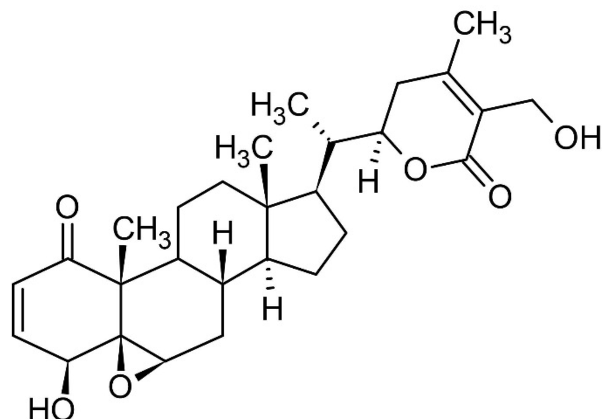


Figure 2.4. The chemical structure of Withaferin A

2.5.3 Mechanisms of carcinogenesis and the inhibitive potential role of WS in CRC growth and proliferation

Withaferin A, withanone, and the alcoholic extract of WS leaves have all been shown to be cytotoxic to cancer cells and to have properties that may make them useful as anticancer medications, including radio-sensitization, immunomodulation, anti-inflammation, antimetastatic, and antiangiogenic effects [65]. Both radiation therapy and chemotherapy, have substantial adverse effects such as nausea, vomiting, alopecia, pulmonary fibrosis, mucosal ulcers, hepatic and cardiovascular toxicity, etc. Cancer treatment may benefit significantly from medications that can eliminate these adverse reactions [66].

Through gene silencing and pathway analysis, Widolo et al. [67] reported that WS and its constituent parts kill cancer cells via at least five separate routes, including *p53* signaling, GM-CFS signaling, death receptor signaling, apoptosis signaling, and the G2-M DNA damage regulation pathway. The most typical signaling was *p53*. It has been reported that tumor necrosis factor-alpha (TNF-α), interleukin (IL)-10, and glutathione (GSH) levels are all lowered by aqueous root extracts of WS (0.05–0.4

mg/ml), which also regulate peripheral blood mononuclear cell (PBMC) and leukemic THP-1 cell viability [66].

Withaferin A suppressed IL-6, COX-2, TNF-α and pro-survival biomarkers (Notch1, NF-B, and p-Akt) in APC Min/+ and dextran sodium sulfate/axoxymethane models and prevented CRC in both models. The treatment of withaferin A reduced cancer proliferation in a transgenic adenomatous polyposis coli (APCMin/+) mice model [68].

In colorectal cancer cell lines SW-480, SW-620, and HCT-116, withaferin A suppressed Notch-1 and inhibited the Akt/NF-B/Bcl-2 pathway. In colon cancer cells, withaferin A inhibited rapamycin signaling factors p4E BP1 and pS6K and stimulated c-Jun-NH2-kinase-mediated apoptosis [69]. It has been reported that Withaferin A triggered G2/M arrest in HCT116 and SW480 cancer cell lines [70]. Withaferin A also caused Mad2 and Cdc20 deterioration, in addition to miotic delay by disrupting the spindle assembly checkpoint mechanism. In a xenograft mice carcinoma model, Choi and Kim, [71] reported that withaferin A suppressed the development of HCT116 human colon cancer cells. The outcome was mainly accomplished by inhibiting STAT3 transcriptional activity. The effect of WS extract on HT-29 colon cells was investigated. The investigators found that methanolic extract of WS root had an antiproliferative effect due to increased ROS generation and mitochondrial dysfunction [45].

Alnuqaydan et al. [72] investigated the promotion of antiproliferative effects by 5-fluorouracil (5-FU) and withaferin A. In colonic cells, the combined therapy suppressed the β-catenin pathway, which has been linked with cell cycle arrest during the G2M phase. According to the findings, the combination of withaferin A and 5-FU promotes cell viability via ER stress-mediated activation. A four-week therapy

of WS extract has been demonstrated to improve immunological dysfunction and CRC rehabilitation [73]. The immunomodulatory action was found to be beneficial for the management of CRC. Changes in leukocytes, neutrophils, lymphocytes, immune system complexes, and immunoglobulin A (IgA), IgG, and IgM antibodies induced the impact [73], (Table 2.3).

Table 2.3 Anti-colorectal cancer activities of WS's bioactive constituents and their associated mechanisms of action.

Biological mechanism	Bioactive constituents	References
Downregulation of COX-2 (↓COX-2)	27-desoxy-24, 25-dihydrowithaneferin A, 27-O-glucopyranosylviscosalactone B, 4,16-dihydroxy-5 h, 6h-epoxyphysagulin D, Diacetylwithaferin A, Physagulin D (1→ 6)-h-D-glucopyranosyl-(1→4)-h-D-glucopyranoside, Viscosalactone B, Withaferin A, Withanolide sulfoxide, Withanoside IV.	[68,74,75]
Downregulation of NF-κB and PI3K/Akt (↓NF-κB, and PI3K/Akt)	Withaferin A, Withanolide sulfoxide	[68], [69,70]
Downregulation of Bcl-2 (↓Bcl-2)	Withaferin A	[68], [69,70]
Upregulation of Apoptosis (↑Apoptosis)	Withaferin A, Withanolide D, withanone, 4β-Hydroxywithanolide E,	[68], [76], [77]
Upregulation of apoptotic caspse-3 gene (↑Caspase-3)		[68], [76]

2.5.4 Significance of targeted upregulated apoptotic activity in CRC management

Apoptosis is a mechanism that has undergone little change throughout evolution and is crucial for maintaining tissue homeostasis [78]. The process of apoptosis requires specially designed machinery. A proteolytic mechanism that uses a family of proteases known as caspases is the main part of this machinery [79]. The death-receptor-

mediated mechanism and the mitochondrial pathway have been identified as two separate but convergent pathways for caspase activation.

The mitochondrial caspase pathway's starter, caspase-9, is a crucial mediator in the control of apoptosis. These enzymes (caspase) are part of a cascade that is initiated by proapoptotic commands, which leads to the cleavage of an assortment of peptides and the disintegration of the cell. The comprehension of caspase programming is essential for strategically regulating apoptosis for therapeutic benefit [78,79]. The suppression of natural apoptosis has been hypothesized to enhance the incidence of cancer [80,81]. Similarly, it has been noted that a higher prevalence of colorectal adenoma is highly correlated with a lower rate of apoptosis [82]. The capacity to trigger apoptosis in epithelial cells of gastrointestinal origin is one of the potential strategies in chemoprevention [81]. As a result, investigating the apoptotic mechanism is a viable avenue for CRC. When determining the prognosis for individuals with stage II colorectal cancer, the degree of expression of the apoptosis-associated genes *CASP9* and *CASP10* could prove useful. It appears that the carcinogenesis of CRC involves both the death-receptor-mediated and mitochondrial pathways. The lifespan of aberrant mucosa cells is increased by the decreased expression of *CASP9* and *CASP10* [83,84]. As a result, these cells have the potential to undergo further gene mutations and eventually give rise to cancerous cells.

It has been suggested that the activity of withanolides is mediated via control of nuclear factor-kappa B (*NF-kB*) expression because *NF-kB* regulates numerous genes that affect cell division, malignancy, cancer metastases, and inflammation. This suggests that withanolides prevent *NF-kB* activation and *NF-kB*-regulated gene expression, which could help clarify why they can increase apoptosis while preventing invasion [85]. According to a study using the Leukemic Murine mouse model, withanolide D

lowers anti-apoptotic genes (TERT, Bcl-2, and Puma) [66]. By high levels of ROS, dysregulation of Bax/Bcl-2 expression, and concurrent disruption of mitochondrial membrane potential ($\Delta\Psi m$), a novel fraction of proteins isolated from WS roots could stimulate mitochondria-mediated apoptosis in triple-negative breast cancer cells (MDA-MB-231) at Half-maximal inhibitory concentration (IC_{50}) dose of 92 g/ml. Also reported [86] were Caspase-3 stimulation, G2/M cell cycle arrest, and nuclear lamin protein cleavage. Furthermore, the crude water extract (0.5%) of WS modified the signaling cascade including pro-apoptotic and tumor-promoting proteins, which helped to inhibit tumor growth [66].

The association between caspase-9 and colorectal cancer is still poorly understood to date. Studying its association with clinicopathological characteristics and longevity may provide insightful data for predicting survival and choosing additional treatment strategies. The WS ethanolic extract is cytotoxic (99.7 g/ml) on cancerous cells, induces apoptosis, and inhibits angiogenesis and cell migration [87]. The possible anticancer action of the extracts of WS is due to the increased autophagy induction and apoptotic effects of the plant. Withaferin A causes apoptosis and Mad2 and Cdc20, a crucial component of the Spindle Checkpoint Complex, are degraded by proteasomes. By restoring correct anaphase initiation and maintaining a greater number of viable cells, further overexpression of Mad2 partially reverses the harmful effect of Withaferin A. It is hypothesized that Withaferin A kills cancer cells by delaying the mitotic exit and then causing chromosome instability [70]. Although various WS compositions have highly promising anti-cancer properties in both in vitro and in vivo applications there are currently no authorized therapeutic candidates. For the discovery of novel anti-cancer pharmaceuticals, conducting clinical trials with WS phytochemicals/formulations is urgently necessary.

2.5.5 Anti-angiogenic and anti-migratory potential of WS

Angiogenesis is essential for the survival, development, and metastasis of cancer cells. Sajida [87] demonstrated that the genes and proteins involved in tumor angiogenesis, such as VEGF, angiogenin, and MMP 2, were downregulated, inhibiting angiogenesis. Some ingredients derived from medicinal plants have demonstrated strong anti-angiogenic activity. Conferone caused oxidative stress and cell suicide by inhibiting angiogenesis in HT-29 CRC cells through an altered secretome that included vascular endothelial growth factor, Angiopoietin-1, and 2 factors [88].

Additionally, Withaferin A demonstrated antiangiogenic efficacy in the mouse model with reduced p-ERK and p-Akt levels. By reducing the expression of MMP in HeLa and PC3 cells, this molecule also prevented the invasion of cancer cells [89]. At lower concentrations, WS extract has been implicated with significantly reducing cell migration, and thus demonstrating its anti-migratory ability [87]. It has thus been reported to have anti-metastatic potential since it significantly reduces MMP expression. Another investigation utilizing bioinformatics and biochemical strategies revealed that phytochemicals present in WS decreased the levels of the migration-promoting proteins hnRNP-K, VEGF, and MMP, making them potential alternative therapies for the management of metastatic cancer [65].

It has further been elucidated that Withanone and withaferin A in combination, had powerful antimigratory and antiangiogenic effects *in vitro* and were specifically lethal to cancer cells. These actions were corroborated by molecular studies of marker proteins, such as MMPs, which are essential for the invasion and metastasis of cancer [65,67,90]. Thus, it may be drawn that; the antimetastatic activity is achieved by targeting multifunctional RNA-binding protein, hnRNP-K, and withanone-rich

combination of withanone and withaferin A restricts cancer cell migration, and angiogenesis *in vitro* and *in vivo.*

2.6.1 The biodiversity of *Aloe* plant species

Aloe species are abundant and diverse in African landscapes, which frequently translates to their regular use as a source of phytotherapeutic medicine to support human health and wellbeing. They have also occasionally been mentioned as having utility in ethnoveterinary medicine by African ethnic groups [91]. *Aloe* L. (Asphodeloideae) is a blooming succulent genus with about 500 species, including trees, shrubs, and perennials. The genera is primarily native to Africa, with species also found in the Arabian Peninsula and Jordan, as well as on various islands off the African coast, including Madagascar [92].

While species overlap exists throughout regions, Southern Africa (Angola, Botswana, Lesotho, Malawai, Mozambique, Namibia South Africa, Swaziland, Zambia, and Zimbabwe) has the greatest biodiversity of *Aloe* species, with roughly 290 species reported [93]. East Africa (Burundi, Djibouti, Eritrea, Ethiopia, Kenya, Rwanda, Somalia, South Sudan, Tanzania, and Uganda) has a remarkable *Aloe* biodiversity, with around 200 species, while Madagascar and the Indian Ocean Islands have about 90. The Arabian Peninsula is home to roughly 50 species, and central and western African regions are home to nearly 30 species [92,93]. The perennial, drought-resistant, and succulent nature of the plant genus *Aloes* allows it to colonize dry areas. It can endure prolonged desiccation because its thick leaves can store a significant amount of water [94].

2.6.1.1 Ethnomedicinal utility of the plant genus *Aloe*

Since 1500 BCE, *Aloe* species have been crucial to history's medical and economic development, and the gel found inside their leaves has been utilized to treat both human and animal ailments [95]. Herbalists from a wide range of African cultural groups have used plants in the genus *Aloe* for a wide range of medicinal applications. Since ancient times, traditional medicine has made extensive use of the species *Aloe vera* (L.) Burm. f. (Asphodelaceae) [96]. Its mucilaginous fluid is administered to wounds and cuts in rural areas of the country to suppress infections and promote healing [97]. *Aloe* plant spp. has been reported to exhibit anti-inflammatory, immunostimulatory, and cellular growth-stimulating properties [98]. Further, their extracts have been reported to have antibacterial, antiviral, antifungal effects, antiprotozoal, purgative, cell proliferation, anticancer properties, and toxicological activities for utility in pharmacology [98–101]. The methanol extract of *A. vera* gel contains an assortment of active ingredients with antimicrobial activities that can be employed as antimicrobial agents in new therapeutic preparations for the treatment of infectious illnesses in humans [99].

One of the most often employed techniques in both traditional and modern medicine is skin healing and tissue regeneration. *In vitro* research, followed by experimental and clinical trials, verified empirical data [101]. According to Liang et al., adding gel to wound dressing would be an easy and consistent technique to use *A. vera.* Although inflammation is a normal part of healing, *Aloe's* anti-inflammatory properties appear to promote tissue recovery [102]. Although aloe leaf gel has been used for many years, little is known about the pharmacological effects of its constituent parts [96]. It has been stated that the leaf gel of many *Aloe* species exhibited anti-inflammatory qualities, and research suggests that this property may be specifically related to TLR4-

mediated reactions. It has also been shown that *Aloe* species at a gel concentration of 0.2% significantly reduced the NF-B activity with LPS but had no discernible effect on FSL-1. Moreover, it appeared that *Aloe* gel extracts were more effective at inhibiting LPS's ability to activate the TLR4 pathway [96]. A recent assessment of *Aloe* and skin protection activities found that the constituents have not been sufficiently investigated to draw definite conclusions about their effects [103] and this warrants more investigative research studies for clarity, understanding, and validation.

2.6.1.2 The biodiversity and botanical description of AS

The Asphodelaceae family's largest genus, *Aloe*, contains more than 400 species that range in size from small shrubs to large trees and are found throughout dry regions of Africa, India, and other regions [97]. The diversity of *Aloe* is greatest in South Africa. AS (Asphodelaceae) shrubs are common in open grasslands and bushlands in countries in Africa like Ethiopia, Sudan, Kenya, and Tanzania. Typically, they have an acaulescent rosette of glaucous green leaves that expand and have a glossy sheen. The tips of the leaves are usually somewhat recurved (Figure 2.5). On their leaves, especially the undersides, young plants frequently have dots. Up to 20 spreading branches, each with a cylinder raceme of pink-red flowers, can be seen on the tall (1 m) erect inflorescence [93,97,104].

Figure. 2.5. This photo illustrates the occurrence of AS naturally growing in the wild without human interference in Kenya [105].

2.6.1.3 Bioactive compounds/substances present in AS

Aloe species have been used in ethnopharmacology, and studies on their phytochemical and pharmacological properties have produced several active ingredients. Herbalists have historically employed them to manage a variety of illnesses [97]. *Aloe* species are abundant natural sources of bioactive substances, with anthraquinones making up most of them [101]. Preliminary phytochemical analysis of AS has indicated the presence of terpenes, flavonoid, and tannin, (Table 2.4) in leaves [59] and naphthoquinones in roots (Figure 2.6) [106]. Anthracenedione (9,10-anthracenedione) is the core of anthraquinones (Figure 2.7), and anthrones found in the leaves of *Aloe* (Figure 2.8), which are structurally related to anthracene [107]. Sometimes, they are referred as 9,10-dioxoanthracene. Often, anthraquinones are found in their glycosidic forms. These components give plants their pigment and are frequently used as natural dyes [107]. Documented evidence demonstrates that vitamins A, B, B_2, B_6, B_{12} and E are present in aloe species, making it a potent anti-inflammatory and antioxidant [3]. Enzymatic amylase, lipase, and carboxypeptidase present in the plant's extracts help in the synthesis of fatty acids, sugars, and starch [100]. Gels have been reported to contain mineral ions, such as Mg^{2+}, Zn^{2+}, Ca^{2+}, K^+, Na^+, Fe^{2+}, P^-, Mn^{2+}, Cu^{2+} and Mo^{4+} [101].

Table 2.4 Bioactive compounds present in AS.

Plant species	Bioactive compounds (Roots)	Bioactive compounds in leaves	Ref
Aloe secundiflora	Anthraquinones (Chrysophanol, Helminthosporin, Aloe-emodin, Aloesaponarin II and Aloesaponarin I), laccaic acid D, methyl ester and asphodelin. Naphthoquinones (5-hydroxy-3,6-dimethoxy-2-methylnaphthalene-1,4-dione and 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione)	Phenols such as anthrones (aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives), chromones and phenylpyrones, Alkaloids, Saponin, Tannins, Flavonoids (nthoxanthins, flavanones, flavanols, flavans, and anthocyanidin), Steroids, Cardiac Glycosides, Aloeresin, Anthraquinones Aloin, Hydro-xyaloins, Polyphenols, Terpenoids	[59], [108], [109], [94], [59], [106], [107]

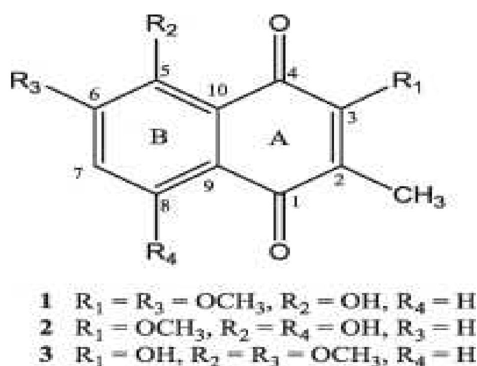


Figure 2.6. This figure shows the chemical structure of naphthoquinones common in the roots of A. secundiflora [106].

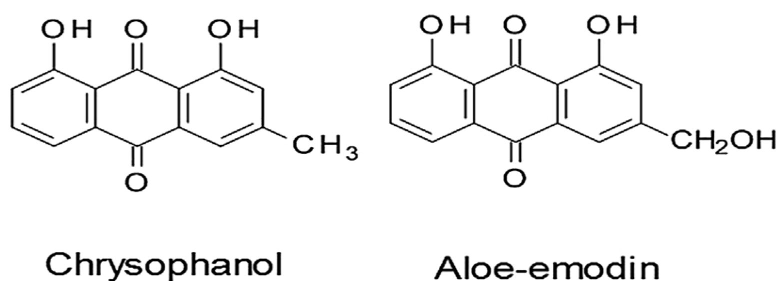


Figure 2.7 Chemical structures of Anthraquinones; Chrysophanol and aloe-emodin common in the roots of AS [107]

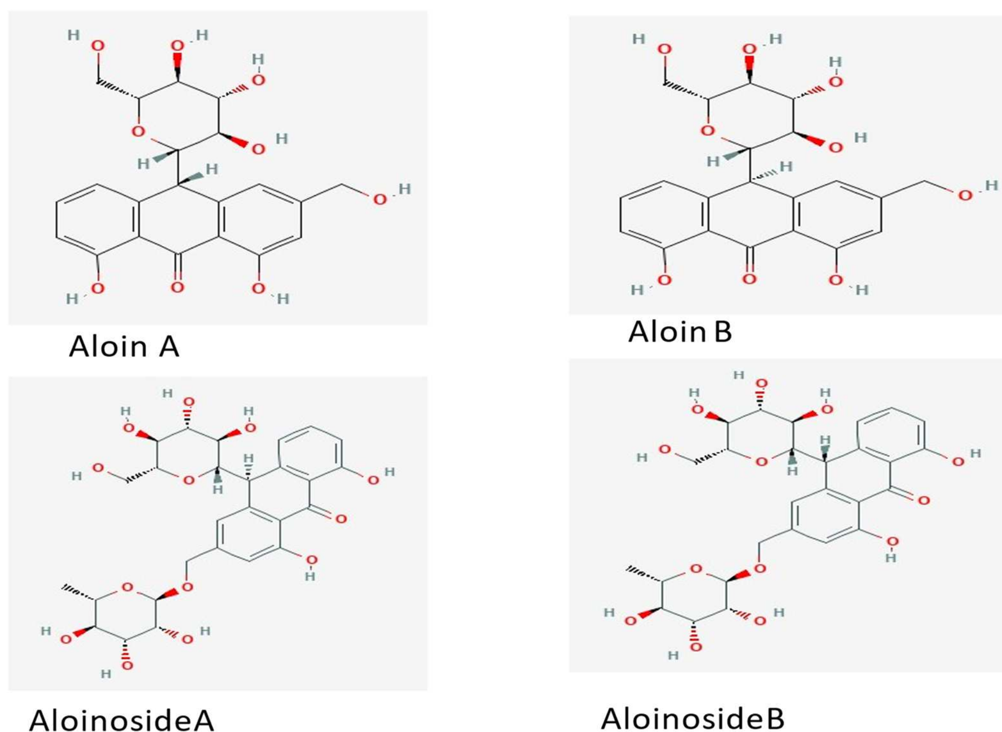


Figure 2.8 Chemical structures of aloin derivatives (anthrones) commonly found in the leaves of Aloe [110,111]

2.6.1.4 Mechanisms of carcinogenesis and the inhibitive potential role of AS in CRC growth and proliferation

Aloe sp. is beneficial in treating a variety of malignancies that affect multiple organs, including the colon, according to a review by Singab et al. [112]. For the treatment of gastrointestinal malignancies, *Aloe* is seen as a possible medication [101]. Aloe-inhibitory emodin's effect on the proliferation and migration of gastric tumor cell lines is dose-dependent. Aloin ($C_{21}H_{22}O_9$), a yellow chemical, is a combination of the diastereoisomers aloin A and aloin B. Aloin (AL), an anthrone C-glucoside with a molecular weight of 418, is the primary phytoconstituent of aloes. Aloin is used in pharmacology for many different aspects, among them as a laxative [113].

AL has been reported with effective activity in reducing tumor angiogenesis and growth, by preventing STAT3 activation in CRC cells in *in vivo* and *in vitro* experimental treatments [114]. In the experiment, the authors reported that Western blotting revealed AL prevented endothelial cells from phosphorylating STAT3 and

activating the EGF receptor (VEGFR) 2. Moreover, the activation of STAT3-regulated antiapoptotic (Bcl-xL), cell proliferation (c-Myc), and angiogenic (VEGF) proteins as well as the constitutively activated STAT3 protein was all down-regulated in response to AL in human SW620 cancer cells. In line with the findings, AL significantly decreased tumor sizes and weight in mice xenografts while inhibiting tumor cell viability and inducing cell apoptosis *in vitro*. This was done without plainly causing any harm [114]. The human gastric cancer MKN45 cell line showed anticancer activity for aloe-emodin and emodin [115].

Chrysophanol, sometimes referred to as 1,8-dihydroxy-3-methyl-anthraquinone and chrysophanic acid is a naturally occurring anthraquinone in *Aloe* plants [116]. Its toxicity aggressive human cancer cell lines were studied by Yao *et al.,* (HepG2, HCT-8, A549, SGC7901 and MDAMB-231), who demonstrated that there was no detectable cytotoxicity to the cells [117]. Chrysophanol did, however, exhibit a time-dependent suppression of HepG2 cell viability, with the highest inhibition at 10 μm and a small waning of the potency of the inhibition at concentrations between 20 and 60 μm. Chrysophanol exposure to HL-7702 cells, however, did not appear to cause any cytotoxicity at concentrations between 0 and 100 μm. In another research, Pandith et al. discovered that chrysophanol at doses of 10, 50, and 100 g/ml inhibited the proliferation of cancer cells [118]. Chrysophanol had little impact on the CRC cell line and breast cancer cell line (MCF-7, T47D), according to other authors (HCT-116) [119]. The treatment's dosage could unquestionably determine the recorded minimal effect. Chrysophanol caused considerable increases in the enzymes extracellular signal-regulated kinase (ERK1/2), p90 ribosomal protein S6 kinase (P90RSK), and protein kinase B (AKT), as well as ROS creation and mitochondrial malfunction [116].

The pro-angiogenic factor vascular endothelial growth factor (VEGF) is important for the development of tumor vascularity [120]. The main VEGF receptor and the key player in VEGF-induced angiogenesis pathways is vascular endothelial growth factor receptor 2 (VEGFR2) [121]. When dormant endothelial cells are stimulated, VEGFR2 signaling causes several downstream mediators to become active, which then promotes cell proliferation, migration, invasion, and differentiation into capillary-like structures [122]. Recent research has shown that VEGFR2-mediated signaling, particularly signal transducer, is frequently linked to a worse prognosis and is highly implicated in a wide range of human malignancies and *STAT3*, which is a transcription activator [123,124].

One of the latent self-signalling transcription factors in the cytoplasm that is triggered by specific cytokines (such as IL-6) and progenitor cells is *STAT3* (e.g., VEGF). The transcription of responsive genes encoding apoptotic cell death inhibitors (e.g., *Bcl-xL*, *Bcl-2*) and inducers of angiogenesis (e.g., VEGF) is modulated by the activation of *STAT3* homodimerization and nuclear translocation [125]. These genes are involved in cell proliferation, survival, differentiation, programmed cell death, metastatic spread, angiogenesis, human defense evasion, and drug resistance [126]. More recently, the scientific literature has been filled with evidence showing that inhibiting constitutive S*TAT3* signaling effectively prevents tumor growth and induces apoptosis [125,127]. Directly targeting the *STAT3* signaling cascade has been an appealing therapeutic target for pharmacological intervention due to the carcinogenic function of STAT3 and the promise of its inhibition [114]. Recently, plant-based substances with minimal adverse effects were found to block angiogenesis and target *STAT3* [127].

Even in high amounts, AL may be one effective, inexpensive, and safe oral medication used in clinical practice for the treatment and prevention of cancer [114]. Future

research will clarify the therapeutic potential of AL in treating human CRC through both experimental and clinical trials, such as those examining its interaction with conventional chemotherapeutics. In addition, research published *in vitro* and *in vivo* has demonstrated that chrysophanol can control the expression of several genes and proteins, including GRP78, p-eIF2a, CHOP, caspase-12, Drp1, PTP1B, PAI-1, Bcl-2/Bax, and caspase-3. Chrysophanol also influences the signaling pathways for NF-jB, MAPK, PI3K/AKT, and PPARc [116].

The primary flavan skeleton, which is a 15-carbon phenylpropanoid chain (C6-C3-C6 system) that comprises two aromatic rings (A and B) connected by a heterocyclic pyran ring (C), is shared by all flavonoids (Figure 2.9) [128]. Flavonoids have been found to have a wide range of anticancer actions, including modulating the activity of enzymes that scavenge reactive oxygen species (ROS), participating in cell cycle arrest, inducing apoptosis and autophagy, and reducing the proliferation and invasiveness of cancer cells [128]. In terms of maintaining the balance of reactive oxygen species (ROS), flavonoids operate as antioxidants under normal circumstances and as powerful pro-oxidants in cancer cells, where they activate apoptotic pathways and suppress pro-inflammatory signaling pathways. There is growing evidence that several flavonoids have anticancer properties, although the underlying molecular pathways are still not fully understood [128–132].

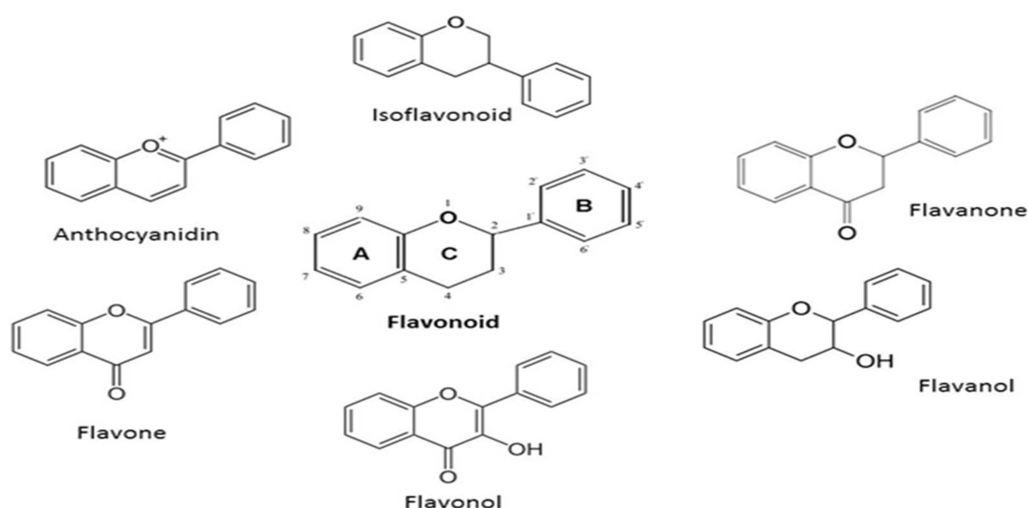


Figure 2.9 Different derivatives of flavonoids commonly found in *Aloe* and have been reported to elicit significant pharmacotherapeutic effects [128].

Due to the presence of phenolic hydroxyl groups, flavonoids can stabilize free radicals and can directly scavenge ROS and chelate metal ions [133]. The activation of antioxidant enzymes, inhibition of pro-oxidant enzymes, and stimulation of the synthesis of antioxidant enzymes and phase II detoxification enzymes are all examples of indirect flavonoid antioxidant actions [133,134]. Flavonoid anticancer actions entail both antioxidant and pro-oxidant activity [134]. Owing to their antioxidant qualities, flavanols have been shown to protect against colon cancer [135]. Quercetin, a flavonoid, has strong chemopreventive effects on cancer [136,137]. By generating ROS, flavonol kaempferol has been reported to activate caspases and induce apoptosis in CRC [138].

It is crucial to understand that AS has similar pharmacotherapeutic characteristics just like other species of the same genus, *Aloe.* They can thus be utilized for potential intervention and management of CRC in predetermined concentrations based on the extensive documented evidence on the pharmacological activity of the plant genus *Aloe*. We advise additional research to establish the proper concentrations and levels

required to evoke positive effects in CRC management in humans, owing to the lack of proven amounts and specified concentration measurements.

2.6.1.5 Antimicrobial activities of AS

2.6.1.5.1 Antibacterial effects of AS

In some developing nations, plants serve as the primary source of treatment for most diseases. Most pathogenic microorganisms have been tested pharmaceutically on about 20% of all plants in the world. As a result, new antibiotics have been introduced onto the market [139]. It has recently been documented that the extracts from AS were actively potent and inhibited the growth of *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Escherichia coli,* and *Erwinia carotovora* bacterial species [94]. Another experimental study has demonstrated that AS leaf extract suppressed growth (No growth values (GUs)) throughout all extract doses against four strains of *Mycobacterium* (*M. fortuitum, M. smegmatis, M. tuberculosis, and M. kansasii*). Interestingly, against *M. tuberculosis*, the extract demonstrated the greatest antimycobacterial action (157 GUs) [59]. In the same study, inhibition zones exceeding 9.00 mm were developed by the extract against the majority of other bacteria, including *P. aeruginosa, E. coli, S. aureus*, and *S. typhi* [59]. Aloe-emodin metabolite has adversely been mentioned as a principal antibacterial agent [140].

It has been published that, with MIC values of 21–23 g/mL, aloesaponarin I and 5-hydroxy-3,6-dimethoxy-2-methylnaphthalene-1,4-dione demonstrated antibacterial efficacy against *M. tuberculosis* [106]. Previously, crude extracts of AS were reported to have considerable antibacterial activity [140] therefore necessitating recent advances in research. The Krebs cycle can be inhibited by the anthraquinone in *Aloe* [141]. Because of this, the cell's ability to produce ATP and NADH is compromised,

which prevents the oxidation of glucose. As a result, the respiratory metabolism of bacteria is impacted, which causes bacterial death [141].

A fowl *in vivo* investigation produced outstanding results. The results of the trial indicated that there was a good chance that the crude AS extract may stimulate the production of IL-6 in a manner comparable to that of purified acemannan, in *A. vera*. The study found that the bacteremia decrease was likely aided by AS's antibacterial activity, and it is plausible that IL-6 together with other components provided immunity [140] and thus reduced poultry mortality and the severity of clinical signs [142]. According to another study, aloe polysaccharide, acemannan in aqueous and ethanolic extracts triggered both a humoral and cell-mediated immune response in poultry upon infection [143]. AS has recently been used routinely in unconventional free-range management approaches to treat poultry typhoid with positive results being reported [142]. There is therefore demonstrated evidence that AS is a competitive antibacterial agent that can be positively exploited pharmaceutically for utility. However, more research is necessary to determine the *in vivo* efficacy of the crude extract, the specific active phytoconstituents, safety levels without inducing toxicity effects (which from studied kidney and liver organs analysis have been considered without considerable negative effects), and the dosage of AS before recommending it for clinical use given the paucity of its studies in humans subjects.

2.6.1.5.2 Antifungal effects of AS

Candida albicans (*C. albicans*) has recently developed a greater resistance to conventionally developed antimicrobials, prompting the quest for a novel antifungal drug [144]. AS has further been documented to positively inhibit the growth of fungal species such as *C. albicans,* and *Fusarium oxysporum* [105] in *in vitro* research studies. According to the published research, AS's crude extract substantially slows down *C.*

albicans growth [105] thus strengthening its potential application in the management of fungal infections. Its crude extract on solid media has demonstrated complete inhibition of *C. albicans.* These findings have further been supported by Rachuonyo et al., who demonstrated that *C. albicans* and other fungi-causing pathogens could be treated with extracts from AS [145]. Mutharia et al., observed that *Penicillium marneffei*, which is a fungus known to infect immunosuppressed hosts, non-AIDS individuals with hematological malignancies, and those on immunosuppressive therapy, is inhibited in its growth when it is exposed to extracts of AS [146]. According to their findings, the extract can be used to prevent superficial mycosis, which is a result of a fungus infection, and aflatoxins, which are caused by *Aspergillus* species, among other mycoses [146].

2.7.1 Botanical description of WU

2.7.1.1 Botanical description and the biodiversity of WU

WU (*W. ugandensis*) belongs to the genus *Warbugia* of the cinnamon family, Canellaceae. There are numerous medicinal plants belonging to the genus *Warburgia* that only occur in the African continent [147]. These evergreen trees, which are mostly found in east and southern Africa, can reach heights of 10 to 27 meters. Its infra-generic classification is still up for debate and thus not as conclusive [147,148]. At present, the genus comprises of four taxa: *Warburgia elongata* Verdc., *Warburgia alutaris* (G.Bertol.) Chiov., *Warburgia tuhlmannii* Engl., and WU. WU consists of two sub species: WU Sprague subsp. ugandensis and WU subsp. longifolia Verdc [148].

WU plant species is referred to by several names, including East African greenheart, Ugandan greenheart, and Kenyan greenheart. It is typically found in the lowland rainforests and upland dry evergreen forests of Eastern and Central Africa, though it

can also be found in secondary bushlands, grasslands, and termitaria in swamp forests [149,150].

2.7.1.2 Traditional medicinal application of some *Warbugia* plant species and WU

Several taxa have a long history of traditional medicinal use, including as an expectorant for dry coughs and a natural antibiotic for treating toothaches, rheumatism, malaria, sinusitis, gastro-intestinal problems, candidiasis, and other gastrointestinal and upper respiratory tract infections [148,151]. The leaves, roots, and stalks are occasionally employed for various herbal preparations, although the powdered bark is most frequently used [152]. The most common way to consume *Warburgia* bark is as a decoction or infusion, however other methods, like smoke inhalation or snuff, have also been observed. Most treatments are monotherapies. However, polyherbal treatments have also been recorded [151]. For instance, the leaves and stalks of *Hibiscus urattensis* L. are occasionally combined with *W. alutaris* and applied topically to treat genital ulcers and inflammation [148,151]. As an additional anti-infective ingredient, the bark is combined with the leaves of *Artemisia afra* Jacq. ex Willd. and the roots of *Acorus calamus* L. A snuff made from the mixed bark powder of *W. alutaris* and *Erythrophleum lasianthum* Corbishley is used to cure headaches [147].

WU has a long history of use in communities where it grows naturally to treat a variety of illnesses and treat various health conditions, including measles, malaria, diarrhea, cough [153], sexually transmitted diseases, toothaches [154], snake bites, common colds, bronchial infections, stomach-aches, fever, oral thrush, muscle pain, internal wounds/ulcers, and urinary tract infections [150,151,155]. Ethanolic extract from freeze-dried leaves has demonstrated antifungal efficacy against *Candida albicans* and

Cryptococcus neoformans as well as antibacterial activity against standard strains of *Staphylococcus aureus*, *Escherichia coli, Vibrio cholerae,* and *Bacillus cereus*. Fresh leaves of WU have been demonstrated to have anticandidal action, supporting their use as a traditional herbal therapy to supplement the treatment of fungal infections in HIV/AIDS patients [156,157]. The abundance of terpenoids, particularly sesquiterpenoids of the drimane and coloratane types, as well as fatty acid derivatives in the leaf and bark tissues of WU are principally responsible for the plant's medical efficiency [158]. Other significant secondary metabolites discovered in WU, primarily in the leaves, include steroids, saponins, tannins, and alkaloids. Most herbalists in Africa employ the plant's bark, even though WU*'s* roots and leaves are also useful for therapy [150].

2.7.1.3 Biologically active phytoconstituents present in WU

Plant materials of WU have for decades been utilized in traditional African herbal medicine because of the broad-spectrum nature of their biological activity [157]. Numerous unique metabolites have been identified and their biological activities explained because of extensive research into the biochemistry of WU. Metabolites already characterized in the plant include terpenoids, drimane and coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, flavonoids, and tannins among others (Table 2.5). Since plants naturally produce a variety of secondary metabolites, choosing WU as a source for innovative therapies is an appropriate method of CRC intervention [159].

Table 2.5 Biologically active compounds present in WU.

Plant part/organ	Bioactive compounds	Ref
Roots	Terpenoids, drimane and coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, tannins, flavonoids, saponins, steroids, mannitol, polygodial, muzigadial, 9-eoxymuzigadial, Pereniporin, Cinnamolide, cinnamolide-3-acetate, deacetylugandensolide, linoleic acid, 6ß-acetyl-cinnamolide, dendocarbins A, 8-coloratadien-11,12-olide, 7b-hydroxy-4, 9α-11α-dihydroxy, 9α-hydroxycinnamolide, 7α-Acetylugandensolide, Drimendiol,12-Hydroxy-epi-albrassitriol, Pereniporin A,	[149], [157], [150], [160], [58] [59] [147]
Leaves	Terpenoids, drimane and coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, tannins, flavonoids, saponins, steroids, mannitol, polygodial, muzigadial, 9-eoxymuzigadial, Pereniporin, Cinnamolide, cinnamolide-3-acetate, deacetylugandensolide, linoleic acid, 6ß-acetyl-cinnamolide, dendocarbins A, 8-coloratadien-11,12-olide, 7b-hydroxy-4, 9α-11α-dihydroxy, 9α-hydroxycinnamolide, 7α-Acetylugandensolide,	[149], [157], [150], [160], [58], [59]
Stems	Terpenoids, drimane and coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, tannins, flavonoids, saponins, steroids, mannitol, polygodial, muzigadial, neolignanamides, 7-hydroxywinterin, Epipolygodial, 3-Hydroxymuzigadial, 9-Deoxymuzigadial, 6α-9α-Dihidroxy-4(13),7-Coloratadien-11,12-dial, 6α-9α-Dihidroxy-4(13),7-Coloratadien-11,12-dial, Cinnamodial, Salutarisolide or Muzigadiolide, 3,9,11-Trihydroxymuzigadiolide, 11α-Hydroxymuzigadiolide, Dendocarbin L, Dendocarbin M, Bemadienolide, 11α-Hydroxycinnamosmolide, Pereniporin B, 9α-Hydroxycinnamolide, Dendocarbin A, 6α,9α,11α-trihydroxycinnamolide, 6β, 9α,11α-Trihydroxycinnamolide, Linoleic acid, 7α-Acetylugandensolide, 7α-Hydroxy-8-drimen-11,12-olide, Deacetylugandensolide, 6β,12-Dihydroxy-7β-dehydroxy-futronolide, 9α,11α-Dihydroxy-11,12-epoxy-3β,4α,10β-trimethyl-drim-4(5),7(8)-diene-6,12-dione, Drimenin.	[161], [149], [157], [150], [160], [147]

2.7.1.4 Human safety application of WU

Plant extracts from WU have also been linked to cytotoxic [156] and antioxidant [12] effects. Intriguingly, WU extracts have been found to have an oral LD50 > 5000 mg/kg body weight in mice, which is higher than that of other evergreen native tree species. No mortality has been reported at dose levels between 500 and 5000 mg/kg body weight, suggesting safety for usage in humans [162]. Despite the lengthy history of

therapeutic uses of WU, its medicinal benefit has garnered less attention. Ethnopharmacological studies should be encouraged to investigate the most efficient way of plant selection, extraction, chemical synthesis, and dose calculation to identify and reap major benefits from this plant species [22].

2.8 The significance of plant diet and lifestyle on human gut microbiome

The modulation of the composition and metabolic activity of the human GI tract microbiota, which influences health, is becoming widely understood to be a function of dietary (specifically macronutrients) and other environmental factors [163]. In comparison to the number of somatic cells in the body, there are roughly 100 trillion more bacteria in the human gastrointestinal (GI) tract. The gut can also contain yeasts, single-cell eukaryotes, viruses, and tiny parasitic worms in addition to the majority of the microorganisms, which are bacteria [164]. The greatest strategy to maintain a healthy gut microbiota population may be through dietary measures, notably the usage of a variety of fiber.

Approaches like consuming probiotics and prebiotics can help to maintain microbial balance and subsequently improve human health [163]. Even though many dietary polyphenols may have biological effects via antioxidant or anti-inflammatory pathways, polyphenols that infiltrate the colon can be degraded by the intestinal bacteria and generate bioactive products [165,166]. The colonic microbiota's fermentation of fiber and the metabolites that are created as a result are responsible for many of the health benefits associated with it. Organic acids produced during the fermentation of carbohydrates give other bacteria, the gut epithelium, and auxiliary tissues energy [163,167]. The primary by-products of carbohydrate fermentation are short-chain fatty acids (SCFA). These weak acids (pKa 4.8) help decrease the *pH* of the colon, which prevents the growth of pathogenic bacteria [167].

CHAPTER THREE

3. MATERIAL AND METHODS

3.1 Study area

The plant species were collected from two different Counties in Kenya (Africa), which included Nakuru and Baringo Counties. Nakuru County is the 32nd largest County out of the 47 Counties in Kenya. The County serves as a home for Kenya's fourth-largest city, Nakuru. Nakuru received a City Charter status from the President of the Republic of Kenya on December 1, 2021, placing it alongside Nairobi, Mombasa, and Kisumu as one of Kenya's major cities. It is Kenya's third most populated county, after Nairobi County and Kiambu County, according to the 2019 census, with a population of 2,162,202. It is the 19th largest county in terms of size in Kenya with a land area of 7,496.5 km2. It was a part of the Rift Valley Province until August 21, 2010 [168].

Baringo County is the 14th largest County in Kenya. Kabarnet is both its capital and largest town. Lake Baringo, Lake Bogoria, and Lake Kamnarok are in the county. The county's economy is largely agricultural. Maize, pigeon peas, beans, Irish potatoes, sweet potatoes, sorghum, cassava, and finger millet are the principal food crops farmed, while coffee, cotton, macadamia nuts, and pyrethrum are the main income crops. Products made from livestock include hides and skins, meat, mutton, and honey. However, these products don't receive much value addition [169].

Processing of plant organs was done at Egerton University in Njoro, Nakuru County, which is a part of the eastern Mau water-catchment. Njoro is a settlement that is around 25 kilometers southwest of the town of Nakuru. The university is 2,238 meters above sea level and is located at 0°22'11.0"S, 35°55'58.0" E (Latitude: -0.369734; Longitude:35.932779). The region receives 1200 mm of rainfall annually, with a bimodal distribution of long and short periods between April and August and October and December. The range of temperatures is 10.2 to 22.0 °C [170]. Plant extracts

obtained from the targeted organs were then shipped to the University of Pecs, Baranya County, Hungary for phytochemical analysis and subsequent experimental activities. The University of Pécs (UP; Hungarian: Pécsi Tudományegyetem; PTE) is an institution of higher education in Hungary. The fifth-largest city in Hungary is Pécs, located near the Croatian border in the southwest on the Mecsek mountain range. The Roman Catholic Diocese of Pécs is headquartered there, and it serves as the political, social, and economic hub of Baranya County [171]

3.2 Sampling design

The purposive sampling method was employed in the acquisition of targeted plant species and colorectal cancer cell lines (Caco-2 cell line). An experimental design was set up with appropriate research questions and hypotheses formulated.

3.3 Acquisition of Caco-2 cell lines

Caco-2 cell lines were obtained from ATCC and directly supplied to our laboratory (Department of Public Health) by the Department of Biochemistry and Medical Chemistry, University of Pecs. Caco-2 (Caco2) are epithelial cells obtained from carcinoma cells of a 72-year-old White male with colorectal adenocarcinoma. This cell line is a good transfection host and has potential applications in cancer and toxicity research. The Caco-2 cell lines were stored per the manufacturer's instructions [172].

3.4 Collection of the plant

Organs from the WU plant were collected at Egerton University in Njoro - which is part of the eastern Mau water-catchment, Nakuru County. Njoro is a small catchment and is around 25 kilometers southwest of Nakuru town. The university is 2,238 meters above sea level and is located at 0°22'11.0"S, 35°55'58.0" E (Latitude: -0.369734; Longitude:35.932779). The region receives 1200 mm of rainfall annually, with a

bimodal distribution of long and short periods between April and August and October and December. The range of temperatures is 10.2 to 22.0 °C [170].

Organs of AS were collected from Kampi ya Moto, in Rongai Sub County, Nakuru County, and located at 0.1244° S, 35.9431° E, GPS coordinates. The annual temperature in the district is 18.75º C (65.75º F), which is -3.75% lower than the national average for Kenya. 118.62 millimeters (4.67 inches) of precipitation and 221.53 days of rain are typical yearly totals for Kampi Ya Moto. On the other hand, WS organs were obtained from the Perkerra irrigation scheme, Baringo South Sub-County in Baringo County (0° 28' 42" N · 36° 1' 38" E · 0.4786 Latitude, and 36.0274 Longitude). The Irrigation Scheme is located 100 km north of Nakuru town, close to Marigat Township. It derives its name from River Perkerra, which is the only perennial natural river in the area and a source of water for irrigation [173].

3.5 Extraction of plant extracts using organic solvents

The selected plants were shade-dried and ground into fine powder. Serial exhaustive extraction (SEE) was done using three solvents of increasing polarity. In the experiment, 1000g of each plant (WS, AS, and WU) part was put in a flask and serially extracted using hexane, ethyl acetate, and methanol, for 3 days (for each solvent) with frequent shaking to extract polar and nonpolar constituents like phytosterols, polyphenols, flavonoids, terpenoids and different coumarin derivatives of the plants. Crude solvent extracts were filtered through Whatman filter paper of different pore sizes (Nos. 4 and 1) and dechlorophyllated. This process was repeated 3 times until there was a complete extraction of all soluble constituents [174]. The extract (pooled together in all three batches of filtrates) was finally concentrated by evaporating the solvent. Solvent removal was done using a rotary evaporator at temperatures between 40ºC and 50ºC under reduced pressure. The aqueous extract was lyophilized using a

freezer dryer. The dry solvent-free metabolites were kept in tightly stoppered sample bottles, sealed using parafilm tape, and placed in a desiccator at 4ºC in a fridge until use [175]. All the chemicals used in the present study were of high standard purity (analytical grade).

3.6 Dissolving plant extracts in DMSO for bioassays

Dimethyl sulfoxide (DMSO) is a versatile substance that is frequently employed as a solvent in pharmacology and toxicology to improve drug delivery, dissolve a variety of medications, and dissolve herbal extracts [176]. It was used as an inert diluent and the suspending medium for water-insoluble crude plant extracts. 30 mg/mL (stock solution) was prepared using 0.5% DMSO and double distilled phosphate buffer saline (ddPBS) as the dissolving and diluent solvents respectively. The stock solution was then used to make final concentrations of 2 mg/mL, 1 mg/mL, and 0.5 mg/mL for Caco-2 cell line treatment. Dimethyl sulfoxide (DMSO) is utilized as a solvent for many organic and inorganic compounds because of its amphiphilic characteristics, high polarity, and liquid state over a wide temperature range [177]. The essential pharmacological impact of DMSO's ability to penetrate membranes made it the perfect vehicle for our experiment, which aimed to ascertain the anti-proliferative and apoptotic effects of various doses of plant extracts on Caco-2 cell lines.

3.7 Passaging Caco-2 cell lines for treatment

The petri dish/flask containing Caco-2 cells maintained in Caco-2 media (Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 50 μM thioglycerol and 25 mg/ml gentamycin) was gently put inside a lamina flow hood under sterile conditions, at 37 ^{0}C with humified air at 5% CO_2 in the incubator [178]. The dish/flask was opened, and the utilized media was removed. It was washed twice with phosphate-buffered saline

(PBS). PBS-EDTA was poured over and put aside for a few minutes. It was then gently collected with caution. 2mL of trypsin was added to separate and detach Caco-2 cells from clumps and the surface respectively. The surface was covered with trypsin by moving the dish side-side gently on the surface. The dish was put in the thermostat for 5 minutes. After 5 minutes, the dish was removed and after satisfactory visible detachment, Caco-2 media was carefully poured into it. Caco-2 cells are an adherent type, tending to attach to the surface. All the contents of the dish were pipetted into a tube and centrifuged at 125 rpm for 5 minutes. The supernatant was then removed while the Caco-2 cells remained at the bottom of the tube. Fresh media was poured into the tube and the cells were carefully disturbed by pipetting up and down. The suspension was divided into new dishes, the dishes were filled with medium and put in the thermostat for growth. Confluence was monitored up to 70-80% to allow for treatment.

3.8 Treatment of Caco-2 cell lines with plant extract solutions

Passaged Caco-2 cell lines replenished with fresh Caco-2 media were treated with 200µL of the extract solutions of varying concentrations (0.5 mg/mL, 1 mg/mL, 2 mg/mL). Treated cells were then incubated at 37 ^{0}C for 36 hours. After the incubation period, the cells' status was observed under a light microscope before the RNA isolation.

3.9 Isolation of RNA

3.9.1 Reagents and isolation procedure used for RNA extraction

3.9.1.1 Reagents for RNA isolation

The reagents used in efficient RNA extraction constituted the following: ExtraZol Tri-reagent (Nucleotest Bio Kft, #EM30-200), Chloroform (Merck Sigma, #C7559), isopropyl alcohol (Merck Supelco, #1.00997), 75% alcohol (diluted from absolute

ethanol; BioTech Hungary Kft, #1001901000), and DEPC water 0.1% DEPC (Diethylpirocarboxylic acid; Merck Sigma, #D5758). They were all used in compliance and per the manufacturers' instructions for maximum and quality RNA extraction.

3.9.1.2 Isolation procedure

The medium was aspirated from the cell cultures, washed twice with PBS, and treated with trypsin-EDTA. The cell suspension was centrifuged, and the cells were pipetted into a 4 cm^3 centrifuge tube. ExtraZol Tri-reagent solution (1 cm^3) was added and incubated for 5 min at room temperature. Chloroform (0.2 cm^3) was then added and after 2-3 min incubation, the sample was centrifuged at 12000 g for 10 min at 2-8 ºC. The aqueous phase was transferred to a clean tube. 0,2 cm^3 isopropyl alcohol was added. After 10 minutes of incubation, the sample was centrifuged again at 12000 g for 10 minutes at 2-8 ºC. Before centrifugation, the RNA precipitate is often not visible, after centrifugation, it gives a gel-like pellet at the bottom of the tube. After washing off the supernatant, the RNA pellet was washed with 1 cm3 of 75 % alcohol. After vortexing, it was centrifuged at 7500 g for 5 min at 2-8 °C. The supernatant was poured off and the pellet was dried. It was then dissolved in 80 μl RNAse free water (DEPC water). The sample was vortexed and incubated at 55 °C for 10 min. After ascertaining sufficient purity and concentration, the isolated Total RNA was stored at -80 °C until use.

3.9.1.3 RNA concentration and purity assessment using UV Spectroscopy

UV Spectroscopy was used for assessing RNA concentration and purity. The performance of this method was optimized by first treating RNA samples with RNAse-free DNAse to remove contaminating DNA. Other contaminants such as residual proteins and phenol that could interfere with absorbance readings, were carefully

removed during the process. The absorbance of a diluted RNA sample was measured at 260 nm and 280 nm. The nucleic acid concentration was calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. An A260/A280 ratio was used to assess RNA purity, and a ratio of 1.8 2.1 was indicative of highly purified RNA.

3.9.2 Measurement and evaluation of the relative gene expression using qRT-PCR

Real-time RT-PCR combines retroviral enzyme with reverse transcriptase activity with polymerase chain reaction to amplify the quantity of mRNA. The end product of PCR is proportional to the mRNA molecules in the tested sample after the fluorescent dye is bound with nucleic acid amplicon. SYBR green is an intercalating dye in double-stranded DNA molecules. A specific probe is unnecessary when using SYBR green because it can bind with all double-stranded DNA molecules [179].

3.9.3 Protocol and equipment used for qRT-PCR (SYBR Green Protocol)

One-step PCR, including reverse transcription and amplification, was performed using the One-Step Detect SyGreen Lo-ROX one-step RT-PCR kit (Nucleotest Bio Ltd PB25.11-12) on a 96-well plate on a LightCycler 480 qPCR platform (according to the manufacturer's instructions). The thermal program was set as follows: incubation at 42°C for 5 min, followed by incubation at 95°C for 3 min, then 45 cycles (95°C-5s, 56°C-15s, 72°C-5s), and a fluorescent readout was taken at the end of each cycle. Each run was followed by melting curve analysis (95°C – 5s, 65°C – 60s, 97°C∞) to confirm amplification specificity. The reaction mix was as follows: 10 µl Master Mix, 0.4 µl RT Mix, 0.4 µl dUTP, 0.4 µl primers, 5 µl mRNA template supplemented with sterile double distilled water for a total volume of 20 µl. Primers were synthesized by

Integrated DNA Technologies (Bio-Sciences) and sequences were designed using primer express software (Table 3.1)

Table 3.1 Forward and reverse primer sequences adopted and applied in our experimental study.

Primer ID	Forward primer	Reverse primer
COX-2	CGGTGAAACTCTGGCTAGACAG	GCAAACCGTAGATGCTCAGGGA
5-LOX	GGAGAACCTGTTCATCAACCGC	CAGGTCTTCCTGCCAGTGATTC
Bcl2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
Bcl-xL	GCCACTTACCTGAATGACCACC	AACCAGCGGTTGAAGCGTTCCT
Casp9	GTTTGAGGACCTTCGACCAGCT	CAACGTACCAGGAGCCACTCTT
HPRT1	TGCTTCTCCTCAGCTTCA	CTCAGGAGGAGGAAGCC

3.9.4 qRT-PCR result analysis

Using a qRT-PCR high-throughput detection and quantification matrices of target DNA sequences, the relative gene expression of *COX-2, 5-LOX, Bcl-xL, Bcl2* and *CASP9* targeted genes was determined. For internal control, the house-keeping gene used in our experimental study was *HPRT1.* The PCR results were expressed as Cp values, indicating the cross point between the amplification curve and threshold value. The Cp values were used to calculate the fold changes of the target genes from the control sample using 2-ΔΔCp (Livak method) [179].

3.10 Analysis of chemical components from crude plant extracts

3.10.1 High performance/pressure liquid chromatography (HPLC)

High performance liquid chromatography was used to detect and determine individual bioactive compounds from complex methanolic plant extract solutions. Methanol was preferred being a strong polar compound compared to the other extraction solvents, and the cost implication if metabolites would be determined from all extraction solvents. Before injection into the HPLC, each sample was filtered using a 0.45

μm filter (Nylon Membranes, Supelco). The HPLC-DAD system was allowed to warm up before any run, and the baseline was monitored until it became stable before sample analysis. Peak identification was accomplished by comparing the UV absorption spectrum and retention duration to values obtained using standards. By injecting the standard solutions containing reference compounds for 30 minutes, the repeatability of the injection integration was assessed for the standards, and the Relative standard deviation (R.S.D). for the integration area was calculated [180].

3.11 Data analysis

The statistical analysis was calculated using MS Excel (Microsoft Corp. Released 2013. Redmond, WA, US) and IBM SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, US). From the data obtained, Kolmogorov-Smirnov test was used to perform normality analysis while multiparametric Post Hoc test was also applied. ANOVA test was used to compare the means of the variables of interest. Results were considered significant if $p \leq 0.05$ at a 95% confidence interval.

CHAPTER FOUR

4. RESULTS

4.1 Expression of targeted gene in Caco-2 cell lines after treatment with WS (Ethyl acetate, Hexane, and ethyl acetate) extracts

In this section, Caco-2 cell lines were treated with roots and stem extracts of WS at increasing concentrations of 0.00 mg/mL, 0.50 mg/mL, 1.00 mg/mL, and 2.00 mg/mL. Three different extraction solvents were used, and their results are herein provided.

4.1.1 *COX-2* gene expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were treated with ethanolic root and stem extracts of WS at increasing concentrations, *COX-2* genes were progressively downregulated in a dose-dependent manner (Figure 4.1), in both extracts. There was also an observed statistically significant difference in their downregulatory effects in similar proportions (p = 0.001), Table 4.1 & Annexed, Table 1.

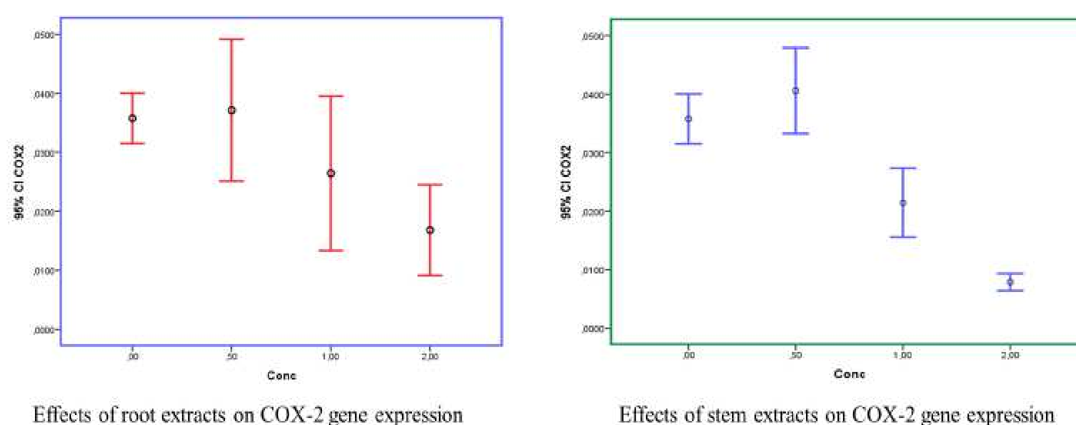


Figure 4.1 *COX-2* gene expressions after treatment with ethanolic root and stem extracts

Table 4.1 A summarized output of the relationship between the expression of target genes and the concentration/dosage of ethanoic root extracts of WS

		Sum of Squares	df	Mean Square	F	Sig.
COX-2	Between Groups	,001	3	,000	16,778	,001
	Within Groups	,000	8	,000		
	Total	,001	11			
5-LOX	Between Groups	,811	3	,270	27,438	,000
	Within Groups	,079	8	,010		
	Total	,890	11			
Bcl2	Between Groups	,000	3	,000	47,425	,000
	Within Groups	,000	8	,000		
	Total	,000	11			
Bcl-xL	Between Groups	5,651	3	1,884	7,261	,011
	Within Groups	2,075	8	,259		
	Total	7,727	11			
CASP9	Between Groups	2,919	3	,973	16,415	,001
	Within Groups	,474	8	,059		
	Total	3,393	11			

a. Treatment = 01-R E Ace (Ethyl acetate)

4.1.2 *CASP9* expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with ethanolic root and stem extracts of WS, the expression of *CASP9* genes was upregulated in a dose-dependent manner, in both extracts. However, the activity of increased expression was higher in root extracts than observed in stem extracts (Figure 4.2). There was a significant difference in their up-regulatory properties (p = 0.001) for both extracts, Table 4.1 & Annexed, Table 1.

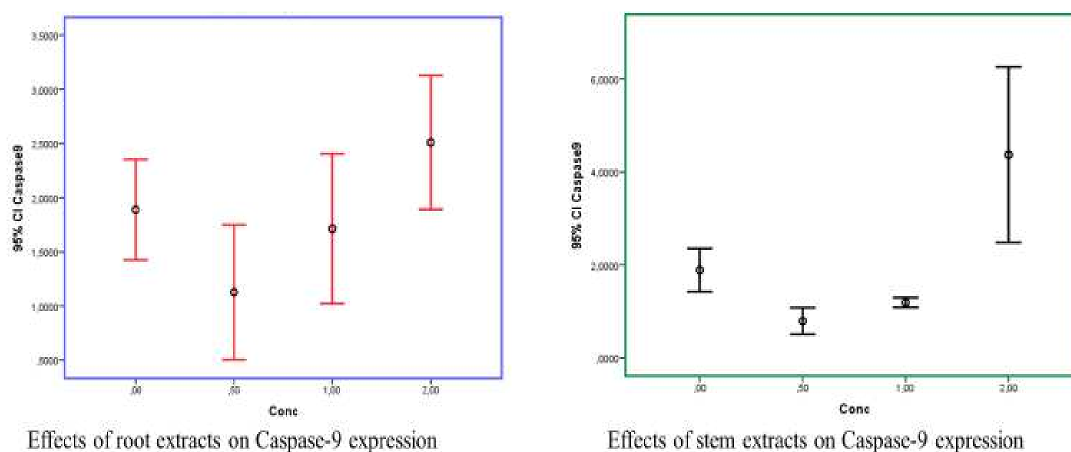
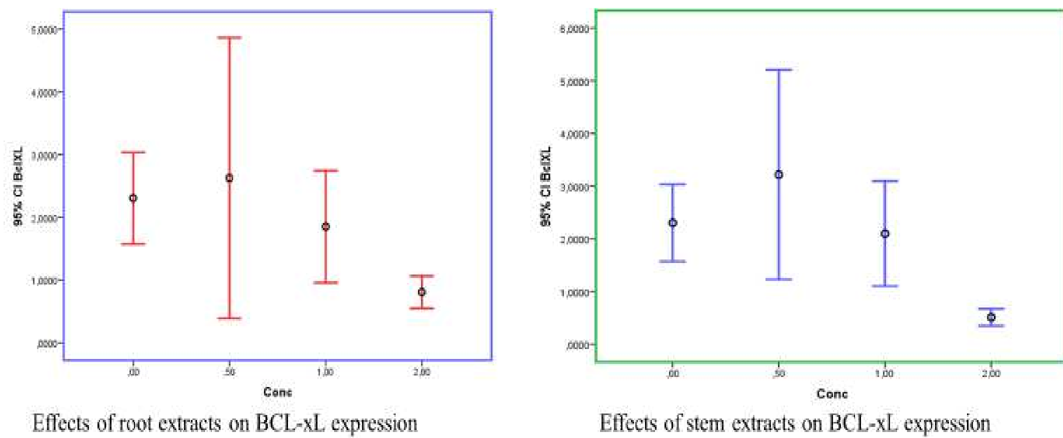


Figure 4.2 *CASP9* expressions after treatment with ethanolic root and stem extracts

4.1.3 *Bcl-xL* expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to ethanolic root and stem extracts of WS, the expression of *Bcl-xL* genes was downregulated in a dose-dependent manner, in both extracts (Figure 4.3). There was a significant difference in their downregulatory properties (p = 0.011, roots and p=0.001, stems) observed in both extracts, Table 4.1 & Annexed, Table 1.



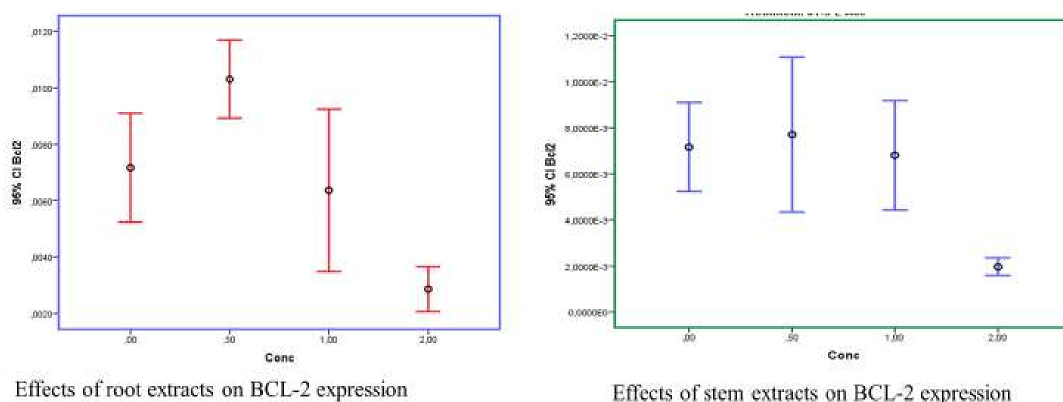
Effects of root extracts on BCL-xL expression

Effects of stem extracts on BCL-xL expression

Figure 4.3 *Bcl-xL* expressions after treatment with ethanolic root and stem extracts

4.1.4 *Bcl2* expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

Upon exposure to ethanolic root and stem extracts of WS, the expression of *Bcl2* genes was downregulated in a dose-dependent manner, in both extracts (Figure 4.4). There was an observed similarity in a significant difference in their downregulatory properties (p = 0.001) in both roots and stem extracts, Table 4.1 & Annexed, Table 1.



Effects of root extracts on BCL-2 expression

Effects of stem extracts on BCL-2 expression

Figure 4.4 *Bcl2* expressions after treatment with ethanolic root and stem extracts

4.1.5 *5-LOX* expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with ethanolic root and stem extracts of WS, the expression of *5-LOX* genes was downregulated in a dose-dependent manner, in both extracts. The activity of decreased expression was higher in root extracts than observed in stem extracts (Figure 4.5). There was a significant difference in their downregulatory potential ($p = 0.001$) observed in both extracts, Table 4.1 & Annexed, Table 1.

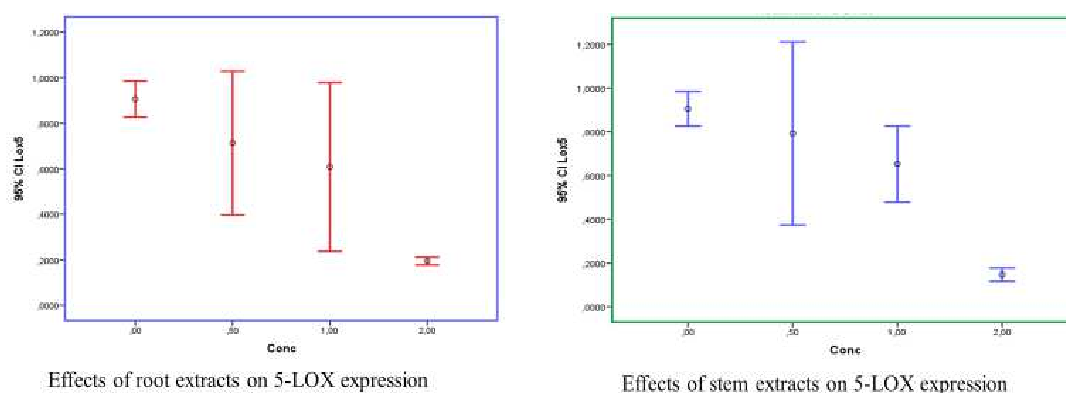


Figure 4.5 *5-LOX* expressions after treatment with ethanolic root and stem extracts

4.2 Pharmacotherapeutic activity of hexanoic extracts

4.2.1 *COX-2* expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were treated with hexanoic root and stem extracts of WS at increasing concentrations, *COX-2* genes were progressively downregulated in a dose-dependent manner (Figure 4.6), in root extracts while there was an observed increased expression with the stem. There was an observed statistically significant difference in the downregulatory properties of root extracts (p=0.007), whereas there was not in stem extracts ($p = 0.531$).

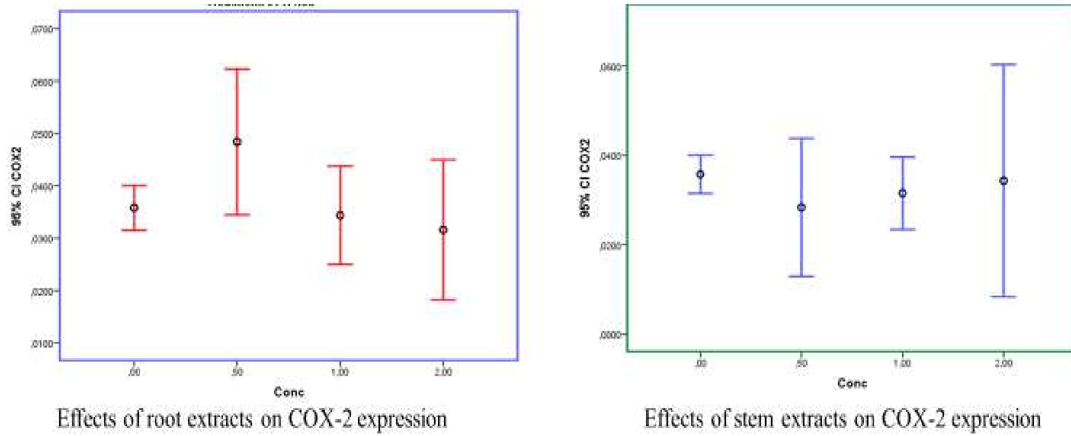


Figure 4.6 *COX-2* expressions after treatment with hexanoic root and stem extracts

4.2.2 *CASP9* expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

After treatment of Caco-2 cell lines with root and stem extracts, *CASP9* enzymatic genes were detected as being variably upregulated in both extracts (Figure 4.7). However, their expression diminished with increasing concentration in root extracts at 2.00 mg/mL. The up-regulatory activities of both extracts on *CASP9* were statistically significant (root, p=0.001, stem, p=0.014).

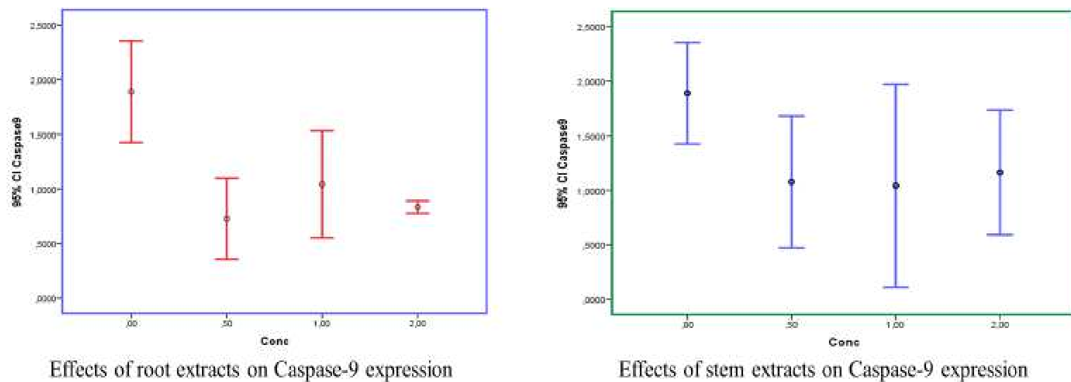


Figure 4.7 *CASP9* expressions after treatment with hexanoic root and stem extracts

4.2.3 *Bcl-xL* expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to hexanoic root and stem extracts of WS, the expression of *Bcl-xL* genes was downregulated only at low (0.5 mg/mL) and high (2

mg/mL) concentrations in both extracts. The expression and activity were highest at the medium (1.0 mg/mL) (Figure 4.8). There was a significant difference in their downregulatory expression (p = 0.008, roots and p=0.032, stems) observed in both extracts.

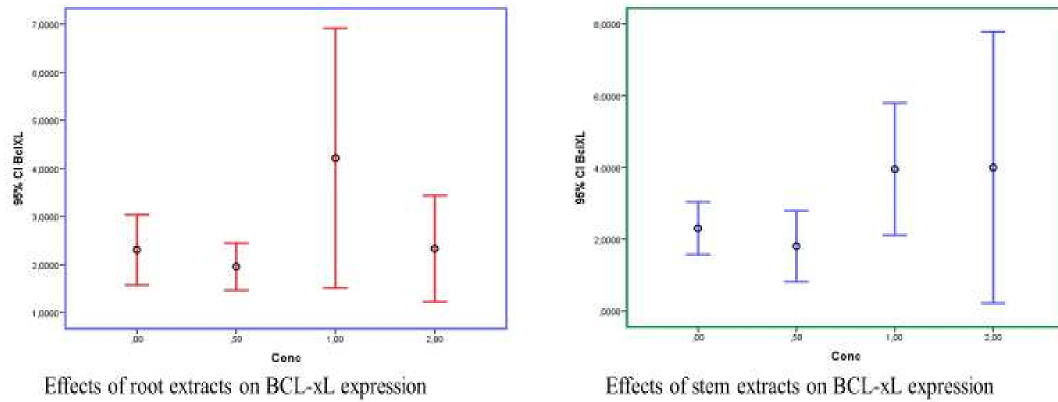


Figure 4.8 *Bcl-xL* expressions after treatment with hexanoic root and stem extracts

4.2.4 *Bcl2* expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to hexanoic root and stem extracts of WS, the expression of *Bcl2* genes was effectively downregulated in root extracts, dose-dependently. In stem extracts, downregulation was expressed only at low (0.5 mg/mL) and high (2 mg/mL) concentrations (Figure 4.9). Root extracts were statistically significant (p = 0.002), while stem extracts were not statistically significant (p=0.372).

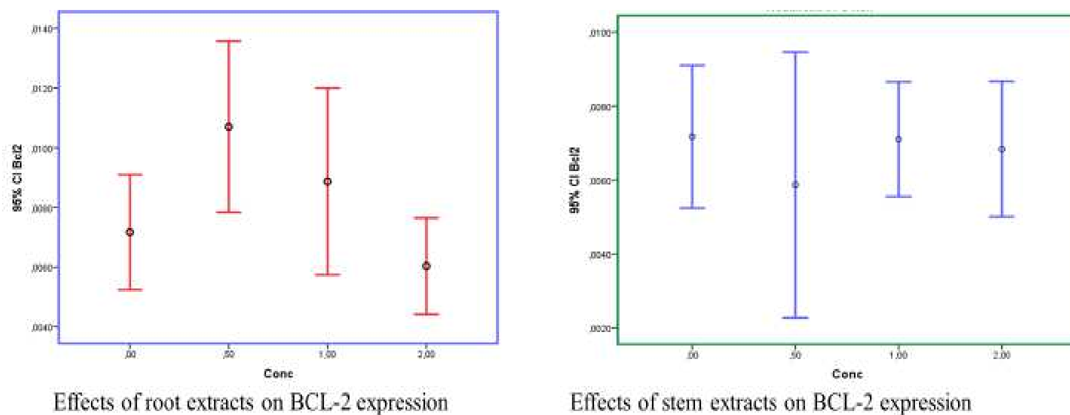


Figure 4.9 *Bcl2* expressions after treatment with hexanoic root and stem extracts

4.2.5 *5-LOX* expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with hexanoic root and stem extracts of WS, the expression of *5-LOX* genes was variably upregulated and downregulated in both extracts in a dose-dependent manner. Downregulation was observed to occur at 1 mg/mL in root extracts and only at high (2 mg/mL) concentrations in stem extracts (Figure 4.10). There was not any significant difference in their downregulatory potential (p = 0.685) in root extracts whereas, there was an observed significant difference in stem extracts (p=0.046).

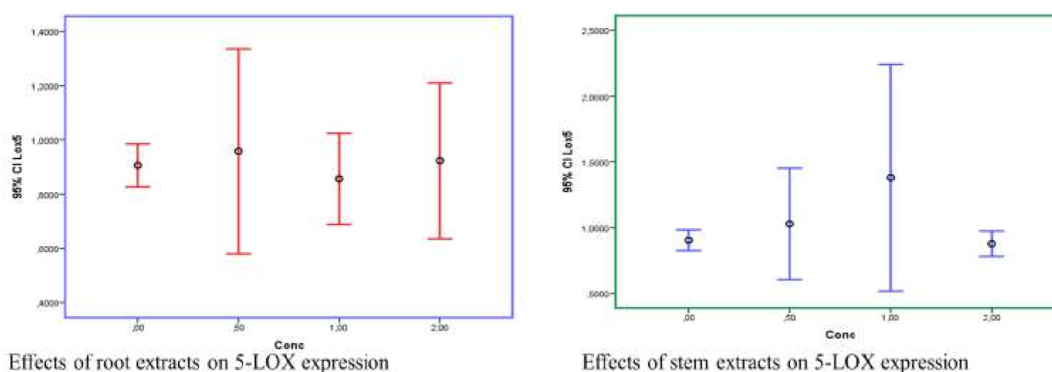


Figure 4.10 *5-LOX* expressions after treatment with hexanoic root and stem extracts

4.3 Pharmacotherapeutic activity of methanolic extracts

4.3.1 *COX-2* expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were treated with methanolic root and stem extracts, *COX-2* genes were progressively downregulated in a dose-dependent manner (Figure 4.11), in both extracts. There was also an observed statistically significant difference in their downregulatory effects from root extracts (p = 0.001) and stem extracts (p=0.010).

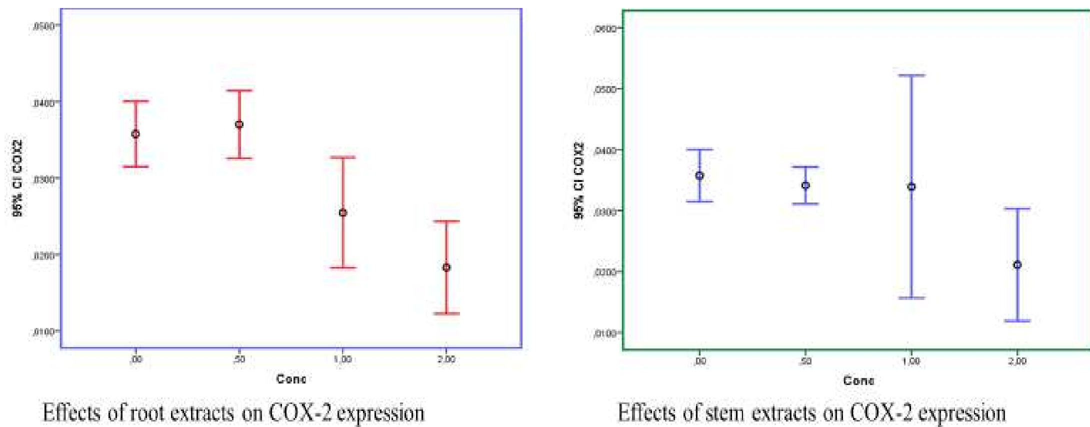


Figure 4.11 *COX-2* expressions after treatment with methanolic root and stem extracts

4.3.2 *CASP9* expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with methanolic root and stem extracts of WS, there was increased (upregulation) expression of *CASP9* genes in a dose-dependent manner, in both extracts (Figure 4.11). There was a notable significant difference in both extracts as p=0.002 from root extracts and p= 0.011 from stem extracts. The highest up-regulatory activity was observed at higher concentrations.

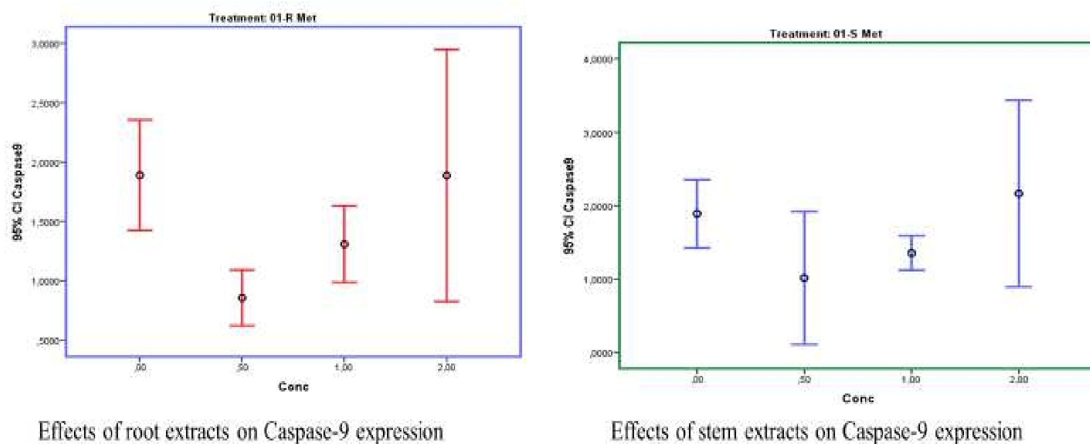


Figure 4.11 *CASP9* expressions after treatment with methanolic root and stem extracts

4.3.3 *Bcl-xL* expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with methanolic root and stem extracts of WS, the expression of *Bcl-xL* genes was downregulated in a dose-dependent manner, in both extracts (Figure 4.12). There was a significant difference in their downregulatory potential ($p = 0.001$, roots and $p=0.001$, stems) observed in both extracts.

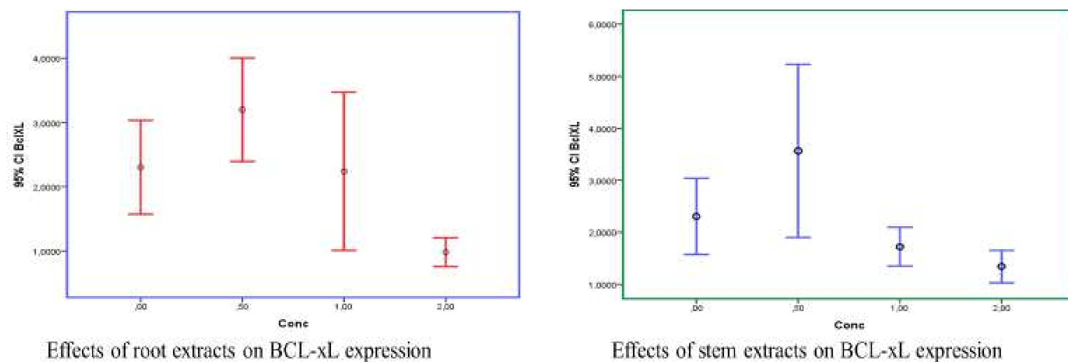


Figure 4.12 *Bcl-xL* expressions after treatment with methanolic root and stem extracts

4.3.4 *Bcl2* expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

The expression of *Bcl2* genes was downregulated in a dose-dependent manner, in both extracts (Figure 4.13). There was a significant difference in their downregulatory effects ($p = 0.007$, roots and $p=0.004$, stems) recorded in both extracts.

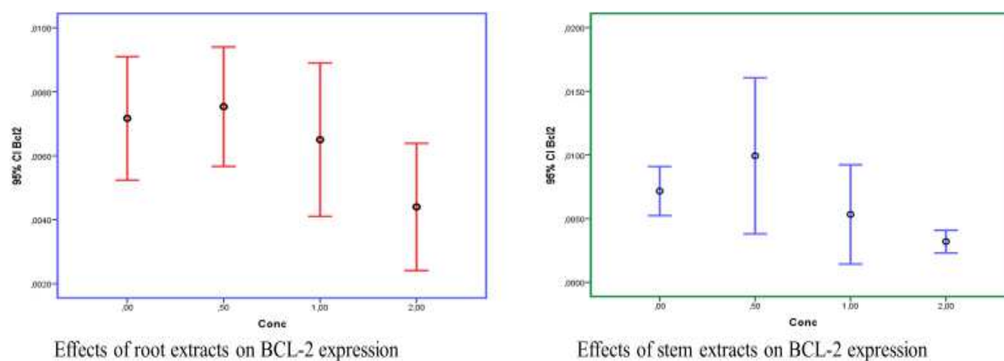


Figure 4.13 *Bcl2* expressions after treatment with methanolic root and stem

4.3.5 *5-LOX* expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to methanolic root and stem extracts of WS, the expression of *5-LOX* genes was downregulated in a dose-dependent manner, in both extracts (Figure 4.14). Similarly, there was a significant difference in their downregulatory properties ($p = 0.001$) observed in both extracts.

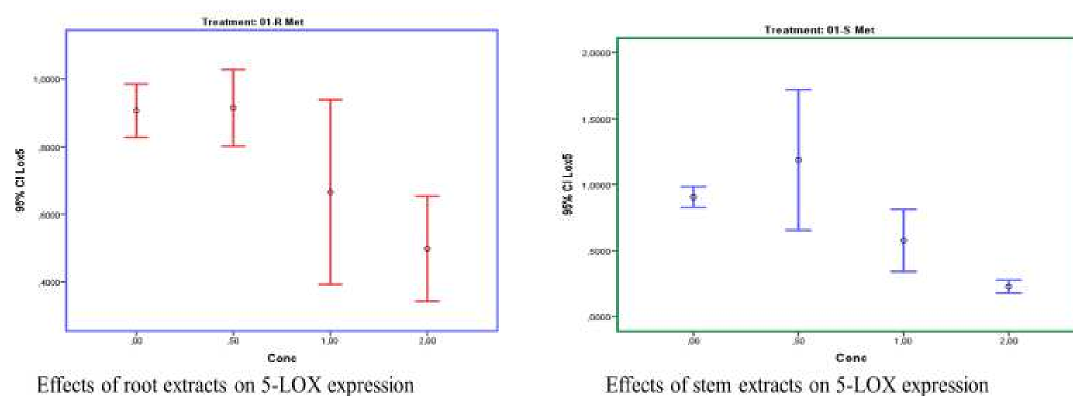


Figure 4.14 *5-LOX* expressions after treatment with methanolic root and stem extracts

5.1 Expression of targeted gene in Caco-2 cell lines after treatment with WU (ethyl acetate, hexane, and methanol) extracts.

In this section, Caco-2 cell lines were treated with root and stem extracts of WU at increasing concentrations of 0.00 mg/mL, 0.50 mg/mL, 1.00 mg/mL, and 2.00 mg/mL. Extracts were obtained using three different extraction solvents (Ethyl acetate, Hexane, and methanol).

5.1.1 *COX-2* gene expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were treated with ethanolic root and stem extracts of WU, *COX-2* genes were increasingly downregulated in a dose-dependent manner (Figure 5.1), in both extracts. However, significant downregulatory effects were observed in

stem extracts statistically ($p = 0.001$) compared to root extracts ($p = 0.379$), Table 5.1 and Annexed, Table 2.

Table 5.1 A summarized output of the relationship between the expression of target genes and the concentration/dosage of ethanolic stem extracts of WU

Targeted gene		**Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
COX-2	Between Groups	,002	3	,001	188,502	,000
	Within Groups	,000	11	,000		
	Total	,002	14			
5-Lox	Between Groups	,049	3	,016	3,593	,050
	Within Groups	,050	11	,005		
	Total	,098	14			
Bcl2	Between Groups	,000	3	,000	22,542	,000
	Within Groups	,000	11	,000		
	Total	,000	14			
Bcl-xL	Between Groups	3,720	3	1,240	12,833	,001
	Within Groups	1,063	11	,097		
	Total	4,782	14			
CASP9	Between Groups	,818	3	,273	6,570	,008
	Within Groups	,457	11	,042		
	Total	1,275	14			

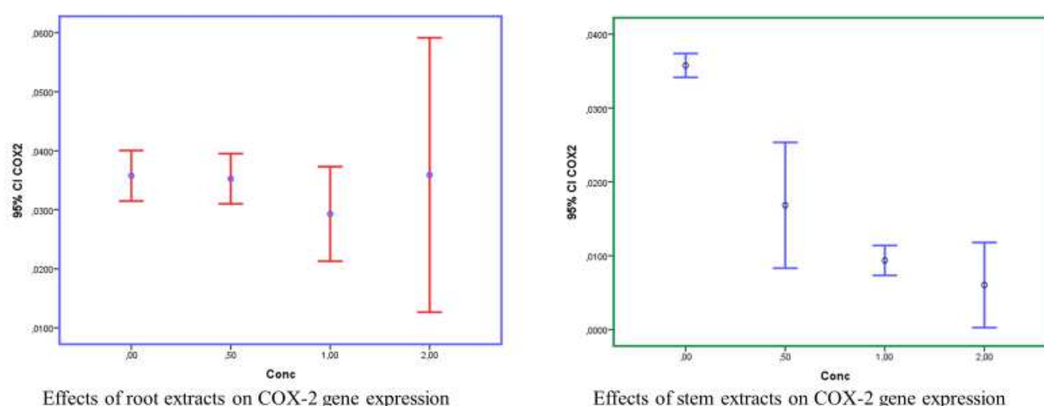
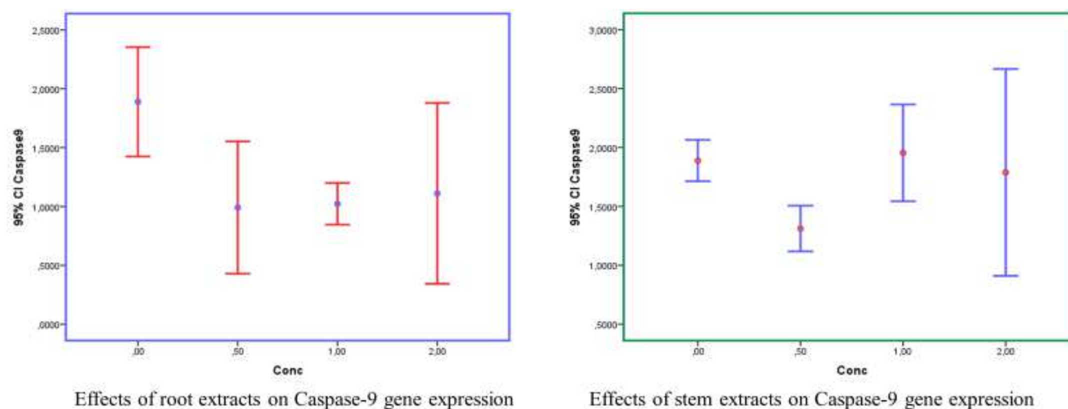


Figure 5.1. Effects of increasing concentrations of ethanolic root and stem extracts on COX-2 gene expression

5.1.2 *CASP9* gene expression after treatment with ethanolic root and stem extracts of increasing dosage concentration

After exposure of Caco-2 cell lines with root and stem extracts at increasing concentrations, *CASP9* enzymatic genes were detected as being variably upregulated in both extracts (Figure 5.2). However, their expression diminished with increasing

concentration in stem extracts at 2.00 mg/mL. The up-regulatory activities of both extracts on *CASP9* were statistically significant (root, p=0.003, stem, p=0.008), Table 5.1 and Annexed, Table 2.



Effects of root extracts on Caspase-9 gene expression

Effects of stem extracts on Caspase-9 gene expression

Figure 5.2 Effects of increasing concentrations of ethanolic root and stem extracts on *CASP9* gene expression

5.1.3 *Bcl-xL* gene expression after treatment with ethanolic root and stem extracts of increasing dosage concentration

After treatment of Caco-2 cell lines with ethanolic root and stem extracts at increasing concentrations from 0.00 mg/mL, 0.50 mg/mL, 1.00 mg/mL to 2.00 mg/mL, *Bcl-xL* genes were observed to be downregulated in a dose-dependent manner up to a concentration of 1.00 mg/mL, with exemption of the stem extracts. Of intrigue, there was a sudden upregulation of gene expression at a concentration of 2.00 mg/mL (Figure 5.3) in root extracts. However, in both extracts, downregulatory effects were statistically significant (p = 0.001), Table 5.1 and Annexed, Table 2.

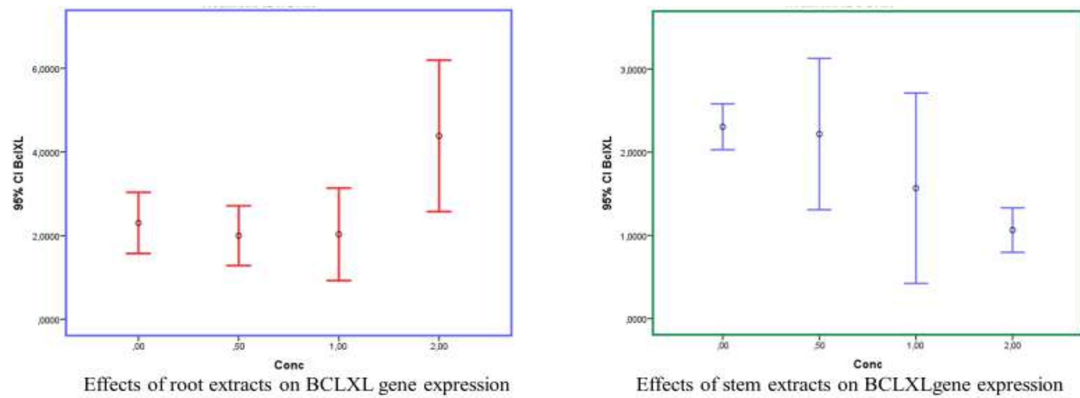


Figure 5.3 Effects of increasing concentrations of ethanolic root and stem extracts on *Bcl-xL* gene expression

5.1.4 *Bcl2* gene expression after treatment with ethanolic root and stem extracts of increasing dosage concentration

After application of appropriate treatments, *Bcl2* genes were observed to be downregulated in a dose-dependent manner with significant effects being in stem extracts compared to root extracts. Of concern, there was a sudden upregulation of gene expression at a concentration of 2.00 mg/mL, (Figure 5.4) in root extracts, like that observed in the expression of *Bcl-xL*. Notwithstanding, downregulatory effects were statistically significant in both extracts ($p = 0.001$), Table 5.1 and Annexed, Table 2.

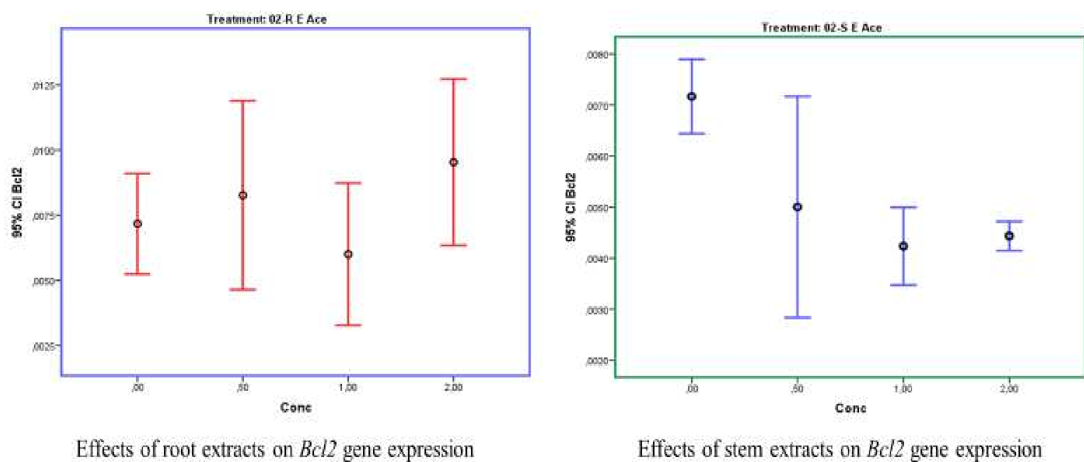


Figure 5.4. Effects of increasing concentrations of ethanolic root and stem extracts on *Bcl2* gene expression

5.1.5 *5-LOX* gene expressions after treatment with ethanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with ethanolic root and stem extracts at increasing concentrations of 0.00 mg/mL, 0.50 mg/mL, 1.00 mg/mL, and 2.00 mg/mL, *5-LOX* genes were interestingly observed to be variably downregulated in a dose-dependent manner in root extracts (Figure 5.5), while being upregulated in stem extracts. Significant up-regulatory properties of stem extracts were observed ($p = 0.05$), while not the same case on downregulatory effects by root extracts ($p = 0.083$), Table 5.1 and Annexed, Table 2.

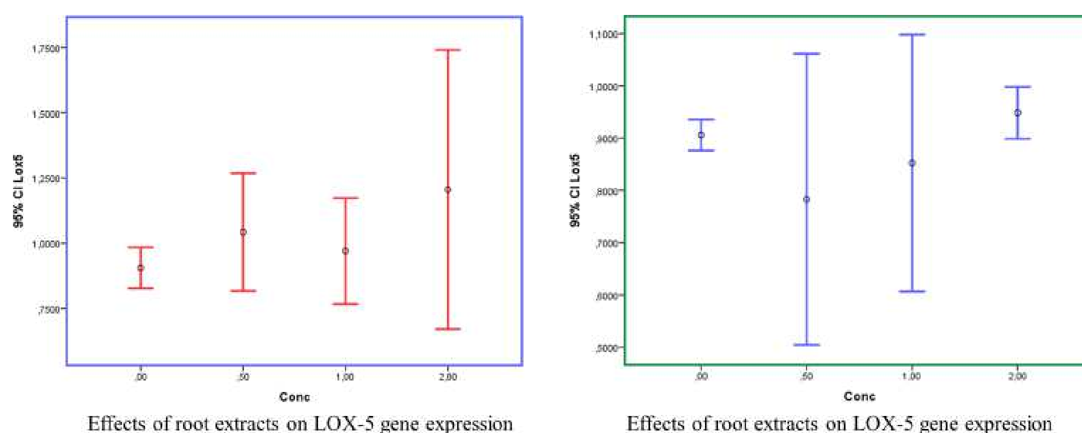
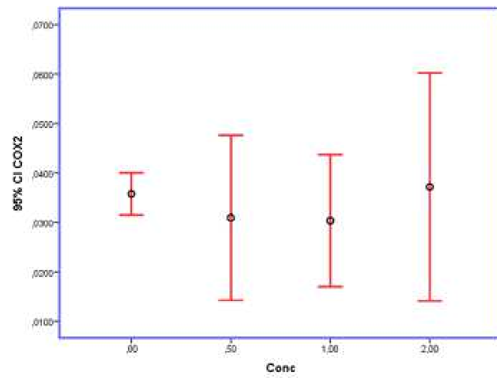


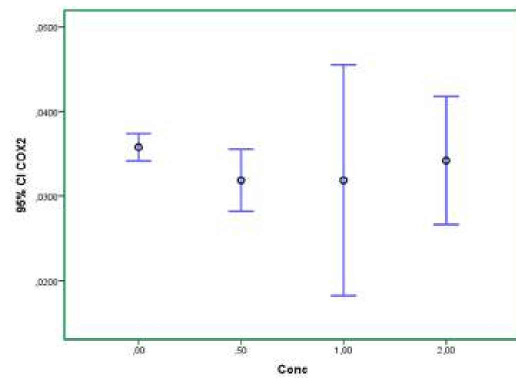
Figure 5.5 Effects of increasing concentrations of ethanolic root and stem extracts on *5-LOX* gene expression

5.2 Pharmacotherapeutic activity of hexanoic extracts

5.2.1 *COX-2* gene expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with hexanoic root and stem extracts of WU, the expression of *COX-2* genes was variably downregulated in lower concentrations and upregulated at a higher dose (2 mg/mL) concentration (Figure 5.6). The downregulatory effetcs observed in root ($p = 0.498$) and stem extracts (p=0.211) were not statistically significant.





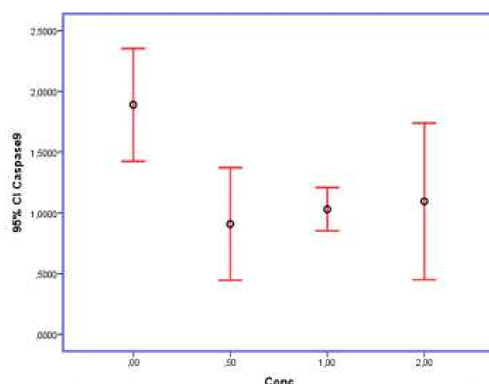
Effects of root extracts on COX-2 gene expression

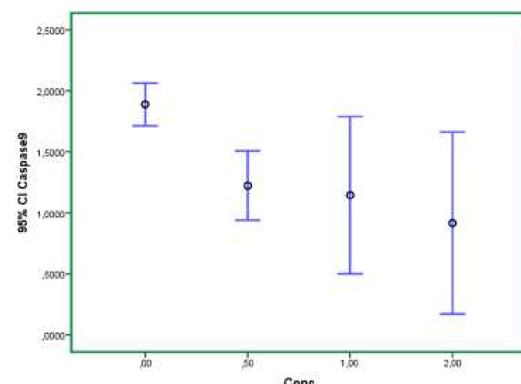
Effects of stem extracts on COX-2 gene expression

Figure 5.6 *COX-2* gene expressions after treatment with hexanoic root and stem extracts

5.2.2 *CASP9* gene expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

After treatment of Caco-2 cell lines with root and stem extracts, *CASP9* genes were observed to be upregulated in root extracts while their expressions were decreased on treatment with stem extracts (Figure 5 .7). Both effects were statistically significant (roots, p=0.001 and stems p=0.001). However, only root extracts demonstrated the required up-regulatory effects.





Effects of root extracts on Caspase-9 gene expression

Effects of stem extracts on Caspase-9 gene expression

Figure 5.7 *CASP9* gene expressions after treatment with hexanoic root and stem extracts

5.2.3 *Bcl-xL* gene expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to hexanoic root and stem extracts of WU, the expression of *Bcl-xL* genes was upregulated in root extracts. However, their expressions were downregulated by stem extracts dose-dependently (Figure 5.8). There was a significant difference in their up-regulatory expression by root extracts ($p = 0.044$) and corresponding downregulatory effects by stem extracts ($p=0.029$). However, stem extracts affected the required downregulatory response.

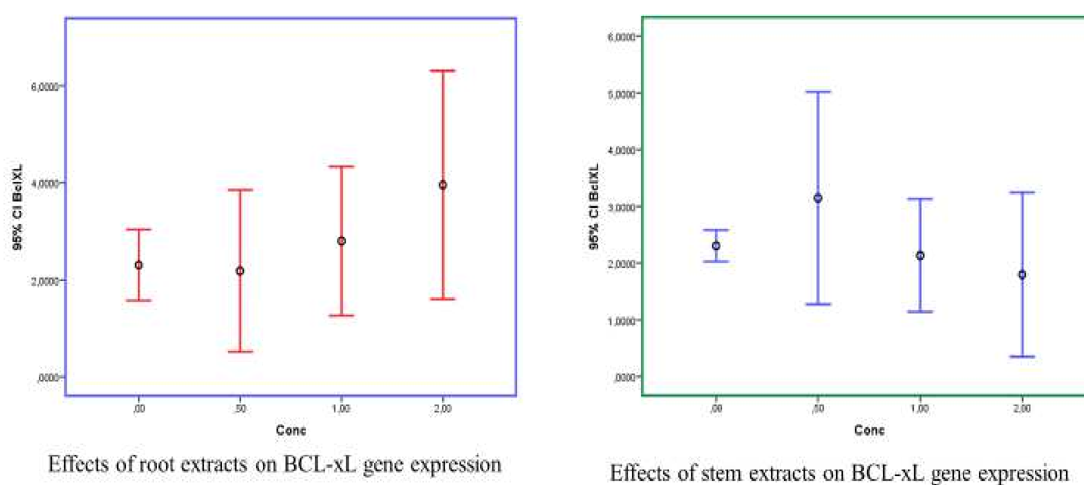
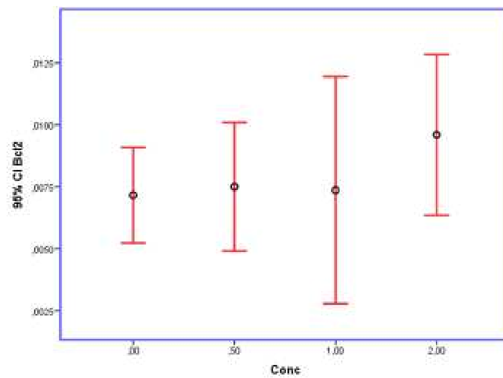


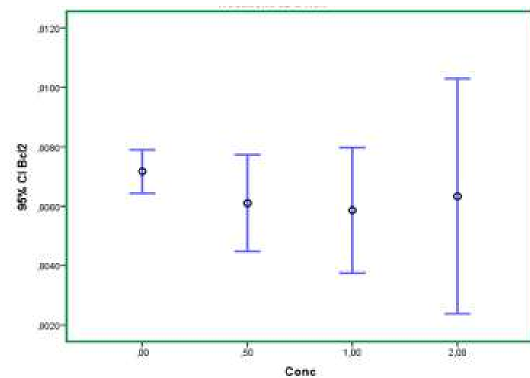
Figure 5.8. *Bcl-xL* gene expressions after treatment with hexanoic root and stem extracts

5.2.4 *Bcl2* gene expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

The expression of *Bcl2* genes was upregulated in root extracts. However, their expressions were downregulated by stem extracts dose-dependently (Figure 5.9). The results were not statistically significant in both extracts ($p = 0.157$, roots) and ($p=0.234$, stems). However, stem extracts elicited the required downregulatory responses as opposed to root extracts.



Effects of root extracts on BCL-2 gene expression

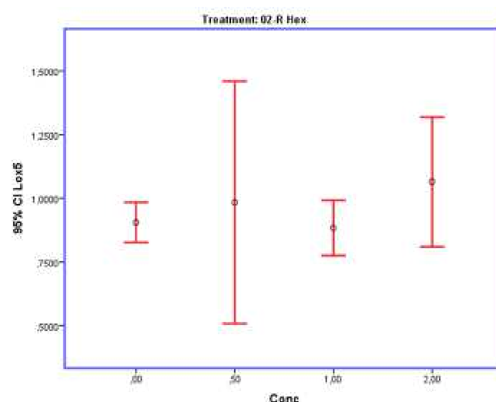


Effects of stem extracts on BCL-2 gene expression

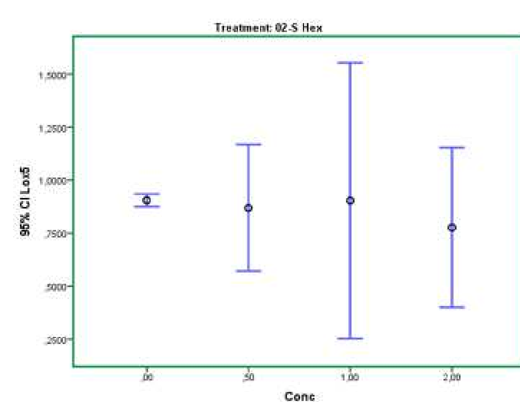
Figure 5.9. *Bcl2* gene expressions after treatment with hexanoic root and stem extracts

5.2.5 *5-LOX* gene expressions after treatment with hexanoic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with hexanoic root and stem extracts of WU, the expression of *5-LOX* genes was variably upregulated and downregulated in both extracts in a dose-dependent manner. Increased expression was observed to occur at 1 mg/mL and (2 mg/mL) in root extracts. Downregulatory effects were observed in stem extracts with minimal increase in expression and activity at 1 mg/mL (Figure 5.10). Beneficial effects can be derived from stem extracts only at a high concentration. In both cases, the effects elicited were not statistically significant (*p*=0.259, roots extracts and *p*=0.614, stem extracts).



Effects of root extracts on 5-LOX gene expression



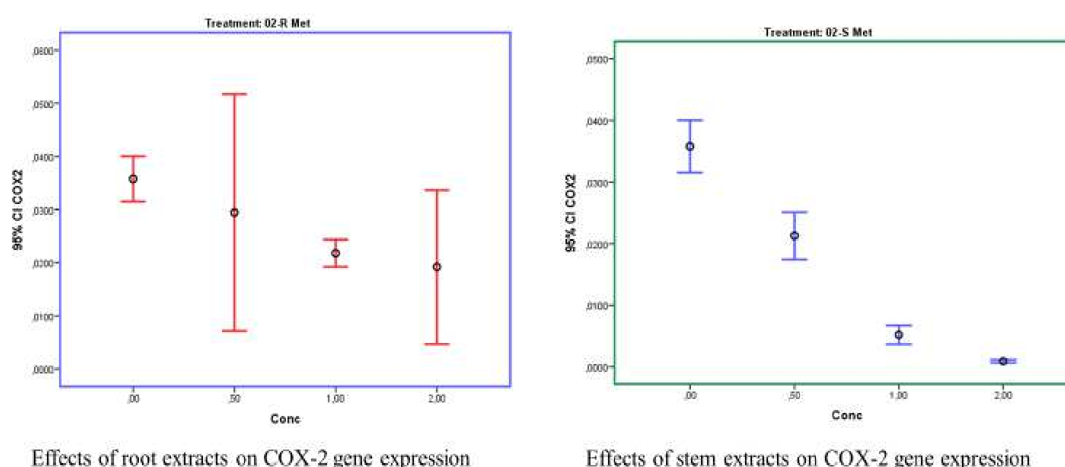
Effects of stem extracts on 5-LOX gene expression

Figure 5.10 *LOX* gene expressions after treatment with hexanoic root and stem extracts

5.3 Pharmacotherapeutic activity of methanolic extracts

5.3.1 *COX-2* gene expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were treated with methanolic root and stem extracts, *COX-2* genes were progressively downregulated in a dose-dependent manner (Figure 5.11), in both extracts. Effects of their expressions were also observed to be statistically significant ($p = 0.021$) and stem extracts (p=0.001), Table 5.2 and Annexed Table 3.



Effects of root extracts on COX-2 gene expression

Effects of stem extracts on COX-2 gene expression

Figure 5.11 *COX-2* gene expressions after treatment with methanolic root and stem extracts

Table 5.2 A summarized output of the relationship between the expression of target genes and the concentration/dosage of methanolic stem extracts of WU

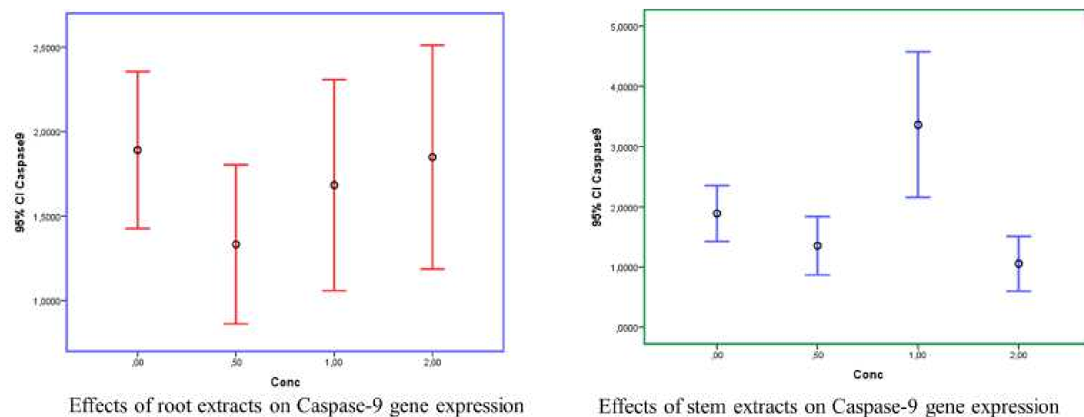
		Sum of Squares	df	Mean Square	F	Sig.
COX2	Between Groups	,002	3	,001	531,036	,000
	Within Groups	,000	8	,000		
	Total	,002	11			
Lox5	Between Groups	1,077	3	,359	46,241	,000
	Within Groups	,062	8	,008		
	Total	1,139	11			
Bcl2	Between Groups	,000	3	,000	84,439	,000
	Within Groups	,000	8	,000		
	Total	,000	11			
Bcl-xL	Between Groups	8,727	3	2,909	93,019	,000
	Within Groups	,250	8	,031		
	Total	8,977	11			

CASP9	Between Groups	9,454	3	3,151	36,713	,000
	Within Groups	,687	8	,086		
	Total	10,140	11			

Treatment = 02-S Met (*W. ugandensis*, Stem, Methanol)

5.3.2 *CASP9* gene expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with methanolic root and stem extracts of WU, there was increased (upregulation) expression of *CASP9* genes in a dose-dependent manner in root extracts (Figure 5.12), with high activity observed in all doses. However, up-regulatory effects were variant in stem extracts, with important effects only observed at lower concentrations. Up-regulatory effects were not statistically significant (p=0.059) in root extracts, whereas they were observed to be (p= 0.001) in stem extracts. The highest up-regulatory activity was observed at higher concentrations in root extracts, Table 5.2, and Annexed, Table 3.



Effects of root extracts on Caspase-9 gene expression   Effects of stem extracts on Caspase-9 gene expression

Figure 5.12 *CASP9* gene expressions after treatment with methanolic root and stem extracts

5.3.3 *Bcl-xL* gene expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

Upon treatment of Caco-2 cell lines with methanolic root and stem extracts of WU, the expression of *Bcl-xL* genes was downregulated in a dose-dependent manner, in both extracts (Figure 5.13). However, in root extracts, there was a slight shift in

increased expression at a high concentration (2 mg/mL). Nonetheless, the expressions were statistically significant (p = 0.002, roots and p=0.001, stems) observed in both extracts, Table 5.2, and Annexed, Table 3.

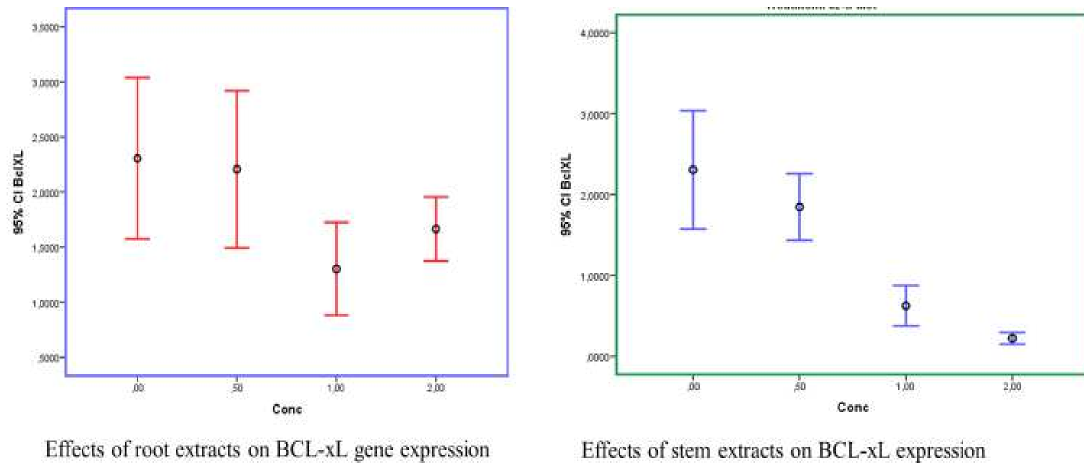
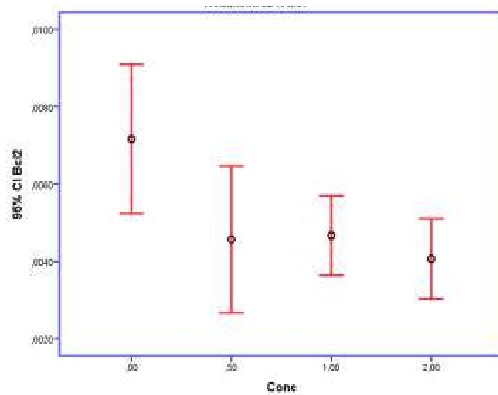


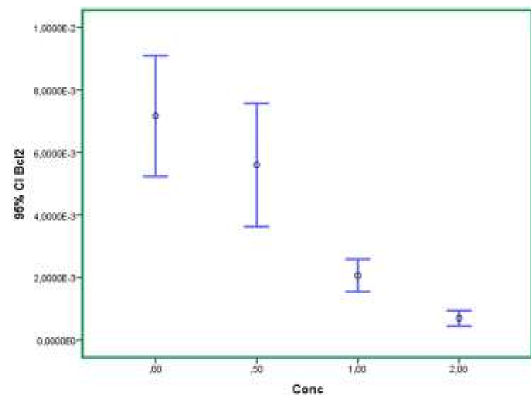
Figure 5.13 *Bcl-xL* gene expressions after treatment with methanolic root and stem extracts

5.3.4 *Bcl2* gene expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

The expression of *Bcl2* genes was downregulated in a dose-dependent manner, in both extracts (Figure 5.14). However, in root extracts, there was a slight shift in increased expression at a high concentration (2 mg/mL). Nonetheless, the observed expressions were statistically significant (p = 0.002, roots and p=0.001, stems) in both extracts, Table 5.2, and Annexed, Table 3.



Effects of root extracts on BCL-2 gene expression

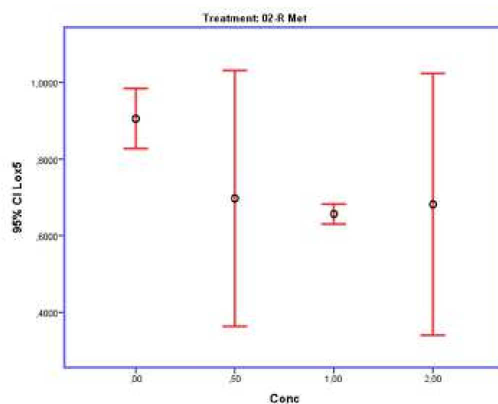


Effects of stem extracts on BCL-2 gene expression

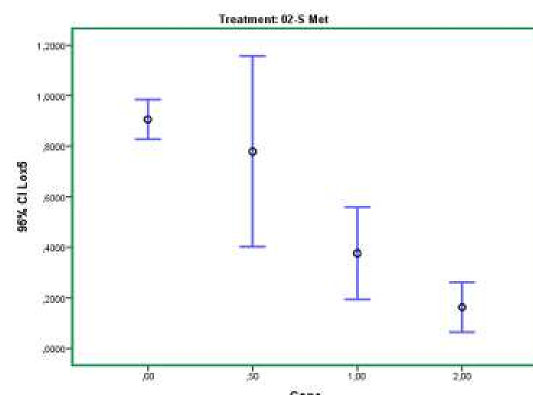
Figure 5.14 *Bcl2* gene expressions after treatment with methanolic root and stem extracts

5.3.5 *5-LOX* gene expressions after treatment with methanolic root and stem extracts of increasing dosage concentration

When Caco-2 cell lines were exposed to methanolic root and stem extracts of WU, the expression of *5-LOX* genes was downregulated in a dose-dependent manner, in both extracts (Figure 5.15). However, stem extracts exhibited the most significant beneficial activities compared to root extracts. At a high concentration, root extracts exhibited a slight increase in promoting gene expression. Both extracts elicited statistically significant effects (p=0.048, roots extracts and p=0.001, stem extracts), Table 5.2, and Annexed, Table 3.



Effects of root extracts on the expression of 5-LOX



Effects of stem extracts on the expression of 5-LOX

Figure 5.15 *5-LOX* gene expressions after treatment with methanolic root and stem extracts

6.1 Expression of targeted gene in Caco-2 cell lines after treatment with AS (hexane, ethyl acetate and methanol) leaf extracts

In this section, Caco-2 cell lines were treated with leaf extracts of AS at increasing concentrations of 0.00 mg/mL, 0.50 mg/mL, 1.00 mg/mL, and 2.00 mg/mL. Extracts were obtained using three different extraction solvents (hexane, ethyl acetate, and methanol).

6.1.1 *COX-2* expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

When Caco-2 cell lines were treated with hexanoic, ethanoic and methanolic leaf extracts, *COX-2* genes were downregulated variably with increasing dosage across the three different solvents (Figure 6.1 & Figure 6.2). In hexanoic treatments, downregulation was progressive with high activity observed at 0.50 mg/mL. The effects were not statistically significant (p=0.794), but sufficient to elicit beneficial activities. In ethanolic extracts, downregulatory effects were only observed at a high dose concentration (2.0 mg/mL). With regards to methanolic extracts, downregulatory effects were minimal in all doses applied. Just like in hexanoic extracts, the effects of using ethanolic and methanolic extracts were not statistically significant either (p=0.69 and p=0.0942).

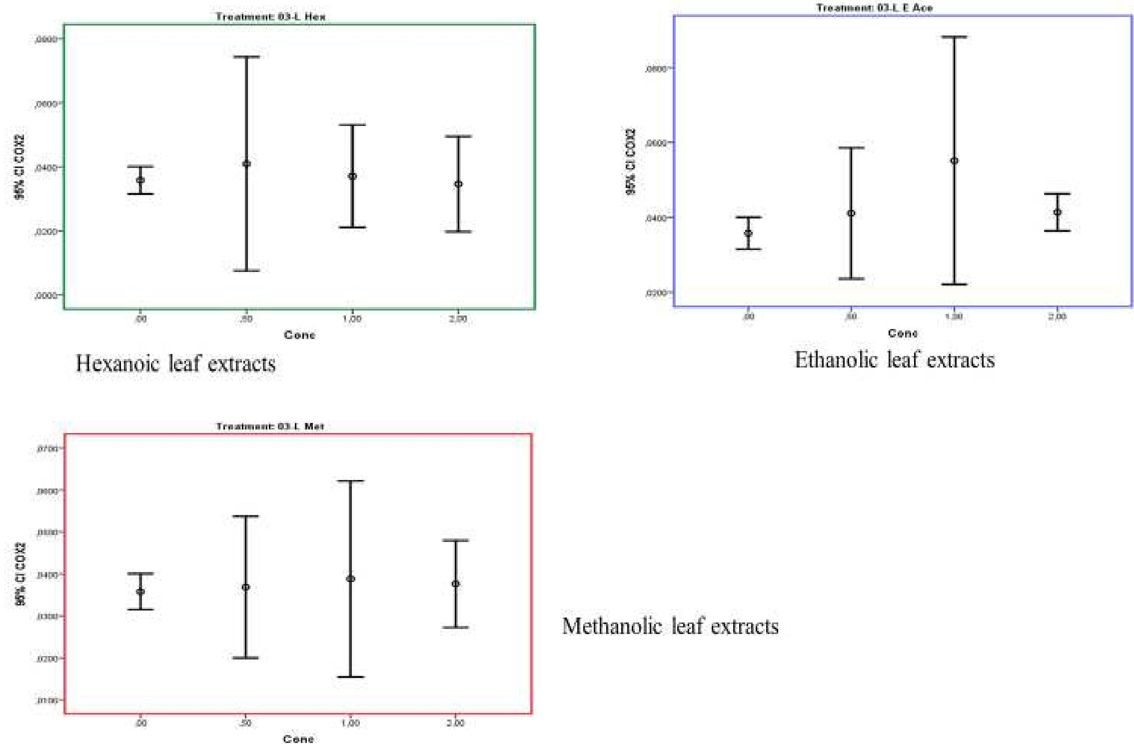


Figure 6.1 *COX-2* expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

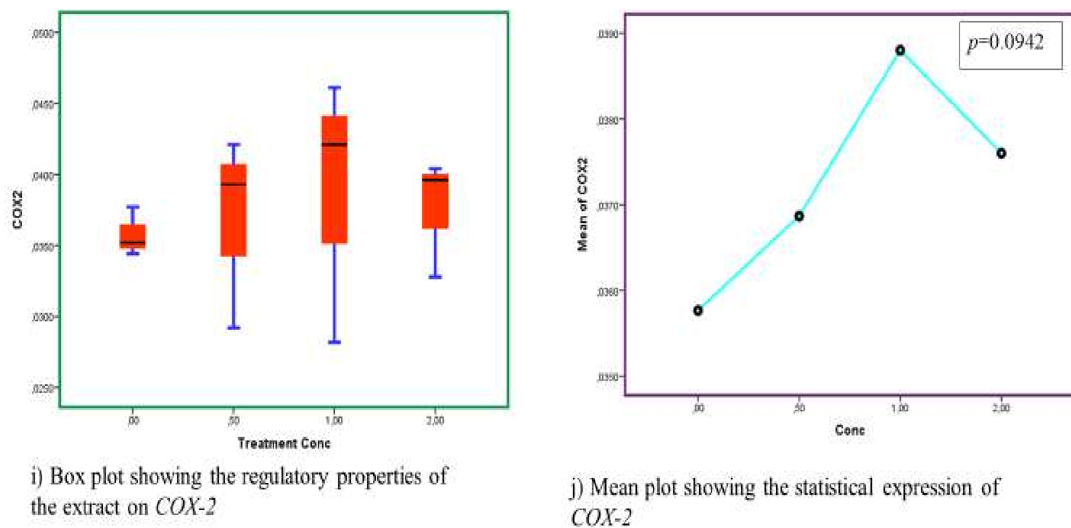


Figure 6.2. Varied regulatory properties observed in *COX-2* after treatment with AS methanolic leaf extracts. Sub-figures i & j represent box and mean lots respectively

6.1.2 *CASP9* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

The expression of *CASP9* genes was variant across the different solvents with significant beneficial effects observed at high concentrations in hexanoic and ethanolic extracts. Expressions were decreased at lower concentrations with meaningful upregulation occurring at high concentrations (Figure 6.3 & Figure 6.4). However, in both cases, the effects were statistically significant (*p*=0.017, hexane and *p*=0.001, ethyl acetate). In methanolic extracts, the expressions were progressively upregulated in a dose-dependent manner. The effects were statistically significant (*p*=0.001) and of a better efficacy compared to hexanoic and ethanolic extracts.

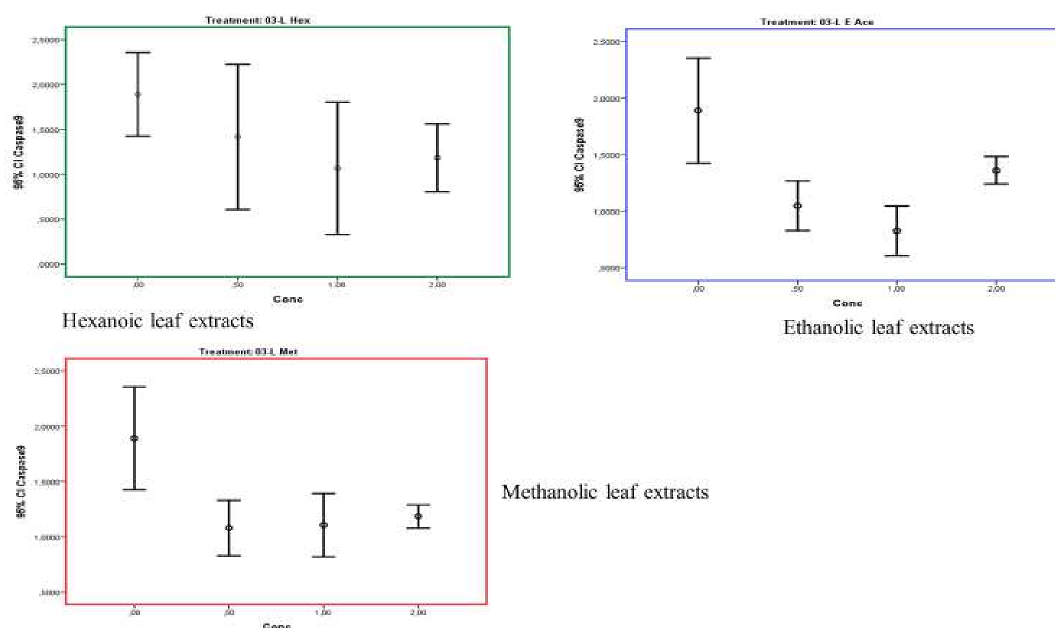


Figure 6.3 *CASP9* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

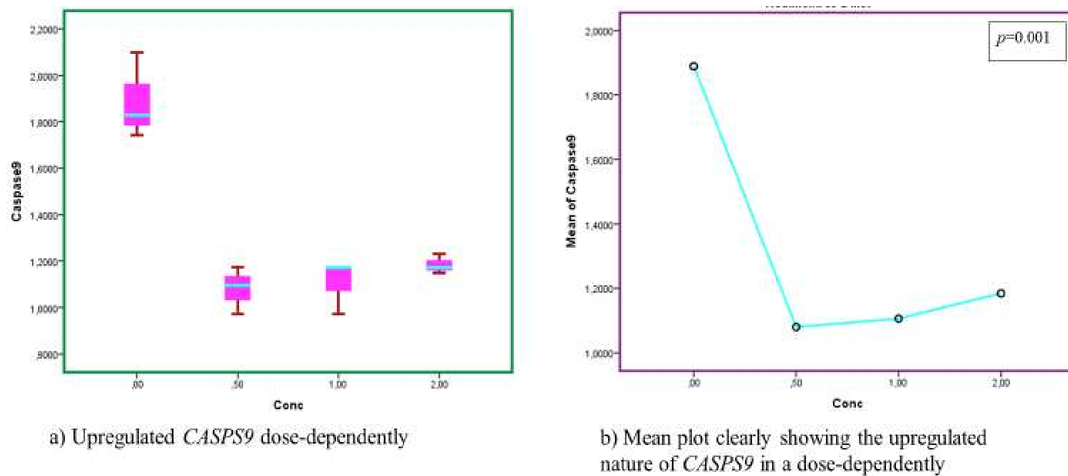


Figure 6.4. Gradual upregulatory activities were observed in *CASP9* after treatment with AS methanolic leaf extracts as clearly shown in sub-figures a & b, box, and mean plot respectively. The regulatory characteristics were statistically significant.

6.1.3 *Bcl-xL* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

In all treatments, downregulatory effects were progressively dose-dependent with higher activities observed at 0.50 mg/mL. The effects were statistically significant in ethanolic extracts (p=0.001), but not in hexanoic (p=0.129) and methanolic (p=0.330) extracts. Nonetheless, all extract treatments exhibited significant downregulatory and beneficial properties as required to stimulate downregulation (Figure 6.5, Figure 6.6).

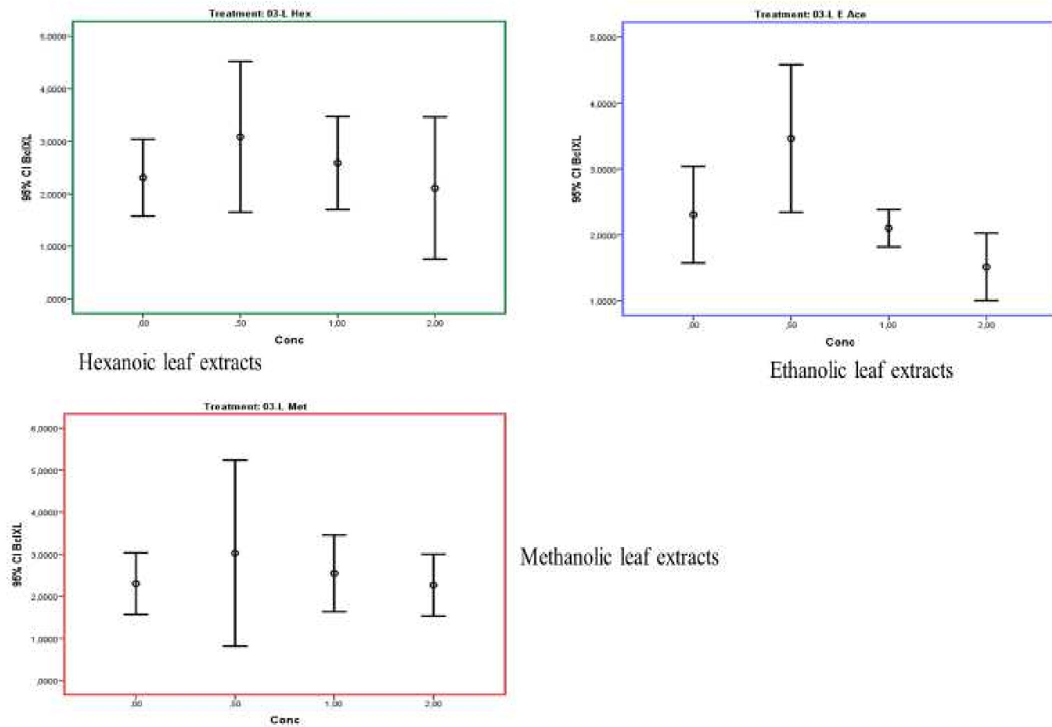


Figure 6.5 *Bcl-xL* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

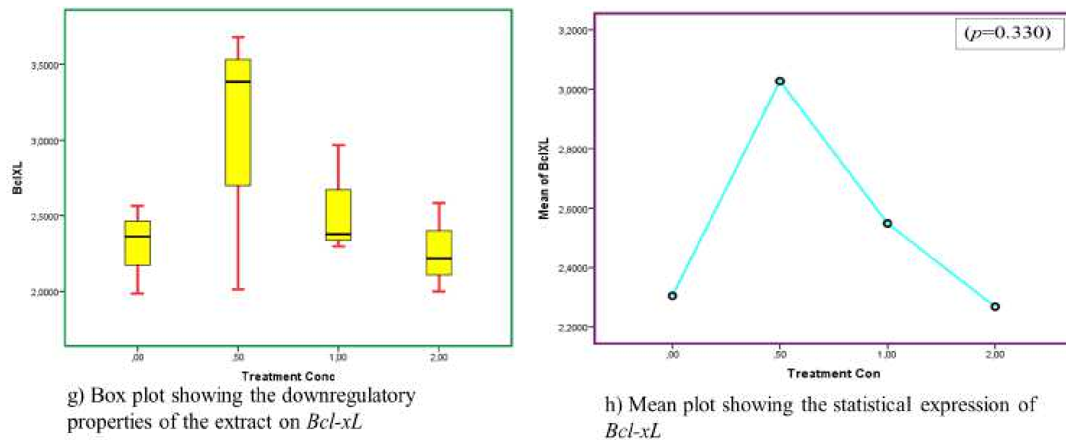


Figure 6.6 Downregulatory properties recorded in Bcl-xL after treatment with AS methanolic leaf extracts. Sub-figures g & h represent box and mean lots respectively.

6.1.4 *Bcl2* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

In all extract treatments, the decreased expression of *Bcl2* was observed to be dose-dependent. The effects were not statistically significant in all extraction solvents; ethanolic extracts (p=0.192), hexanoic extracts (p=0.316), and methanolic extracts (p=0.235). Nonetheless, all extract treatments exhibited significant downregulatory and beneficial properties as required to inhibit cellular growth (Figure 6.7 & Figure 6.8).

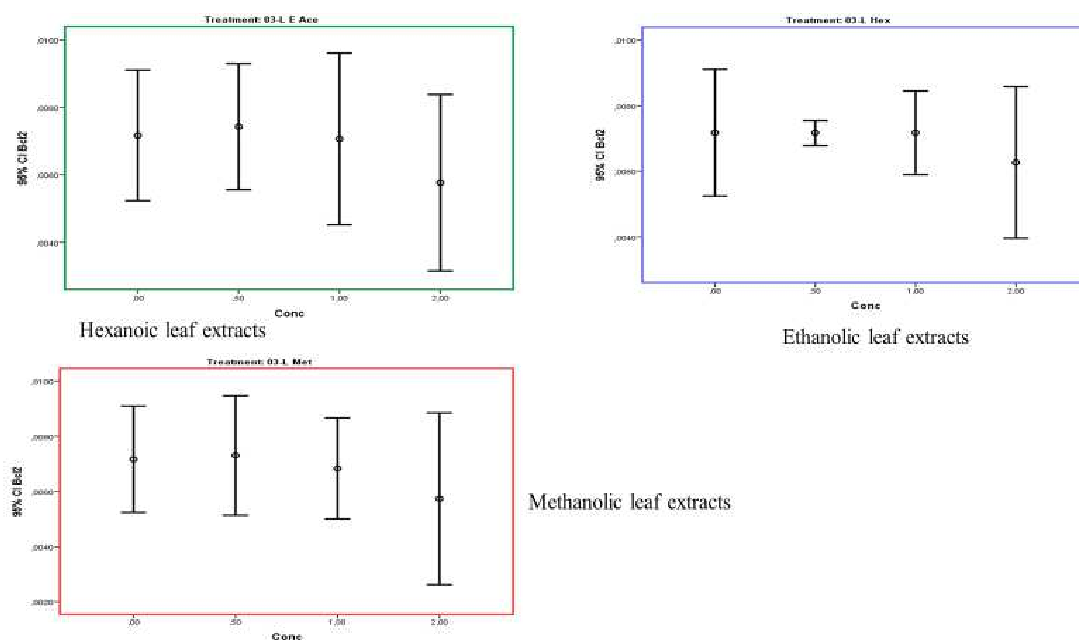
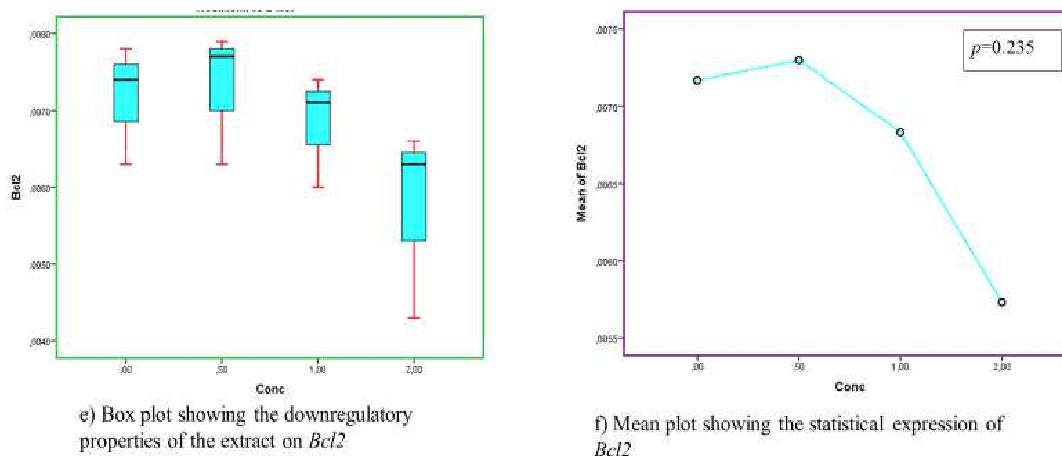


Figure 6.7 *Bcl2* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents



e) Box plot showing the downregulatory properties of the extract on *Bcl2*

f) Mean plot showing the statistical expression of *Bcl2*

Figure 6.8. Downregulatory properties recorded in *Bcl2* after treatment with AS methanolic leaf extracts. Sub-figures e & f represents box and mean lots respectively

6.1.5 *5-LOX* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

In all treatments, downregulatory effects were progressively dose-dependent with greater beneficial effects being observed in ethanolic and methanolic extracts. Effects were statistically significant in ethanolic and methanolic extracts (p=0.001) at almost equal proportions. Effects from hexanoic extracts were not statistically significant (p=0.569) extracts. However, the downregulatory properties exhibited were sufficient to elicit beneficial effects against CRC cellular growth (Figure 6.9, Figure 6.10). A mechanistic graphic depiction of the relative gene expressions of *CASPS9, 5-LOX, Bcl2/Bcl-xL*, and *COX-2* with increasing AS extract concentration is shown in (Figure 6.11).

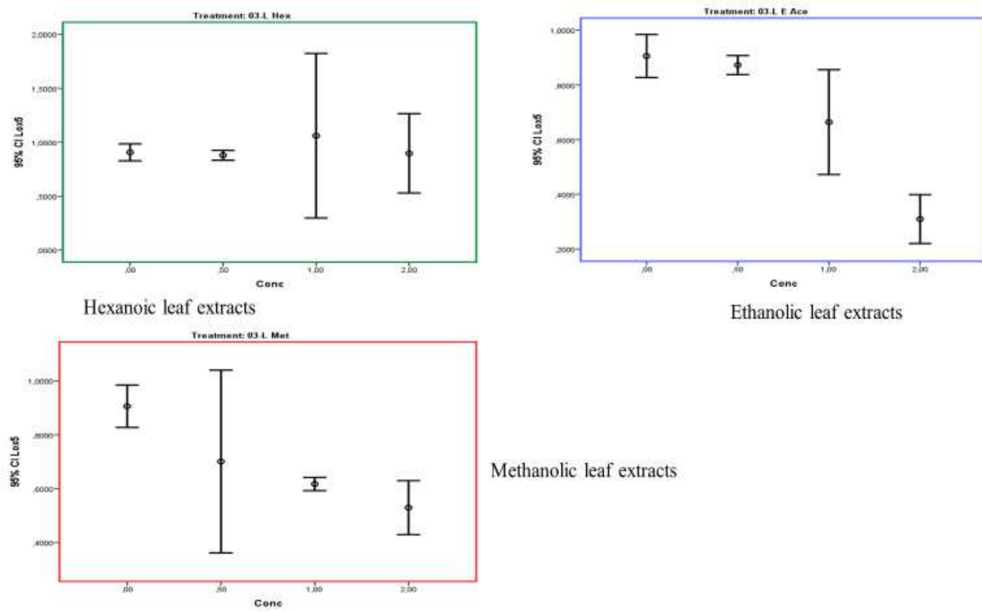


Figure 6.9. *5-LOX* gene expressions after treatment with extracts of varying concentrations obtained using hexane, ethyl acetate and methanol solvents

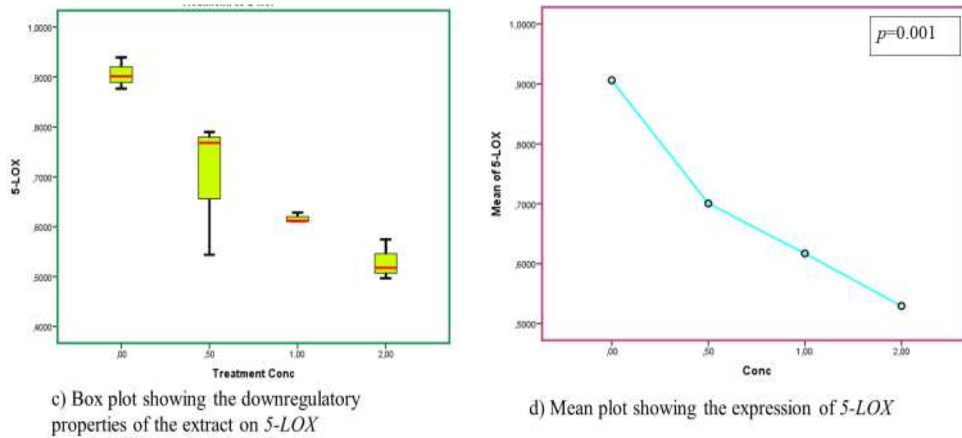


Figure 6.10. Downregulatory effects were observed in *5-LOX* upon treatment with AS leaf extracts as clearly shown in sub-figures c & d. The modulatory expressions were statistically significant. (c) Box plot, (d) Mean plot

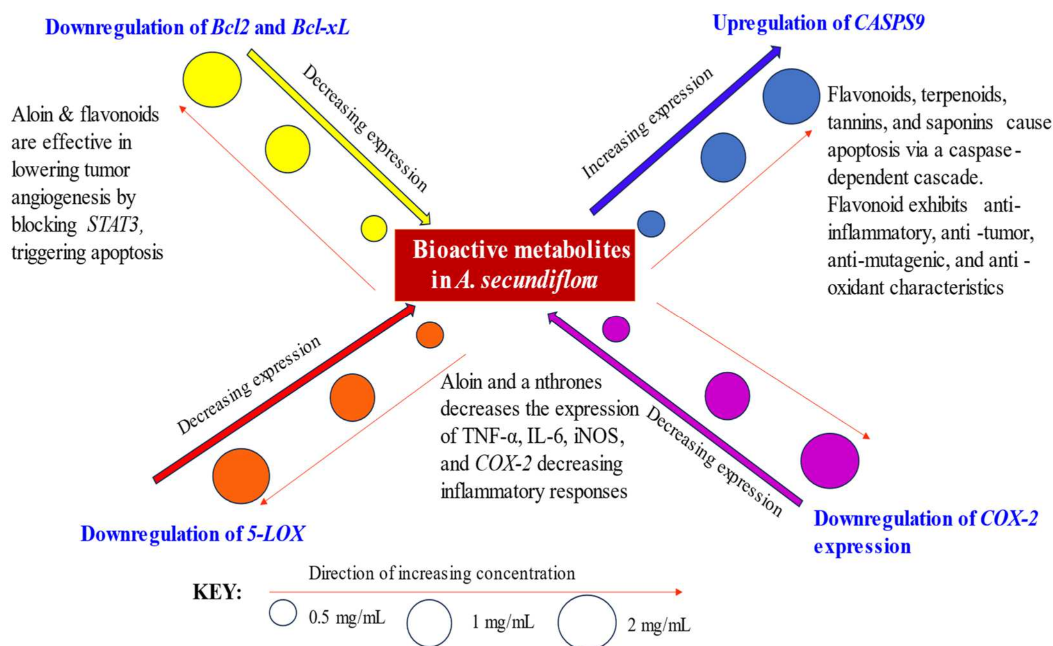


Figure 6.11. A summarized mechanistic schematic presentation of *CASP9, 5-LOX, Bcl2/Bcl-xL,* and *COX-2* relative expressions with increasing AS extract concentration.

7. Phytoconstituents present in WS, WU and AS

Methanol as an extraction solvent in the present study is a strong polar compound compared to the other two extraction solvents. The phytoconstituents present in the plant organs were therefore determined based on this polarity principle for a generalized determination of the metabolites present. Tables 7.1, Table 7.2, and Table 7.3 show the metabolites successfully established to be present from the target samples containing the methanolic extracts.

Table 7.1 A summarized table of the phytoconstituents present in WS.

Plant part/organ	Bioactive compounds
Roots	Withanolide A, Withaferin A, Withanolide sulfoxide, 27 Hydroxy withanone and choline
Stems	Choline, Somniferine, Withanine, Withanoside I, Quercetin, chlorogenic acid and catechin and Ashwagandhine

Table 7.2 A summarized table of the phytoconstituents present in WU.

Plant part/organ	Bioactive compounds
Roots	Drimane, ugandensial, warburganal, flavonoids, saponins, steroids,
Stems	Terpenoids, drimane, coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, tannins, flavonoids, saponins, steroids,

Table 7.3 A summarized table of the phytoconstituents present in AS.

Plant species	Bioactive compounds in leaves
Aloe secundiflora	Aloenin, nthoxanthins, flavanones, Anthraquinones, Aloina and terpenoid

CHAPTER FIVE

5. DISCUSSION

5.1 Phytotherapeutic potential of WS, WU and AS on selected target genes in Caco-2 cell line

In this study, I attempted to analyze the effect of WS extracts. This section focuses on the phytotherapeutic activities and effects of plant extracts from different organs, on the expression of *COX-2, CASP9, Bcl-xL, Bcl2,* and *5-LOX* genes in Caco-2 cell lines. The effects are analyzed, observed, and compared among 3 different extraction solvents; hexane, ethyl acetate, and methanol as elucidated in the methodology section, and the most effective solvent (s) determined for recommendation.

5.1.1 WS and WU

5.1.1.1 Phytotherapeutic effects of roots and stem extracts on *COX-2* expression

Cyclooxygenase 1 & 2 (COX-1 & COX-2) are rate-limiting enzymes involved in the conversion of arachidonic acid into inflammatory prostaglandins. Chronic inflammation increases the risk of cancer [181]. During inflammation, *COX-2* is strongly inducible. *COX-2* selective inhibitors are thought to have the same anti-inflammatory, anti-pyretic, and analgesic actions as nonselective inhibitor NSAIDs with minimal or no gastrointestinal side effects [181]. Being enzymes involved in the synthesis of lipid prostaglandins, they are significant in the body's metabolic activities [182]. While *COX-2* is impacted and plays a significant role in inflammatory processes, *COX-1* modulates homeostasis [183]. In the colon, *COX-2* is expressed at low levels, but under stressful circumstances, it may be affected by cytokines, tumor necrosis factors, growth factors, and lipopolysaccharides. Elevated *COX-2* levels are associated with the development and spread of colon cancer [35]. Results from our experimental study demonstrate that upon exposure of Caco-2 cell lines on root and stem extracts of WS and WU, *COX-2* genes were increasingly downregulated in a

dose-dependent manner, with greater efficacious benefits being adequately observed in ethanolic and methanolic solvents. The significant benefit in the inhibition of *COX-2* observed in our study is attributable to the abundant presence of the biochemical metabolites already established in both roots and stems of WS and WU.

In WS, 27-desoxy-24, 25-dihydrowithaneferin A, 27-O-glucopyranosylviscosalactone B, 4,16-dihydroxy-5 h, 6h-epoxyphysagulin D, Diacetylwithaferin A, Physagulin D (1→ 6)-h-D-glucopyranosyl-(1→4)-h-D-glucopyranoside, Viscosalactone B, Withaferin A, Withanolide sulfoxide and Withanoside IV have all been implicated in suppressing the expression of COX-2, (Table 2.2) [68,74,75].

In WU, terpenoids, sesquiterpenoids, tannins, flavonoids, saponins, muzigadial, steroids, polygodial and mannitol, have all been widely implicated in inhibiting the expression of *COX-2,* (Table 2.4) [162,181,184–190]. The downregulatory effect observed is therefore potentially caused by these anti-inflammatory metabolites present in the roots and stems of WU utilized in our study. The concentration of metabolites especially drimane and coloratane sesquiterpenoids have been reported to be abundant in the stems [150], and this could explain the efficacious significant difference realized in our results with stem extracts. Notably, the roots and stems of WS and WU could therefore serve as promising alternative phytotherapeutic *COX-2* inhibitors to synthetic agents that are widely reported to exhibit adverse effects [3].

5.1.1.2 Phytotherapeutic effects of roots and stem extracts on *CASP9* expression

CASP9, the initiator of the mitochondrial caspase pathway, is an important mediator in the control of apoptosis. These enzymes (Caspases) are part of a cascade that is activated by proapoptotic mandates and results in the dissociation of a variety of peptides and cell fragmentation. Understanding caspase programming is critical for selectively modulating apoptosis for medicinal purposes [78,79]. Apoptosis is a vital

physiological process that involves the selective removal of cells in a range of biological events [191]. It has been argued that suppressing spontaneous apoptosis increases the risk of cancer [80,81]. Comparably, a greater incidence of colorectal adenoma has been reported to be highly linked with a decreased rate of apoptosis [82]. Our results demonstrated that upregulation of *CASP9* in Caco-2 cells occurred variably upon exposure to roots and stem extracts, presenting WS and WU as attractive stimulators of apoptotic effects. We observed that, upon exposure of Caco-2 cell lines on root and stem extracts of WS and WU, *CASP9* genes were increasingly upregulated in a dose-dependent manner, with greater efficacious benefits being adequately recorded in ethanolic and methanolic extracts for root and stem extracts. With minimal emphasis, the highest up-regulatory activity was observed at higher concentrations in root extracts in WU compared to stem extracts. This means that root extracts elicited significant beneficial effects in comparison with stem extracts. Hexane demonstrated significant extraction potential in root extracts only.

One potential chemo-prevention strategy is the ability to induce apoptosis in gastrointestinal epithelial cells [81]. The suppression of natural apoptosis has been hypothesized to enhance the incidence of cancer [80,81]. Similarly, it has been noted that a higher prevalence of colorectal adenoma is highly correlated with a lower rate of apoptosis [82]. The capacity to trigger apoptosis in epithelial cells of gastrointestinal origin is one of the potential strategies in chemo-prevention [81]. As a result, investigating the apoptotic mechanism is a viable avenue for colorectal cancer. When determining the prognosis for individuals with stage II colorectal cancer, the degree of expression of the apoptosis-associated genes *CASP9* and *CASP10* could prove useful. It appears that the carcinogenesis of colorectal cancer involves both the death-receptor-mediated and mitochondrial pathways. The lifespan of aberrant mucosa cells

is increased by the decreased expression of *CASP9* and *CASP10* [83,84]. As a result, these cells have the potential to undergo further gene mutations and eventually give rise to cancerous cells.

It has been suggested that the activity of withanolides is mediated via control of nuclear factor-kappa B (NF-kB) expression because NF-kB regulates numerous genes that affect cell division, malignancy, cancer metastases, and inflammation. This suggests that withanolides prevent NF-kB activation and NF-kB-regulated gene expression, which could help clarify why they can increase apoptosis while preventing invasion [85]. According to a study using the Leukemic Murine mouse model, withanolide D lowers anti-apoptotic genes (TERT, Bcl-2, and Puma) [66]. Under high levels of ROS, dysregulation of *Bax/Bcl2* expression, and concurrent disruption of mitochondrial membrane potential ($\Delta\Psi m$), a novel fraction of proteins isolated from WS roots could stimulate mitochondria-mediated apoptosis in triple-negative breast cancer cells (MDA-MB-231) at an IC_{50} dose of 92 g/ml. Also reported [86] were *CASP3* stimulation, G2/M cell cycle arrest, and nuclear lamin protein cleavage. Furthermore, the crude water extract (0.5%) of WS modified the signaling cascade including pro-apoptotic and tumor-promoting proteins, which helped to inhibit tumor growth [66].

The association between *CASP9* and colorectal cancer is still poorly understood to date. Studying its association with clinicopathological characteristics and longevity may provide insightful data for predicting survival and choosing additional treatment strategies. The WS ethanolic extract is cytotoxic (99.7 g/ml) on cancerous cells, induces apoptosis, and inhibits angiogenesis and cell migration [87]. The possible anticancer action of the extracts of WS is due to the increased autophagy induction and apoptotic effects of the plant. Withaferin A causes apoptosis and Mad2 and Cdc20, a crucial component of the Spindle Checkpoint Complex, are degraded by proteasomes.

By restoring correct anaphase initiation and maintaining a greater number of viable cells, further overexpression of Mad2 partially reverses the harmful effect of Withaferin A. It is hypothesized that Withaferin A kills cancer cells by delaying the mitotic exit and then causing chromosome instability [70]. Although various WS compositions have highly promising anti-cancer properties in both *in vitro* and *in vivo* applications there are currently no authorized therapeutic candidates. For the discovery of novel anti-cancer pharmaceuticals, conducting clinical trials with WS phytochemicals/formulations is urgently necessary. Withaferin A exposure has been demonstrated to enhance the amount of late apoptotic cells and the aggregation of cells in the subG1 arrest in the cell cycle. It has been linked with induction of apoptosis via PARP and caspase-3 cleavage [68–70,76].

Natural phytoconstituents also common in WU such as terpenoids, drimane and coloratane sesquiterpenoids [192], flavonoids [192], tannins [193], cinnamolide and its derivatives [194], and saponin [195–197] have been reported to induce and promote apoptosis via Caspase-dependent cascade. Considering the above, studying the apoptotic pathway of associated genes in a dose-dependent manner, as is the case in our novel study, is a promising treatment option for CRC.

5.1.1.3 Phytotherapeutic effects of roots and stem extracts on *Bcl-xL* & *Bcl2* expression

Knowing the affinity and concentration of each member of the Bcl2 family is necessary to comprehend the main interactions that take place between them. These variables determine the dominant interactions, which determine whether mitochondrial outer membrane permeabilization (MOMP) takes place [198]. *Bcl2* is located in the mitochondrion, endoplasmic reticulum (ER), and the nuclear envelope, while *Bcl-xL* is found in the nuclear envelope, extranuclear membranes such as the

mitochondrion, and the cytoplasm [199]. *Bcl2* and *Bcl-xL* have intricate mechanisms of action, with many proposed associations with other proteins, and the significance of any particular interaction in the final phenotype at the cellular level is unclear [200]. *Bcl-xL* knockdown with siRNA and prexasertib therapy has been shown to significantly increase apoptosis. Furthermore, the combination of prexasertib with navitoclax has been shown to have a high antitumor impact and cause apoptosis in malignant cells via downregulating *Bcl-xL* [191]. The Bcl-2 protein family, which includes anti-apoptotic and pro-apoptotic members, is the best-defined protein family involved in the modulation of apoptotic cell death. Anti-apoptotic members of this family, such as *Bcl2* and *Bcl-xL*, inhibit apoptosis in two ways sequestering proforms of death-driving cysteine proteases (caspases) or preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor). After entering the cytoplasm, cytochrome c and AIF directly activate caspases, which cleave several cellular proteins, resulting in apoptosis [191]. Our study demonstrated that stem extracts from WS and WU significantly downregulated the expression of *Bcl-xL* and *Bcl2* in a dose-dependent manner. Both genes uniquely expressed similar modulatory expressions. However, their similarity in expression is attributable to being gene-members of the same anti-apoptotic family [198]. The decreased expression of *Bcl-xL* and *Bcl2* correlates to increased apoptotic effects, critical in the antiproliferation of cancerous cells. In principal summary, *Bcl-xL* and *Bcl2* mediate apoptosis [201] and might represent a promising target for CRC therapy as demonstrated through our analyzed laboratory results. The characteristic downregulation of *Bcl-xL* and *Bcl2* by the treatment extracts reported in our study is attributed to the occurrence of a wide range of apoptosis-stimulating active metabolites discussed earlier as being present in WS and WU. In both plants, ethyl acetate and

methanol extraction solvents yielded metabolites with the most significant *Bcl-xL* and *Bcl2* inhibitory benefits. In hexanoic extracts (using hexane extraction solvent), those derived from roots of WS, and stem extracts of WU were the only satisfactorily efficacious in downregulation of *Bcl2* dose-dependently.

In CRC, 27-desoxy-24, 25-dihydrowithaneferin A, 27-O-glucopyranosylviscosalactone B, Withaferin A, Withanolide sulfoxide, and Withanoside IV have been linked to reduced *Bcl2* expression [68]. Withaferin A has been shown to increase the number of late apoptotic cells as well as cell aggregation in the subG1 cell cycle arrest. Furthermore, it reduces the amounts of anti-apoptotic proteins like Bcl2 and Bcl-xL while promoting apoptosis via PARP and caspase-3 cleavage [68–70,76].

Data in the present research study shows that *Bcl-2/Bcl-xL* inhibition enhances their decreased expression, which consequently results in decreased CRC (Caco-2) cell line growth. Until there is a better quantitative understanding of how Bcl-2 family proteins govern apoptosis in cells, pharmacological manipulation of the Bcl-2 family activities will be restricted. Our research nonetheless emphasizes the necessity of further investigating Bcl-2 family connections, as well as their pharmacological modulation using the widely available WS and WU phytoconstituents *in vivo,* following our successful *in vitro* application. We demonstrate for the first time that *Bcl2/Bcl-xL* and their modulation in the corresponding intracellular signaling pathways play a pivotal role in the potential activity of WU against CRC cells. Following the comparative nature of the solvents deployed in the extraction of plant metabolites, the deployment of ethyl acetate and methanol as extraction solvents for effective extraction of *Bcl-xL* and *Bcl2* modulatory phytoconstituents is highly recommended from this unique study.

5.1.1.4 Phytotherapeutic effects of roots and stem extracts on *5-LOX* expression

Lipoxygenases (LOXs) are extensively distributed in plants, mammals, fungi, and some bacteria. They are non-heme, iron-containing enzymes. The first two steps in the production of the peptide-LTs and the chemoattractant factor LTB4 are catalyzed by the 5-LOX, a Ca2+- and ATP-dependent enzyme. Granulocytes, monocytes/macrophages, mast cells, and B lymphocytes are among the myeloid cells that express the *5-LOX* protein genome [202,203]. Several LOXs produce metabolites in the arachidonic acid pathway that appear to promote carcinogenesis, hinting that targeting these pathways may be effective in slowing the progression of CRC and other types of cancers [204–206]. 5-LOX and its products 5(S)-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-S-HETE) and leukotriene (LT)-B_4 are among these LOXs and metabolites [205]. 5-LOX is thus a dioxygenase that transforms arachidonic acid to 5-S-HETE, which is then transformed by LTA_4 hydrolase to LTB_4 [207].

Our results in WS demonstrated that ethanolic and methanolic (roots and stems) extracts significantly downregulated the expression of *5-LOX* in a dose-dependent manner. However, in hexanoic extracts, the effects were variably expressed with an increasing concentration of either plant organ extract used. It is therefore deduced that ethyl acetate and methanol are excellent extraction solvents for maximum extraction of beneficial metabolites capable of downregulating the expressive properties of *5-LOX*.

In WU, results demonstrated that methanolic root and stem extracts significantly downregulated the expression of *5-LOX* genes in a dose-dependent manner compared to the other extraction solvents (Ethyl acetate and hexane), (Figure 5.15). However, stem extracts exhibited the most significant beneficial activities compared to root extracts. At a high concentration, root extracts exhibited a slight increase in promoting

gene expression. Both extracts elicited effects that were statistically significant with displayed efficacy. On the other hand, ethanolic extracts interestingly yielded downregulatory (root extracts in lower concentration) and up-regulatory (stems in a dose-dependent manner) effects. This observation suggested that root extracts constituted the right metabolite components effective in eliciting beneficial effects in suppressing the expression of *5-LOX* in minimal concentrations (0.5-1.0 mg/mL). Regarding hexanoic extracts, downregulatory effects were observed in stem extracts with minimal increase in expression and activity at 1 mg/mL. This result suggests that beneficial effects can be derived from stem extracts only at a high concentration if extractions are done using hexanoic extraction solvents. We suggest that this difference in activity be further studied to decipher and determine the specific metabolites involved in the downregulation of *5-LOX* present in either of the plants' organs. WU neolignanamides have been identified as active components responsible for their anti-inflammatory and anti-proliferative effect, with the potential to decrease *5-LOX* expression [161].

Although the sequence of events linking cancer development and *5-LOX* gene expression is uncertain, it appears that *5-LOX* expression is occasionally elevated during neoplastic transformation [205]. In *in vivo* and *in vitro*, *LOX* inhibitors decrease cancer cell proliferation and induce death via the mitochondrial route [206,207]. Other authors have also implicated WS ethanolic extracts with a 65% inhibition potential of *5-LOX* with an IC_{50} value of 0.92 mg/mL [202]. Together, the findings of this study and other research point to the potential value of *5-LOX* overexpression and 5-LOX signaling pathway inhibition as targets for the prevention and treatment of CRC. Natural *5-LOX* inhibitors present in WS and WU are thus validated in this study for application in the treatment of human CRC. The highest recommendations are given

on the use of ethyl acetate and methanol for WS efficacious activities, while methanol was most efficacious in WU, and thus highly recommended.

5.1.1.6.1 Cytotoxicity and safety properties of WS

During the treatment of oncologic disorders, the impact of anticancer medications on healthy cells is a major source of worry. In various cancer treatments, most chemotherapeutic agents have an identical effect on normal and malignant cells, which is the primary cause of minor to severe adverse outcomes [45]. Researchers have undertaken very little research to fully grasp this significant problem. Withanolides have been investigated for their potential cytotoxicity toward normal cells as well as their powerful anticancer properties [45].

A combination of Withanolide A and Withaferin A offers multimodal activities against CRC cell lines. These chemical components are reported to trigger the cytotoxicity of cells in various cancerous cell lines [55]. The proposed cytotoxicity mechanisms comprise activating the extrinsic and intrinsic apoptosis signal cascades, induced by intensified generation of nitric oxide reactive and oxygen species within the cancerous cells [3]. The standardized extract of WS did not exhibit any toxicity at a concentration of 2000 mg/kg, according to a specific acute and sub-acute toxicity investigation [45,208]. Another comparable in vivo investigation [209] reported no harmful effects from the hydroalcoholic extract of WS roots. The WS root extract did not have any maternal or fetal toxicity in mice, according to an investigation on gestational developmental adverse effects [210].

According to research by Baig et al. [211], withanolides have a lower cytotoxic effect on normal cells than they do on cancerous cells. For example, with an IC_{50} of 99.7 g/ml, the extract exhibited cytotoxic action against lung cancer cells [87]. In a different investigation, WS extract's anti-proliferative efficacy additionally demonstrated lower

cytotoxicity in normal cells as compared to DU145 prostate cancer cells [73]. Similar findings were made regarding the cytotoxic activity of WS extract on normal Vero cells compared to the human MDA-MB-231 breast carcinoma cell line [212]. According to Nishikawa et al. (2015), withaferin A had negligible cytotoxic properties on normal fibroblasts (TIG-1 and KD cells) but had anticancer properties on prostate cancer cells (PC-3 and DU-145). Withaferin A and withanone therapy in combination have shown improved cytotoxic ability against tumor cells. The restriction of growth and invasion in the cancerous cells was brought about by the downregulation of hnRNP-K, VEGF, and MMP-3 [65]. *Withania* and its numerous species have also been examined for their harmful effects [213]. According to the study of 69 clinical and preclinical research, WS roots were shown to be effective as well as safe. It has been demonstrated to be non-toxic and effective clinically for human health and wellness, from its use in antiquity to its present applications [214].

5.1.1.6.2 Cytotoxicity and safety properties of WU

Plant extracts from WU have also been linked to cytotoxic [156] and antioxidant [12] effects. Intriguingly, WU extracts have been found to have an oral LD50 > 5000 mg/kg body weight in mice, which is higher than that of other evergreen native tree species. No mortality has been reported at dose levels between 500 and 5000 mg/kg body weight, suggesting safety for usage in humans [162].

Despite the lengthy history of therapeutic uses of WU, its medicinal benefit has garnered less attention. Ethnopharmacological studies should be encouraged to investigate the most efficient way of plant selection, extraction, chemical synthesis, and dose calculation to identify and reap major benefits from this plant species [22]. For this reason, the present study aimed to evaluate among others, these properties from WU, ethanolic, hexanoic, and methanolic roots and stem extracts on the expression of the five well-elaborated targeted genes, dose-dependently. This study undoubtedly presents the plant as a viable alternative to synthetic inhibitors with favorable benefits in CRC therapeutic management. As it is, however, there is little information available on this important plant species, particularly *in vivo* and *in vitro* CRC experimental trials. This work offers a novel paradigm for a potential mechanism of action for existing well-known chemicals, as efficacious anti-CRC treatments by their regulatory effects on specific gene expression as demonstrated in this study. As a result, this is the first study of its kind to assess the plants' *in vitro* use at various dose levels for application *in vivo* and clinical trials, with significant therapeutic effects hypothesized in CRC management in humans.

6. Genome regulatory effects of AS leaves extracts

6.1 Phytotherapeutic effects of AS' extracts on *COX-2* expression

Investigations into the botanical compounds and pharmacological activities of *Aloe* species have led to the discovery of various active compounds. *Aloe* species have been employed in ethnopharmacology. In the past, herbal practitioners have used them to treat a range of diseases. Anthraquinones comprise most of the bioactive compounds found in aloe species, which are plentiful natural sources. As has been highlighted before in section 2, the preliminary chemical compound analysis of AS has demonstrated the abundant occurrence of aloin (AL), terpenes, flavonoids, tannins, and other useful phytoconstituents in the leaves (Table 2.4). By blocking *STAT3* activation in CRC cells, aloin is an effective therapeutic for lowering tumor angiogenesis and growth in both in vivo and in vitro experimental settings [114]. With regards to *COX-2* expression, as has been discussed in Section 5.1.1.1, cancer risk is increased by persistent inflammation [181] while strong induction of *COX-2* occurs during inflammation.

This study demonstrates that hexanoic extracts downregulated the expression of *COX-2* in a dose-dependent manner. Further, hexane elicited better *COX-2* inhibitory effects compared to the other solvent extractants. In ethanolic extracts, downregulatory effects were only observed at a high dose concentration while in methanol, downregulatory effects were quite minimal across all doses applied. Of significance to note is that one of the crucial enzymes involved in the manufacture of prostaglandins, and is linked to inflammatory processes, is the COX-2 enzyme. Relief from these inflammatory conditions may result from inhibition of cyclooxygenase and, consequently, of the prostaglandin biosynthetic pathway [215].

Uniquely, this is one of the very 1st studies that directly link AS leaf extracts with downregulatory activities of *COX-2* in CRC cell lines, with potential applications for CRC management. Given our comparative analysis of the 3 solvents deployed for extraction, it is therefore recommended that hexane is the most effective extraction solvent in obtaining efficacious metabolites for *COX-2* inhibitory purposes in CRC management.

6.2 Phytotherapeutic effects of AS' extracts on *CASP9* expression

As discussed in section 5.1.1.2, an essential mediator in the regulation of apoptosis is *CASP9*, the starter of the mitochondrial caspase pathway. Caspase enzymes are a component of a cascade that is triggered by proapoptotic signals and causes the fragmentation of cells and the dissociation of several peptides. It has been reported by other authors that aloin effectively reduced tumor sizes and weight in mice xenografts while reducing tumor cell viability and causing apoptosis in vitro [114]. The findings of this study showed that methanolic extracts demonstrated the highest efficacy with significant activity in the upregulation of *CASP9,* in a dose-dependent manner. However, in ethanolic and hexanoic solvents, the expression of the *CASP9* genes varied, with significant positive effects only being shown at high concentrations. Methanol is thus highly recommended for use as an efficient extraction solvent for bioactive ingredients with demonstrated upregulatory effects of *CASP9* in CRC treatment. It is further highlighted that this is one of the 1st research studies to evaluate and document the activity of AS's active ingredients in the modulation of CRC *CASP9* genes responsible for programmed cell death (apoptosis), for deployment in CRC treatment.

6.3 Phytotherapeutic effects of AS' extracts on *Bcl-xL* and *Bcl2* expression

The best-defined protein family involved in the regulation of apoptotic cell death is the Bcl-2 protein family, which comprises members that are both anti- and pro-apoptotic. The anti-apoptotic members of this family include, among others, *Bcl2* and *Bcl-xL*, as was previously mentioned in Section 5.1.1.3. In the present study it has exclusively been demonstrated that in all treatments, downregulatory effects of the extracts (ethanolic, hexanoic, and methanolic) on *Bcl-xL* and *Bcl2* were progressively influenced dose-dependently. All extract treatments exhibited significant downregulatory and beneficial properties as required to stimulate inhibitory properties on genetic expression. Other investigators have also asserted that aloin is effective in lowering tumor angiogenesis and growth by blocking *STAT3* activation in CRC cells, which in turn controls the expression of the antiapoptotic protein *Bcl-xL* gene [114]. One of the latent self-signalling transcription factors in the cytoplasm is *STAT3* (e.g., VEGF), which is activated by cytokines (like IL-6) and progenitor cells. The stimulation of *STAT3* homodimerization and nuclear translocation modulates the transcription of responsive genes encoding apoptotic cell death inhibitors (e.g., *Bcl-xL, Bcl2*) and inducers of angiogenesis (e.g., VEGF) [125]. These genes play roles in human defense evasion, angiogenesis, metastatic spread, cell survival, differentiation, and programmed cell death [126]. In recent years, there has been an abundance of research demonstrating that blocking constitutive *STAT3* signaling substantially inhibits tumor development and triggers apoptosis [125,127]. It is therefore imperative to understand that *Bcl2* & *Bcl-xL* inhibition induces beneficial apoptotic effects which consequently decreases CRC tumor growth. This is therefore the 1st study that has successfully evaluated the downregulatory effects of AS's extracts using different extraction solvents on the expression of *Bcl2* & *Bcl-xL* in colorectal cancer cell lines.

In line with these fundamental findings, ethanolic, hexanoic, and methanolic leaf extracts of AS are strongly recommended for considerable deployment for further *in vivo* trials and subsequent clinical trials for the therapeutic management of CRC in humans, with substantial beneficial effects postulated.

6.4 Phytotherapeutic effects of AS' extracts on *5-LOX* expression

Inhibiting the expression of *5-LOX*, which is upregulated in colorectal cancer, could be helpful in both the prevention and therapy of the disease [216]. It has been shown that eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes, among others, act as powerful autocrine and paracrine regulators of cell biology when arachidonic acid, a polyunsaturated fatty acid, is metabolized by either the COX pathway or the LOX pathway. The proliferation and invasiveness of tumor cells as well as the suppression of immune surveillance are only a few of the physiological and pathological responses that these chemicals are known to affect [217]. Targeting arachidonic acid pathways maybe useful in delaying the progression of CRC and other types of malignancies since LOXs produce metabolites in the arachidonic acid pathway that seem to promote carcinogenesis.

The results of this study exclusively demonstrated that in all extract treatments, downregulatory properties were observed to occur in a dose-dependent manner, but with greater beneficial effects being observed in ethanolic and methanolic extracts. In addition, it was also observed that hexanoic extracts, though with lower effects compared to ethanolic and methanolic extracts, had significant effects, sufficient to elicit beneficial effects of decreasing the expression of *5-LOX*. Although prostaglandins (PGs) and other Cox-derived metabolites have received most of the attention, new research indicates that leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs), two products catalyzed by LOX, also have a significant biological

impact on the initiation and progression of human cancers. In several human cancer cell lines and tissues, including those of the colon [216,218,219], an increase in the expression of *5-LOX* and their metabolites has been found. This over-expression has been reported to be significantly linked to tumor cell proliferation, resistance to apoptosis, and angiogenesis [220,221]. Additionally, it was discovered that the direct suppression of *5-LOX* or *12-LOX* significantly reduced the development of tumor cells [217,220–222].

The strong association between *5-LOX* expression level and extract dosage concentration was our most significant discovery. This finding is consistent with other research, which showed that gastric or colorectal [220] tumors with higher *5-LOX* and *COX-2* levels grew deeper and larger. Additionally, it lends credence to the idea that *5-LOX* and *COX-2* share features of expression and function that are proangiogenic and anti-apoptotic [223] as well as substrate preference in human cancer. Like COX-2 and PGs, 5-LOX enzymes and their products may operate on tumor cells by inhibiting apoptosis, increasing cell proliferation, and stimulating angiogenesis, according to several experimental investigations [221,223]. The degree of *5-LOX* expression and LTB4 synthesis in cancer cell lines was found to be correlated with the dose- and time-dependent reduction in cell viability and induction of apoptosis caused by *5-LOX* inhibitors recently [221]. A key mechanism of 5-*LOX* activities on proliferation and apoptosis was the release of endothelial growth factor (VEGF) and mRNA levels by *5-LOX* activity in malignant mesothelial cells [221].

The wellbeing of mankind is significantly impacted by the molecules in natural products, which have an evolutionary structure. Numerous secondary metabolites are synthesized by the biosynthetic machinery of nature, and each one of them exhibits unique biological characteristics that make it desirable to use them as pharmaceutical

structural models or as health products. It is crucial to understand that AS contains similar pharmacotherapeutic characteristics just like other identified species of the genus, *Aloe.* They can thus be utilized for potential intervention and management of CRC in predetermined concentrations based on the extensive documented evidence on the pharmacological activity of the plant genus. The presence of innumerable biomolecules in AS signifies the usefulness of incorporating this plant as a potential anti-CRC agent with certainty of phytotherapeutic effects. Despite the positive effects recorded in the ethnoveterinary application of AS particularly in chicken, this is the first unique research study detailing its potential application in human health, with specific emphasis on CRC. This is of course given the abundant presence of bioactive phytoconstituents common in the leaves and roots of perennial, evergreen, and succulent plants.

Overwhelming evidence is in congruent support of our findings, concerning the benefits of suppressing the expression of *5-LOX*. AS is a promising phytotherapeutic plant, however, based on my thorough research, this is the first study to assess the levels of expression and clinicopathologic significance of the *5-LOX* gene on CRC using multiple extraction solvents of different polarities on AS. From the outcome of our comparative assessment on the extraction solvent of choice, we strongly recommend the application of methanol and ethanolic solvents for maximum inhibitory benefits of *5-LOX* expression. AS leaf extracts are therefore unreservedly perfect natural *5-LOX* inhibitors whose exploitation for therapy is of paramount importance in CRC management.

Thus, our findings imply that *5-LOX* overexpression may have a significant impact on the emergence of CRC. Therefore, blocking this metabolic pathway might be a timely and useful therapeutic strategy for both the prevention and treatment of CRC. Finally,

this important plant may be investigated as a raw ingredient for making conventional medications

6.5 Cytotoxicity and safety medical application properties of AS

AS has been reported in other studies to be safe in medical applications. At high amounts, aloin has been demonstrated to be efficacious, and a safe oral medication used in pharmacotherapy practice for the treatment and prevention of cancer [114]. Chrysophanol, also known as 1,8-dihydroxy-3-methyl-anthraquinone and chrysophanic acid, is an anthraquinone that is found in *Aloe* plants naturally [116]. Yao et al. investigated the cytotoxicity of it on six aggressive human cancer cell lines (HepG2, HCT-8, A549, SGC7901, and MDAMB-231) and found no evidence of cytotoxicity [117]. Chrysophanol did, however, demonstrate a time-dependent suppression of HepG2 cell viability, with the greatest inhibition occurring at a concentration of 10 m and a slight decreasing of the inhibitory effect occurring at concentrations between 20 and 60 m. However, exposure to chrysophanol at concentrations between 0 and 100 m did not seem to have any negative effects on HL-7702 cells. However, AS constitutes anthraquinones, and these metabolites have significant drawbacks if used excessively, despite being safe in small dosages and for brief periods. AS are thus by documented evidence safe for human medical application in appropriate pharmaceutical proportions.

7 CONCLUSION AND RECOMMENDATIONS

7.1 CONCLUSION

In this study, natural plant components offer an exciting new form of anticancer therapy with a sufficient cutting-edge method of action. Phytoconstituents of plant origin have been shown to have an overwhelming therapeutic activity to treat a variety of infectious diseases, in contrast to synthetic medicines that are frequently observed

to correlate with various negative effects. Due to the adverse effects of chemotherapeutic medicines, as highlighted above, phytochemicals and their derivatives are now being explored for use more frequently for treatment and prevention of CRC. Side effects appear after healthy, normal cells are damaged which opens the door for new phytotherapeutic strategies to serve as effective and safe substitutes. Indirectly or directly, plant-based natural products account for around 50% of all regularly used chemotherapeutic therapies, making them a prolific source of new anti-colon cancer medications.

Of significance to note, several of the pharmaceuticals that are currently in the market were manufactured from plant sources, which are employed as a good supply of drugs. Plants and products derived from plants continue to be crucial components of the medical and healthcare systems in developing nations. In non-industrialized communities, the use of medicinal plants (herbs) to treat ailments is practically universal and frequently less expensive than using costly conventional medications. As depicted from the extensive scope of the present study, the three native plants (WS, WU & AS) deployed for the study, demonstrate significant pharmacotherapeutic properties, crucial for urgent consideration in the fight against the deadly sting of CRC. The following specific conclusions are thus derived:

1. The 1st null hypothesis was rejected and concluded that extraction of hexanoic, ethanolic, and methanolic extracts from WS, WU, and AS is possible.

   Regarding this hypothesis, obtaining plant extracts from the three targeted plants was efficiently done utilizing the three extraction solvents determined for this study.

2. The 2nd null hypothesis was also rejected and concluded that active phytochemical compounds are present in extracts derived from different extraction solvents.

   Given this hypothesis, active biological metabolites were found present in the different plant extracts, and their presence resulted in modulating the expression of targeted genes tested and described in this study.

3. The 1st alternate hypothesis was accepted. It was hence concluded that active ingredients from plant extracts have phytotherapeutic properties influencing the expression of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines, eliciting beneficial effects.

   Regarding this hypothesis, phytoconstituent compounds present in the plant extracts resulted in upregulation and downregulation of the specific targeted genes. Beneficial effects were established through the upregulation of *CASP9* genes and the downregulation of *COX-2, 5-LOX, Bcl-xL,* and *Bcl2.*

4. Further, the 2nd alternate hypothesis was also accepted and concluded that it was possible to determine the modulatory expressions of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* after treatment with different plant extracts.

   Regarding this hypothesis, expressions of genes were notably observed and appropriately recorded upon treatment exposure. Different plant extracts, extracted using different extraction solvents modulated gene expressions variably.

5. Finally, the 3rd alternate hypothesis was accepted. It was then concluded that methanol and ethyl acetate can extract metabolites exhibiting high regulatory effects on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* in Caco-2 cell lines.

Considering this hypothesis, exposure of target genes to methanolic and ethanoic extracts exhibited significant beneficial effects intended for inhibition of Caco-2 cellular proliferation, through upregulation of *CASP9* genes and downregulation of *COX-2, 5-LOX, Bcl-xL,* and *Bcl2.*

7.2 RECOMMENDATIONS

1. Utilization of WS's root and stem extracts in *in vivo* and subsequent clinical trials following successful *in vitro* trials on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes.
2. Utilization of WU's root and stem extracts in *in vivo* and subsequent clinical trials following successful *in vitro* trials on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes.
3. Utilization of AS leaf extracts in *in vivo* and subsequent clinical trials following successful *in vitro* trials on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes.
4. Application of methanol and ethyl acetate independently as extraction solvents of choice for maximum beneficial effects in modulating the expressions of *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes. Extraction solvents have different polarities and therefore dissolve plant metabolites based on their polar strengths.
5. Further research to determine the specific metabolites eliciting phytotherapeutic modulatory effects on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* is particularly recommended.

8. NOVELTY OF THE RESEARCH

1) To the best of our knowledge, this is the 1st study to evaluate the effects of WU root and stem extracts on CRC. The highlight of this is in using different dosage concentrations for optimum effect assessment.
2) To the best of our knowledge, this is the 1st study to compare the effects of WU extracts obtained using 3 different extraction solvents (hexane, ethyl acetate, and methanol) all of different polarities, to determine their efficacy in modulating the targeted genes for considerable CRC management.
3) To the best of our knowledge, this is the 1st study to report on the positive effects of WU root and stem extracts on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes, appropriate for CRC management.
4) To the best of our knowledge, this is the 1st study to evaluate and compare the effects of WS root and stem extracts using 3 different extraction solvents. Again, the highlight of this is in using different dosage concentrations for optimum effect assessment.
5) To the best of our knowledge, this is the 1st study to report on the positive effects of WS root and stem extracts on *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* targeted genes in a dose-dependent manner for considerable CRC management.
6) To the best of our knowledge, this is the 1st study to evaluate the effects of AS leaves on human CRC. Again, the highlight of this is in using different dosage concentrations for optimum effect assessment.
7) This is the 1st unique study to compare in a dose-dependent manner, the effects of AS leaf extracts using 3 extraction solvents (hexane, ethyl acetate, and methanol).
8) Finally, and to the best of our knowledge, this is the 1st unique study to report on the positive effects of AS on human *COX-2, 5-LOX, Bcl-xL, Bcl2,* and *CASP9* genes.

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ANNEXES

LIST OF PUBLICATIONS RELATED TO DISSERTATION

1. **John M. Macharia**, Ruth W. Mwangi, István Szabó, Afshin Zand, Zsolt Kaposztas, Tímea Varjas, Nóra Rozmann, Bence L. Raposa, Regulatory activities of *Warbugia ugandensis* ethanolic extracts on colorectal cancer-specific genome expression dose-dependently, Biomedicine & Pharmacotherapy, Volume 166, 2023, 115325, ISSN 0753-3322, https://doi.org/10.1016/j.biopha.2023.115325
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4. **Macharia, John M.,** Veronica Ngure, Barnabás Emődy, Bence Király, Zsolt Káposztás, Nóra Rozmann, Attila Erdélyi, and Bence Raposa. 2023. Pharmacotherapeutic Potential of *Aloe secundiflora* against Colorectal Cancer Growth and Proliferation. *Pharmaceutics* 15,no.5:1558. https://doi.org/10.3390/pharmaceutics15051558.
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8. **John M. Macharia**, Lu Zhang, Ruth W. Mwangi, Nora Rozmann, Zsolt Kaposztas, Tímea Varjas, Miklós Sugár, Huda Alfatafta, Márton Pintér, Raposa L. Bence, (2022) Are chemical compounds in medical mushrooms potent against colorectal cancer carcinogenesis and antimicrobial growth? https://doi.org/10.1186/s12935-022-02798-2.

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1. **John M. Macharia**, Grace W. Gakenye, Nóra Rozmann, David Onchonga, Ruth W. Mwangi, Zsolt Kaposztas, John M. Mathenge, Dorina Pusztai, Marton Pinter, Miklos Sugar, Bence L. Raposa (2022). An empirical assessment of the factors influencing acceptance of COVID-19 vaccine uptake between Kenyan and Hungarian residing populations: A cross-sectional study, *BMC Springer. Scientific Reports,* https://doi.org/10.1038/s41598-022-26824-5,
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11. Mwangi, R. W., **Macharia, J. M**., Wagara, I. N., & Bence, R. L. (2021). The medicinal properties of Cassia fistula L: A review. *Biomedicine and Pharmacotherapy*, *144*, 112240. https://doi.org/10.1016/j.biopha.2021.112240
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STATISTICAL ANAYLSIS: Post Hoc test analysis

Table 1 An expanded Post Hoc test analysis of multiple comparisons showing the concentration of dosage application in relation to genome expressions from ethanolic root extracts of WS.

Dependent Variable	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
COX-2	,00	,50	-,0013667	,0032593	,974	-,011804	,009071
		1,00	,0093333	,0032593	,081	-,001104	,019771
		2,00	,0189667*	,0032593	,002	,008529	,029404
	,50	,00	,0013667	,0032593	,974	-,009071	,011804
		1,00	,0107000*	,0032593	,045	,000262	,021138
		2,00	,0203333*	,0032593	,001	,009896	,030771
	1,00	,00	-,0093333	,0032593	,081	-,019771	,001104
		,50	-,0107000*	,0032593	,045	-,021138	-,000262
		2,00	,0096333	,0032593	,071	-,000804	,020071
	2,00	,00	-,0189667*	,0032593	,002	-,029404	-,008529
		,50	-,0203333*	,0032593	,001	-,030771	-,009896
		1,00	-,0096333	,0032593	,071	-,020071	,000804
5-Lox	,00	,50	,1938333	,0810688	,156	-,065777	,453444
		1,00	,2985333*	,0810688	,026	,038923	,558144
		2,00	,7113667*	,0810688	,000	,451756	,970977
	,50	,00	-,1938333	,0810688	,156	-,453444	,065777
		1,00	,1047000	,0810688	,592	-,154911	,364311
		2,00	,5175333*	,0810688	,001	,257923	,777144
	1,00	,00	-,2985333*	,0810688	,026	-,558144	-,038923
		,50	-,1047000	,0810688	,592	-,364311	,154911
		2,00	,4128333*	,0810688	,004	,153223	,672444
	2,00	,00	-,7113667*	,0810688	,000	-,970977	-,451756
		,50	-,5175333*	,0810688	,001	-,777144	-,257923
		1,00	-,4128333*	,0810688	,004	-,672444	-,153223
Bcl2	,00	,50	-,0031333*	,0006272	,005	-,005142	-,001125
		1,00	,0008000	,0006272	,601	-,001208	,002808
		2,00	,0043000*	,0006272	,001	,002292	,006308
	,50	,00	,0031333*	,0006272	,005	,001125	,005142
		1,00	,0039333*	,0006272	,001	,001925	,005942
		2,00	,0074333*	,0006272	,000	,005425	,009442
	1,00	,00	-,0008000	,0006272	,601	-,002808	,001208
		,50	-,0039333*	,0006272	,001	-,005942	-,001925
		2,00	,0035000*	,0006272	,002	,001492	,005508
	2,00	,00	-,0043000*	,0006272	,001	-,006308	-,002292
		,50	-,0074333*	,0006272	,000	-,009442	-,005425
		1,00	-,0035000*	,0006272	,002	-,005508	-,001492

Bcl-xL	,00	,50	-,3214333	,4158796	,865	-1,653226	1,010360
		1,00	,4535667	,4158796	,705	-,878226	1,785360
		2,00	1,4957000*	,4158796	,029	,163907	2,827493
	,50	,00	,3214333	,4158796	,865	-1,010360	1,653226
		1,00	,7750000	,4158796	,314	-,556793	2,106793
		2,00	1,8171333*	,4158796	,010	,485340	3,148926
	1,00	,00	-,4535667	,4158796	,705	-1,785360	,878226
		,50	-,7750000	,4158796	,314	-2,106793	,556793
		2,00	1,0421333	,4158796	,133	-,289660	2,373926
	2,00	,00	-1,4957000*	,4158796	,029	-2,827493	-,163907
		,50	-1,8171333*	,4158796	,010	-3,148926	-,485340
		1,00	-1,0421333	,4158796	,133	-2,373926	,289660
CASPS9	,00	,50	,7624667*	,1987753	,021	,125918	1,399015
		1,00	,1761333	,1987753	,812	-,460415	,812682
		2,00	-,6210333	,1987753	,056	-1,257582	,015515
	,50	,00	-,7624667*	,1987753	,021	-1,399015	-,125918
		1,00	-,5863333	,1987753	,071	-1,222882	,050215
		2,00	-1,3835000*	,1987753	,001	-2,020048	-,746952
	1,00	,00	-,1761333	,1987753	,812	-,812682	,460415
		,50	,5863333	,1987753	,071	-,050215	1,222882
		2,00	-,7971667*	,1987753	,016	-1,433715	-,160618
	2,00	,00	,6210333	,1987753	,056	-,015515	1,257582
		,50	1,3835000*	,1987753	,001	,746952	2,020048
		1,00	,7971667*	,1987753	,016	,160618	1,433715

*. The mean difference is significant at the 0.05 level.

a. Treatment = 01-R E Ace (W. somnifera, root, Ethyl acetate)

Table 2 An expanded Post Hoc test analysis of multiple comparisons showing the concentration of dosage application in relation to genome expressions from ethanolic stem extracts of WU

Dependent Variable	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
COX-2	,00	,50	,0189333*	,0014680	,000	,014515	,023351
		1,00	,0264000*	,0014680	,000	,021982	,030818
		2,00	,0297333*	,0014680	,000	,025315	,034151
	,50	,00	-,0189333*	,0014680	,000	-,023351	-,014515

		1,00	,0074667*	,0016952	,005	,002365	,012568
		2,00	,0108000*	,0016952	,000	,005698	,015902
	1,00	,00	-,0264000*	,0014680	,000	-,030818	-,021982
		,50	-,0074667*	,0016952	,005	-,012568	-,002365
		2,00	,0033333	,0016952	,257	-,001768	,008435
	2,00	,00	-,0297333*	,0014680	,000	-,034151	-,025315
		,50	-,0108000*	,0016952	,000	-,015902	-,005698
		1,00	-,0033333	,0016952	,257	-,008435	,001768
5-LOX	,00	,50	,1228000	,0474398	,100	-,019972	,265572
		1,00	,0534333	,0474398	,682	-,089339	,196206
		2,00	-,0425667	,0474398	,807	-,185339	,100206
	,50	,00	-,1228000	,0474398	,100	-,265572	,019972
		1,00	-,0693667	,0547788	,601	-,234226	,095493
		2,00	-,1653667*	,0547788	,049	-,330226	-,000507
	1,00	,00	-,0534333	,0474398	,682	-,196206	,089339
		,50	,0693667	,0547788	,601	-,095493	,234226
		2,00	-,0960000	,0547788	,344	-,260859	,068859
	2,00	,00	,0425667	,0474398	,807	-,100206	,185339
		,50	,1653667*	,0547788	,049	,000507	,330226
		1,00	,0960000	,0547788	,344	-,068859	,260859
Bcl2	,00	,50	,0021667*	,0004341	,002	,000860	,003473
		1,00	,0029333*	,0004341	,000	,001627	,004240
		2,00	,0027333*	,0004341	,000	,001427	,004040
	,50	,00	-,0021667*	,0004341	,002	-,003473	-,000860
		1,00	,0007667	,0005013	,454	-,000742	,002275
		2,00	,0005667	,0005013	,679	-,000942	,002075
	1,00	,00	-,0029333*	,0004341	,000	-,004240	-,001627
		,50	-,0007667	,0005013	,454	-,002275	,000742
		2,00	-,0002000	,0005013	,977	-,001709	,001309
	2,00	,00	-,0027333*	,0004341	,000	-,004040	-,001427
		,50	-,0005667	,0005013	,679	-,002075	,000942
		1,00	,0002000	,0005013	,977	-,001309	,001709
Bcl-xL	,00	,50	,0872000	,2197870	,978	-,574260	,748660
		1,00	,7380333*	,2197870	,028	,076574	1,399493
		2,00	1,2418000*	,2197870	,001	,580340	1,903260
	,50	,00	-,0872000	,2197870	,978	-,748660	,574260
		1,00	,6508333	,2537882	,104	-,112954	1,414621
		2,00	1,1546000*	,2537882	,004	,390812	1,918388
	1,00	,00	-,7380333*	,2197870	,028	-1,399493	-,076574
		,50	-,6508333	,2537882	,104	-1,414621	,112954
		2,00	,5037667	,2537882	,251	-,260021	1,267554
	2,00	,00	-1,2418000*	,2197870	,001	-1,903260	-,580340
		,50	-1,1546000*	,2537882	,004	-1,918388	-,390812
		1,00	-,5037667	,2537882	,251	-1,267554	,260021
CASPS9	,00	,50	,5775000*	,1440674	,009	,143922	1,011078
		1,00	-,0652000	,1440674	,968	-,498778	,368378
		2,00	,1008333	,1440674	,895	-,332744	,534411
	,50	,00	-,5775000*	,1440674	,009	-1,011078	-,143922
		1,00	-,6427000*	,1663547	,012	-1,143353	-,142047
		2,00	-,4766667	,1663547	,063	-,977319	,023986
	1,00	,00	,0652000	,1440674	,968	-,368378	,498778
		,50	,6427000*	,1663547	,012	,142047	1,143353
		2,00	,1660333	,1663547	,754	-,334619	,666686
	2,00	,00	-,1008333	,1440674	,895	-,534411	,332744
		,50	,4766667	,1663547	,063	-,023986	,977319
		1,00	-,1660333	,1663547	,754	-,666686	,334619

*. The mean difference is significant at the 0.05 level.

a. Treatment = 02-S E Ace (*W. ugandensis*, stem, Ethyl acetate)

Table 3 An expanded Post Hoc test analysis of multiple comparisons showing the concentration of dosage application in relation to genome expressions from methanolic stem extracts of WU

Dependent Variable	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
COX-2	,00	,50	,0145000*	,0009787	,000	,011366	,017634
		1,00	,0305667*	,0009787	,000	,027433	,033701
		2,00	,0348667*	,0009787	,000	,031733	,038001
	,50	,00	-,0145000*	,0009787	,000	-,017634	-,011366
		1,00	,0160667*	,0009787	,000	,012933	,019201
		2,00	,0203667*	,0009787	,000	,017233	,023501
	1,00	,00	-,0305667*	,0009787	,000	-,033701	-,027433
		,50	-,0160667*	,0009787	,000	-,019201	-,012933
		2,00	,0043000*	,0009787	,010	,001166	,007434
	2,00	,00	-,0348667*	,0009787	,000	-,038001	-,031733
		,50	-,0203667*	,0009787	,000	-,023501	-,017233
		1,00	-,0043000*	,0009787	,010	-,007434	-,001166
5-LOX	,00	,50	,1267333	,0719533	,356	-,103687	,357153
		1,00	,5295333*	,0719533	,000	,299113	,759953
		2,00	,7431000*	,0719533	,000	,512680	,973520
	,50	,00	-,1267333	,0719533	,356	-,357153	,103687
		1,00	,4028000*	,0719533	,002	,172380	,633220
		2,00	,6163667*	,0719533	,000	,385947	,846787
	1,00	,00	-,5295333*	,0719533	,000	-,759953	-,299113
		,50	-,4028000*	,0719533	,002	-,633220	-,172380
		2,00	,2135667	,0719533	,070	-,016853	,443987
	2,00	,00	-,7431000*	,0719533	,000	-,973520	-,512680
		,50	-,6163667*	,0719533	,000	-,846787	-,385947
		1,00	-,2135667	,0719533	,070	-,443987	,016853
Bcl2	,00	,50	,0015667*	,0004631	,039	,000084	,003050
		1,00	,0051000*	,0004631	,000	,003617	,006583
		2,00	,0064667*	,0004631	,000	,004984	,007950
	,50	,00	-,0015667*	,0004631	,039	-,003050	-,000084
		1,00	,0035333*	,0004631	,000	,002050	,005016
		2,00	,0049000*	,0004631	,000	,003417	,006383
	1,00	,00	-,0051000*	,0004631	,000	-,006583	-,003617
		,50	-,0035333*	,0004631	,000	-,005016	-,002050
		2,00	,0013667	,0004631	,071	-,000116	,002850
	2,00	,00	-,0064667*	,0004631	,000	-,007950	-,004984
		,50	-,0049000*	,0004631	,000	-,006383	-,003417
		1,00	-,0013667	,0004631	,071	-,002850	,000116
Bcl-xl	,00	,50	,4598000	,1443907	,051	-,002590	,922190
		1,00	1,6786667*	,1443907	,000	1,216277	2,141056
		2,00	2,0810333*	,1443907	,000	1,618644	2,543423
	,50	,00	-,4598000	,1443907	,051	-,922190	,002590
		1,00	1,2188667*	,1443907	,000	,756477	1,681256
		2,00	1,6212333*	,1443907	,000	1,158844	2,083623

	1,00	,00	-1,6786667*	,1443907	,000	-2,141056	-1,216277
		,50	-1,2188667*	,1443907	,000	-1,681256	-,756477
		2,00	,4023667	,1443907	,090	-,060023	,864756
	2,00	,00	-2,0810333*	,1443907	,000	-2,543423	-1,618644
		,50	-1,6212333*	,1443907	,000	-2,083623	-1,158844
		1,00	-,4023667	,1443907	,090	-,864756	,060023
CASPS9	,00	,50	,5361667	,2392107	,192	-,229870	1,302204
		1,00	-1,4734000*	,2392107	,001	-2,239437	-,707363
		2,00	,8339667*	,2392107	,034	,067930	1,600004
	,50	,00	-,5361667	,2392107	,192	-1,302204	,229870
		1,00	-2,0095667*	,2392107	,000	-2,775604	-1,243530
		2,00	,2978000	,2392107	,618	-,468237	1,063837
	1,00	,00	1,4734000*	,2392107	,001	,707363	2,239437
		,50	2,0095667*	,2392107	,000	1,243530	2,775604
		2,00	2,3073667*	,2392107	,000	1,541330	3,073404
	2,00	,00	-,8339667*	,2392107	,034	-1,600004	-,067930
		,50	-,2978000	,2392107	,618	-1,063837	,468237
		1,00	-2,3073667*	,2392107	,000	-3,073404	-1,541330

*. The mean difference is significant at the 0.05 level.

Treatment = 02-S Met (Treatment = 02-S Met (W. ugandensis, Stem, Methanol))

Submission of the doctoral dissertation and declaration of the originality of the dissertation

The undersigned,
Name: Macharia John Macharia
Maiden name: Macharia
Mother's maiden name: Janet C. Chumo
Place and time of birth: Kericho District, Kenya, 17th April 1984.

on this day submitted my doctoral dissertation entitled: "Evaluation and determination of the phytotherapeutic properties of selected plants and their bioactive metabolites on targeted genes in colorectal cancer management", to the PR6-1/O-42, Oncology - Health Sciences Programme (Preventive Oncology Subprogramme) of the Doctoral School of Health Sciences, Faculty of Health Sciences, University of Pécs.

Names of the supervisor(s):

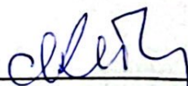
1. Dr. habil. Raposa László Bence
2. Dr. habil. Káposztás Zsolt

At the same time, I declare that
- I have not submitted my doctoral dissertation to any other Doctoral School (neither in this country nor abroad),
- my application for degree earning has not been rejected in the past two years,
- in the past two years I have not had unsuccessful doctoral procedures,
- my doctoral degree has not been withdrawn in the past five years,
- my dissertation is independent work, I have not presented others' intellectual work as mine, the references are definite and full, on preparation of the dissertation I have not used false or falsified data.

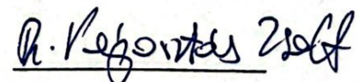
Furthermore, I declare that I contribute to the request of DOI identification of my doctoral dissertation.

Dated: Pécs, 9th November 2023.

signed by Candidate



Supervisor



Co-supervisor

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