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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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INTRODUCTION

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. 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. 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.

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. 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.

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. 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. 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. 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).

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.

MATERIAL AND METHODS

Study area

The plant species were collected from two different Counties in Kenya (Africa), which included Nakuru and Baringo Counties. Processing of plant organs was done at Egerton University in Njoro, Nakuru County, which is a part of the eastern Mau water-catchment. 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.

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.

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. Organs of AS were collected from Kampi ya Moto, in Rongai Sub County, Nakuru County. On the other hand, WS organs were obtained from the Perkerra irrigation scheme, Baringo South Sub-County in Baringo County

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. This process was repeated 3 times until there was a complete extraction of all soluble constituents. 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 40^{o}C and 50^{o}C 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 4^{o}C in a fridge until use.

Reconstitution of plants extracts

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. 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.

Treatment and isolation of RNA in Caco-2 cell lines

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.

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.

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. Purification of RNA was done using UV Spectroscopy.

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).

Analysis of chemical components from crude plant extracts

High performance liquid chromatography (HPLC) 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.

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.

RESULTS

***Withania somnifera* L.**

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 ($p = 0.001$). 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 ($p = 0.001$). 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. There was a significant difference in their downregulatory properties ($p = 0.011$, roots and $p=0.001$, stems) observed in both extracts. 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. There was an observed similarity in a significant difference in their downregulatory properties ($p = 0.001$) in both roots and stem extracts. 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, 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 ($p = 0.531$). After treatment of Caco-2 cell lines with root and stem extracts, *CASP9* enzymatic genes were detected as being variably upregulated in both extracts. 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, ($p=0.014$). When Caco-2 cell lines were treated with methanolic root and stem extracts, *COX-2* genes were progressively downregulated in a dose-dependent manner, in both extracts. There was also an observed statistically significant difference in their downregulatory effects from root extracts ($p = 0.001$) and stem extracts ($p=0.010$). Metabolites identified were: Withanolide A, Withaferin A, Withanolide sulfoxide, 27 Hydroxy withanone and choline, Choline, Somniferine, Withanine, Withanoside I, Quercetin, chlorogenic acid and catechin and Ashwagandhine.

Warbugia ugandesis

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, in both extracts. However, significant downregulatory effects were observed in stem extracts statistically ($p = 0.001$) compared to root extracts ($p = 0.379$). 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, in root extracts, like that observed in the expression of *Bcl-xL*. Notwithstanding, downregulatory effects were statistically significant in both extracts (p = 0.001). The expression of *Bcl2* genes was upregulated in root extracts. However, their expressions were downregulated by stem extracts dose-dependently. 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. 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. 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. 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, 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. 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. 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). Metabolites identified were: Drimane, ugandensial, warburganal, flavonoids, saponins, steroids, Terpenoids, drimane, coloratane sesquiterpenoids, ugandensial, warburganal, mukaadial, tannins, flavonoids, saponins, steroids.

Aloe secundiflora

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. 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). 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. 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. Metabolites identified were: Aloenin, nthoxanthins, flavanones, Anthraquinones, Aloina and terpenoid.

DISCUSSION

W. somnifera and *W.ugandensis*

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. 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. Being enzymes involved in the synthesis of lipid prostaglandins, they are significant in the body's metabolic activities. While *COX-2* is impacted and plays a significant role in inflammatory processes, *COX-1* modulates homeostasis. 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. 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. In WU, terpenoids, sesquiterpenoids, tannins, flavonoids, saponins, muzigadial, steroids, polygodial and mannitol, have all been widely implicated in inhibiting the expression of *COX-2.* 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, 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.

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. Apoptosis is a vital physiological process that involves the selective removal of cells in a range of biological events. It has been argued that suppressing spontaneous apoptosis increases the risk of cancer. Comparably, a greater incidence of colorectal adenoma has been reported

to be highly linked with a decreased rate of apoptosis. 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. The suppression of natural apoptosis has been hypothesized to enhance the incidence of cancer. Similarly, it has been noted that a higher prevalence of colorectal adenoma is highly correlated with a lower rate of apoptosis. The capacity to trigger apoptosis in epithelial cells of gastrointestinal origin is one of the potential strategies in chemo-prevention. 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.

Aloe secundiflora

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*. 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. 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). These genes play roles in human defense evasion, angiogenesis, metastatic spread, cell survival, differentiation, and programmed cell death. In recent years, there has been an abundance of research demonstrating that blocking constitutive *STAT3* signaling substantially inhibits tumor development and triggers apoptosis. 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.

Inhibiting the expression of *5-LOX*, which is upregulated in colorectal cancer, could be helpful in both the prevention and therapy of the disease. 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. 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, 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. Additionally, it was discovered that the direct suppression of *5-LOX* or *12-LOX* significantly reduced the development of tumor cells. The strong association between *5-LOX* expression level and extract dosage concentration was our most significant discovery.

SUMMARY OF NEW FINDINGS

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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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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