Thesis for the Degree of Doctor of Philosophy

Characterization, anticancer, and anti-inflammatory activities of phytochemicals recovered from *Aspilia africana C.D. Adams*

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(아스필리아 아프리카나의 파이토케미칼 특성, 항암, 항염증 효과)

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Contents

List of Figures

List of Abbreviations

AAE	*Aspilia africana* extract
HWE	Hot Water Extraction
SWE	Subcritical Water Extraction
CDKs	Cyclin-dependent kinases
COCL2	Cobalt (II) chloride
Cox-2	Cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H2O2	Hydrogen peroxide
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NF-kB	Nuclear factor-kB
NO	Nitrc oxide
Nrf-2	Nuclear factor erythroid 2-related factor 2
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PI	Propidium iodide
TNF-α	Tumor necrosis factor alpha
WST-1®	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt

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Abstract

Cancer is a leading cause of death worldwide, accounting for 9.6 million deaths in 2018 from which 783,000 occurred from gastric cancer. Cancer is defined as the uncontrollable growth of cells which results in tumor formation and immune system damage. A number of experiments in anticancer research are mainly focused on retaking control in the regulation of cancer by inducing cancer death via activation of apoptosis that is usually deregulated in most cancers or by inducing the cell cycle arrest to control the cancer progression. Inflammation is frequently linked with cancer progression and development. Chronic inflammation has been linked as well with a wide range of diseases, such as cardiovascular diseases, arthritis, diabetes, pulmonary diseases, Alzheimer's disease, and autoimmune diseases. Macrophages play a crucial role in immunomodulation during inflammation through the production of several cytokines and growth factors. Since the past era, there is a growing interest in the use of medicinal plants for health-related issues. Medicinal plants have been reported to possess a large number of bioactive compounds that are responsible for their biological activities. *Aspilia africana C.D. Adams* (*A. africana*) belongs to the East part of Africa and grows all through tropical Africa. *A. africana*

is a preferred herb in African folk medicine due to its numerous physiological activities including antimicrobial, anti-inflammatory, antioxidant, and possess many important bioactive compounds. In this thesis, we investigated the benefit of the use of green extraction techniques in comparison to conventional extraction techniques for the recovery of bioactive compounds from *A. africana*; We further studied the anti-cancer and anti-inflammatory effects of obtained extracts in human gastric adenocarcinoma AGS cells, murine macrophages RAW264.7 cells, respectively.

Results obtained and presented here in this thesis show that hot water extraction (HWE) and subcritical water extraction (SWE) techniques would be the best extraction techniques for the recovery of bioactive compounds, such as phenolic antioxidants from *A. africana*, as compared with the conventional organic solvent (ethanol). HWE extracts contained the highest concentration of Gallic acid, total phenolic contents (TPC), Total flavonoid contents (TFC) and exhibited the highest antioxidant activity with an IC_{50} nine times lower than that of ethanol extracts, followed by SWE. Our results also demonstrated that *A. africana* arrested the proliferation of AGS cells by arresting the G1 cell cycle phase progression and by inducing apoptosis. Cyclins such as cyclin E1&D1 and CDK2,4&6 that play a major role in G1/S phase evolution were down-regulated where the expression of CDK inhibitors (p21, p27, p18, and p15) was up-regulated. The expression levels of pro-apoptotic proteins such as tBid, Bad, Bak, Bax, Cytochrome c, FLIP, cleaved form of caspases 3,8 &9 and cleaved Parp were increased while anti-apoptotic proteins such as Bcl-2, Bcl-xL were decreased following the treatment of AGS cells with *A. africana*. Additionally, *A. africana* inhibited nitric oxide (NO) production and the expression of important inflammatory enzymes such as iNOS and COX-2, pro-inflammatory cytokines (tumor necrosis factor (TNF) –α, interleukin (IL) - 6, and IL-1β and prevented the activation of PI3K/Akt and NF-kB signaling pathways in macrophages RAW264.7 cells stimulated with lipopolysaccharides (LPS).

CHAPTER ONE

General Introduction

CHAPTER 1

General introduction

1.1 Traditional medical plants

Since the past era, there is a growing interest in the use of medicinal plants for health-related issues. The interest in the research on medicinal plants is mainly focused on the eras of pharmacognosy, phytochemistry, and horticulture. The pharmacognosy research of medical plants consists of bioactivity assays, identification of possible mode of action, and targeted sites of phytomedicinal active compounds. The phytochemistry studies of medicinal plants focus on the characterization of bioactive compounds which are then separated for structure analysis. Medicinal plants have been subjected as well for horticulture research which focuses on finding the possible optimum growth in agriculture.

1.1.1 Phytochemicals and biological activities of medicinal plants

Medicinal plants have been used for centuries by humans as a remedy for various diseases. The benefic effects of medicinal plants mainly result from a combination of secondary metabolites present in them. Each plant species has its unique mode of actions as a result of the distinct taxonomic presence of secondary metabolites combination in each plant species (Wink 2010). Apart from secondary products that vary from plants species to another, all plants species have common primary products such as lipids, proteins, carbohydrates, nucleic acids and heme that are crucial for plants' primary metabolic processes that maintain plant cells (Cseke, Kirakosyan et al. 2016);(Wink and Schimmer 2018).

Although ancient studies did not show the involvement of plant secondary products in the maintenance of plant cells, recent researches have demonstrated that plants

secondary products are essential in plants' ecophysiology (Pagare, Bhatia et al. 2015). It has been reported that these chemicals have a defensive and protective role in plants against pathogen attack, herbivory, and interplant competition. Plant's secondary products also protect plants against abiotic stresses resulting from environmental changes such as a change in water status, change in temperature, UV exposure, change in light levels, and change in mineral nutrients (Mazid, Khan et al. 2011); (Bartwal, Mall et al. 2013). Plant secondary metabolites have been reported to be involved in the regulation of plant growth, in the modulation of gene expression, as well as in signal transduction (Pagare, Bhatia et al. 2015).

It is likely that the variety of ecological functions of plants' secondary metabolites have medicinal benefit for humans. For example, secondary metabolites that are responsible for plant's defense against microbial pathogens through cytotoxicity may be useful as antimicrobial agents in humans thus a number of reports on different medicinal plants as antimicrobial agents (Shetty, Mahin-Syed-Ismail et al. 2016); (Mostafa, Al-Askar et al. 2018); (De Zoysa, Rathnayake et al. 2019). Similarly, secondary metabolites acting against herbivores via neurotoxin activity by acting on the nervous system can be beneficial in humans, for example, sedatives, antidepressants, anesthetics, and muscle relaxants (Gross, Stolz et al. 2019); (Saki, Bahmani et al. 2014); (Rabiei and Rabiei 2017); (Taur and Patil 2011). Secondary metabolites from plants have been reported as well to possess various effects such as antioxidant, anti-inflammatory, antitumor, and antidiabetic (Zhang, Ravipati et al. 2011); (Yildirim, Karakas et al. 2013); (Karakas, Yildirim et al. 2012).

1.1.2 Conventional extraction techniques used for the recovery of bioactive compounds from medicinal plants

Extraction is an important step in the preparation of a sample before analysis by chromatography. A series of factors such as extraction techniques, solvent, and raw materials play an important role in the successful extraction of bioactive compounds (Tiwari 2015). There are two types of extraction techniques that are used:

conventional and non-conventional techniques. Traditional techniques for extracting natural substances from plants require major adaptations to reduce risks to health, safety, and the environment. Consequently, the search for alternative products and processes which are more respectful of human and his environment occupies a primordial place in research and development (Rodríguez-Pérez, Quirantes-Piné et al. 2015).

The main used conventional techniques for the extraction of bioactive compounds are maceration, soxhlet, and hydro-distillation. The maceration principle consists of crushing the used sample into small particles favorizing the sample-solvent mixture. This procedure is made easier by the agitation step which helps to increase the diffusion and removes concentrated solution from the sample surface. This procedure has been used to obtain bioactive compounds and essential oils (Azmir, Zaidul et al. 2013). The soxhlet procedure consists of placing a small amount of dry sample in the equipment in a way that the solvent goes through the sample. The procedure continues repetitively until the extraction is complete. Practical optimization and favorable conditions for the application of this technique are easily found in the literature however this technique requires large quantities of solvent and wide extraction time (Heleno, Diz et al. 2016). Lastly, the hydro-distillation procedure is conducted by using distilled water and it is mainly used to extract volatile elements from foods. This procedure normally takes about 6 to 8 hours and does not involve organic solvents. The main processes are hydro-diffusion, decomposition by heat, and hydrolysis. This a very complete technique that extracts both volatile organic and no volatile organic compounds and separates them in just one step; However, the high temperature used during extraction are susceptible of compounds degradation, which limits the application of this technique (Wu, Wang et al. 2015). The effectiveness of conventional extraction techniques will depend on the choice of organic solvent as well as the polarity of targeted compounds. Due to the variability of compound polarities, it is difficult to develop one single method that can extract efficiently all compounds. An efficient solvent is a solvent with low

toxicity, low boiling point, that provides rapid mass transfer with preservative action. Extraction time, temperature, and type of extract play a major role in the final obtained extract (Da Silva, Rocha-Santos et al. 2016).

1.1.3 Novel extraction techniques for the recovery of bioactive compounds from medicinal plants

The development of clean and green extraction technologies that can replace or minimize the use of organic extraction solvents has been stimulated by the fact of possible security risks that can cause conventional extraction techniques such as solvents toxicity, the possible presence of solvent residues in final extracts, and the low yield of extraction rate. The use of these techniques preserves the stability of compounds that are extracted and reduce the extraction energy (Tiwari 2015). Objectives of green extraction techniques are to perverse our environment and its resources (Pereira and Meireles 2010) by providing the fastest extraction rate, increased heat and mass transfer, more effective use of energy, reduction of equipment size and handling steps (Mustafa and Turner 2011).

Various authors have defined 12 factors as main principles of green chemistry: synthesis of less harmful products that present no or little toxicity to human health; harmless solvents; prevention by avoiding waste; reduced use of atoms by maximizing all starting materials into end product; safe product design that presents non-toxicity and preforms desired functions; use of fresh materials from renewable sources; degradation design for safely degradation of products that are easily degraded in the environment; prevention of pollution by realtime analysis; use of innocuous solvents; the quest for energy efficacy with low impact on the economy and environment; prevention of derivatives formation; catalysis by reagent selectivity; and application of safe chemistry to avoid accidents (Lenardão, Freitag et al. 2003).

Various new substitutes to conventional extraction techniques have been proposed for the extraction of targeted compounds from various sources such as:

-Supercritical fluid extraction (SFE): is characterized by the changes in pressure and temperature that in return transforms gas in the supercritical fluid. Compared to conventional processes that use liquid solvents, supercritical fluids present low viscosity, low surface tension, spread more easily in the solid matrix which favors a rapid penetration of solvent into the solid, therefore, increasing the extraction value (Pouliot, Conway et al. 2014). The SFE consists of two main steps: chemical compounds that are present in the solid matrix are solubilized and then separated in the supercritical fluid. After that the solvent has solubilized the compounds in the solid matrix, the solvent leaves the extractor and due to a reduction of pressure and an increase of temperature, the final extract becomes a solvent-free extract (Da Silva, Rocha-Santos et al. 2016). The SFE is mainly used to extract nonpolar compounds such as carotenoids and lipids due to the same nature of solved that are used for this technique. The only option to extract polar compounds like flavonoids using this technique is to add modifiers such as water, ethanol, methanol, and acetone (Herrero, Castro-Puyana et al. 2013).

-Subcritical water extraction (SWE): Subcritical water can be well-defined as hot water with adequate pressure to preserve the liquid state at critical temperature between 100°C, the normal boiling point of water and 374°C, the critical point of water under critical pressure which varies between 1-22.1 MPa (Gbashi, Adebo et al. 2017). When the temperature increases, the dielectric constant, surface tension, and viscosity decrease, but the diffusivity improves. Adequate pressure can be used to maintain water in its liquid state at high temperature. The constant dielectric of water is 80 at 25°C. Remarkably, when the temperature is elevated up to 250°C and the pressure is at 25 bar, the constant dielectric decreases up to 25, which is almost the same as those of ethanol (Ɛ= 24) and methanol (Ɛ=33) at 25°C. With these conditions, some water proprieties are similar to those of organic solvents which can

dissolve different medium and some low polar compounds (Lee, Kim et al. 2014). The exceptional property of using SWE is that the dielectric constant can be varied over a prolonged choice by just changing the temperature and pressure (Gbashi, Adebo et al. 2017). The mechanism of SWE consists of 4 steps: solute desorption at different active sites in the sample under pressurized and elevated temperature conditions; extracts diffusion into the sample matrix; depending on the matrix, solutes partition from sample matrix into extraction fluid; sample elution and collection by chromatography (Gbashi, Adebo et al. 2017).

-Ultrasound-assisted: Ultrasound-assisted technique concept consists of a sound wave between 20 kHz and 100 MHz. This technique produces cavitation, a phenomenon that involves a bubble's production, growth, and collapse (Azmir, Zaidul et al. 2013). The technique is effective for the extraction of various analytes from various samples because of its capability to accelerate mass and heat transfer through disruption of plant-cell walls resulting in an improved release of desired compounds from different natural resources (Roselló-Soto, Koubaa et al. 2015). The use of the Ultrasound technique is easy, flexible, versatile and less costive compared to other techniques. This technique has been used for the extraction of several biomaterials and molecules such as polysaccharides, peptides, essential oils, proteins, pigments, dyes, and bioactive compounds (Tiwari 2015); (Briones-Labarca, Plaza-Morales et al. 2015).

-Microwave-assisted extraction: Microwaves can be defined as electromagnetic grounds that range between 300 MHz and 300 GHz with two perpendicular magnetic and electric field frequencies. During this technique, the solvent enters inside the matrix of the solid via diffusion resulting in the solute dissolubility to reach a concentration that is limited by the characteristics of the solid (Angiolillo, Del Nobile et al. 2015). Microwave usage is a thriving technology that allows easy and safe access to high temperatures in a reproducible manner; This technique allows the reduction of reaction time, an increase of extraction yield, and the improvement of

purity compared to other conventional heating techniques. This technique can be performed using or not a solvent (Oroian and Escriche 2015). Various compounds including antioxidants, essential oils, flavorings, pigments, and other organic compounds can be successfully extracted using this technique (Li, Fabiano-Tixier et al. 2013).

1.1.4 Use of traditional medicinal plants for drug development

The development of drugs from plants began with the development of chemistry, purification, isolation, and characterization of the plant's active compounds. The crude extracts from medicinal plants, microbes or animals contain structurally wide-ranging and unique chemical components. Natural products have been crucial in biotechnology and pharmaceutical industries, as sources of a wide range of modern medications whether naturally occurring molecules or their derivatives. Therapeutic agents are complex due to the mixture of synthetically prepared chemicals. These agents are called synthetic, semi-synthetic, or natural depending on their origins (sources) (Rodrigues, Reker et al. 2016). The leading source of natural drugs is plants because of the biodiversity, structural, and chemical diversity of their contents. Among medicines that are derived from plants we cite, digoxin from *Digitalis lanata*, morphine from *Opium*, and aspirin from *willow tree bark* (Ji, Larregieu et al. 2016); (Veeresham 2012). It is predicted that more than 60% of all drugs in a developed nation, are natural products or secondary metabolites derived from them (Veeresham 2012). Even though there are challenges in the discovery of novel drugs due to methods that are applied, natural products continue to generate new clinical entrants and medicinal compounds. These compounds such as amphotericin B, azithromycin, romidepsin, and acyclovir remain a vital foundation of novel drugs, particularly in anti-infective therapies (Bérdy 2012); (Gao, Deng et al. 2010); (Lahlou 2013). Several of the compounds have progressed into clinical trials or on the market (Butler 2004). The main goal of all drug discovery developments is to find the principal promising compounds, which can be used as therapeutic agents to treat different

health conditions such as cancer, infections, metabolic diseases, nervous system diseases, and high blood pressure (Lahlou 2013). In the first steps of drug design procedures, scientists isolate and purify key compounds from their sources using different extraction techniques. Then a number of pharmacological and biochemical tests are conducted in order to select key compounds for precise targets. During this step, some of the key compounds are not enough selective for their molecule of targets. Scientists can modify the structures of the key compounds to meet the desired functions. If the modifications are successful, then the key compounds can enter the stage of in vitro and in vivo examinations for selected disease duplicates (Valecha, Looareesuwan et al. 2010). If positive results are obtained, then scientists conduct safety examinations of the key compounds to investigate the mechanisms of drug absorption, metabolism, distribution, and excretion in order to examine how the targets compounds (therapeutic agents) behave once inside the body. After all, if the optimizations and results are still positive, the key compounds may therefore become potential drugs (Lahlou 2013).

1.2 Aspilia africana C.D. Adams (A. africana)

Herbal medicinal plants are gaining an interest in the primary care in health systems in most population due to the increase in cost of existing synthetic anticancer drugs and also their related side effects. To date, a number of plants derived medicines have been demonstrated to execute a crucial role in anticancer therapy by inhibiting important cancer-causing hormones and enzymes, initiating tumor cell death, restoring DNA repair, inducing the production of important enzymes with antioxidant ability, and reinforcing immunity (Raskin, Ribnicky et al. 2002). *Aspilia africana C.D. Adams* (Figure 1) is an herbal medicine which belongs to the family of *Asteraceae.* It is a semi-woody perennial herb that can grow up to two meters tall. The leaves of *A. africana* are three to seven cm wide with a six to twenty-five cm length, opposite to one another, rough with ovate lanceolate form in a creased accordion style (Obute and Adubor 2007). The flowers are yellow. The stem has

many branches and it is rigid at the base. *A. africana* belongs to the East part of Africa but grows as well in tropical Africa (Akobundu 1987). Different parts of the plant such as whole plant, leaves, roots, stems are used for medicinal purpose. It can be administered in different forms: dried, fresh, or extracts. Various methods of extraction (hot or cold) and solvents (distilled water, methanol, chloroform, ethanol) have been applied for extracting bioactive compounds from *A. africana* (Oko and Agiang 2011). The phytochemical analysis showed the presence of important plant secondary metabolites such as Alkaloids, saponins, tannins, phenols, flavonoids, and sterols in *A. africana* leaves. The concentrations of these metabolites vary. Some studies have reported that alkaloids are more abundant followed by saponins, flavonoids, and tannins (Adeniyi and Odufowora 2000); (Abii and Onuoha 2011); (Oko and Agiang 2011) while other report suggested that tannins and saponins are most abundant compounds suggesting that the concentrations of these metabolites depend on processing method used, plant material used and the location of the plant. The presence in *A. africana* of these phytochemicals can be the reason of its wide use in African folk medicine. Different studies have reported the pharmacological activities of *A. africana.* The plant is commonly used traditionally to halt bleeding, it promotes fast healing of sores and wounds, as well as for managing cardiovascular diseases. It is also used to treat anaemia, stomach pains, rheumatism pain, cough, … (Agnihotri, Wakode et al. 2010); (Iwu 2014). *A. africana* has been reported to exert remarkable antimicrobial activities in in vitro studies (Adeniyi and Odufowora 2000); (Oko and Agiang 2011). A study done by (Anibijuwon, Duyilemi et al. 2010) showed that aqueous and ethanolic extracts of *A. africana* leaves exhibited similar antibacterial at concentrations between 12.5 to 100 mg/mL in comparison with tetracycline, chloramphenicol, and ampicillin. *A. africana* was also reported for antifungal activities where *Aspergillus parasiticus, Aspergillus flavus, candida albican*, and *Penicillum notatum* were very sensitive to *A. africana* leaves extracts (Adeniyi and Odufowora 2000); (Oko and Agiang 2011). The hexane extract from *A. africana* leaves was reported to attenuate rheumatism and suppresses inflammation caused by tissue injury (Agnihotri, Wakode et al. 2010). Other

biological activities such as antioxidant, antiseptic activity, anti-ulcerative activity, anti-parasitic, and antiplasmodic activities have been reported for *A.africana* (Waako, Smith et al. 2005); (Watcho, Wansi et al. 2005); (Okoli, Akah et al. 2007); (Okokon, Nwidu et al. 2006).

Figure 1: Flower and leaves of *Aspilia africana C.D. Adams*

1.3 Gastric cancer

Gastric cancer still one of the deadly and most common cancers particularly in old men worldwide. GLOBOCAN data in 2018 showed that stomach cancer ranges in the 5th and 3rd position as the most common and most deadly neoplasm with 783,000 estimate deaths (Bray, Ferlay et al. 2018). According to WHO's report cancer rates are expected to increase in 2020 by 50% to reach 15 million (Organization 2003). Situated in the digestive tract between the small intestine and the esophagus, the stomach secretes gastric acid and enzymes that are necessary during food digestion. Another intrinsic factor is also secreted by the stomach which is crucial for vitamin B12 absorption. The stomach is lined up with glands and columnar epithelial cells that compose the mucous membrane. These cells are subjected to gastritis, an inflammation that can cause peptic abscesses, and eventually gastric cancer (Clinton, Giovannucci et al. 2019).

1.3.1 Classification and Epidemiology

Stomach cancers also called gastric cancers are malignant tumors that develop at the expense of the stomach wall. They are called primary when they originate in the stomach and secondary when they originate from another organ (Canzonieri and Giordano 2019). It's mainly adenocarcinoma, which represents by far the most frequent histological variety (around 90 to 95%), followed in order of frequency by lymphomas (4%), carcinoid tumors (3%) and stromal tumors malignant (2%) (Ku, Nfor et al. 2019). Pathologically, Gastric cancers are classified as signet ring cell carcinoma, adenocarcinoma, and undifferentiated carcinoma. This classification is less used than Lauren's classification which classified Gastric cancer into two main subtypes known as diffuse and intestinal types. Lauren's classification includes macroscopic and microscopic differences (Lauren 1965). It has been hypothesized that the diffuse type takes origin from the normal gastric mucosa, whereas the intestinal types are related to atrophic chronic gastritis together with intestinal metaplasia. The proportion of diffuse and intestinal types differs from continents or

countries. The intestinal type is more common in Europe; It often occurs in high risk areas, like distal stomach and it is usually preceded by an enduring precancerous lesion. Young people are more likely to have the diffuse type (Ahn, Lee et al. 2010). The incidence of Gastric cancer varies geographically. Most of the new cases (50%) are observed in developing countries. Areas with high risk are especially East Asia (Japan and China), South and Central America, and Eastern Europe. The areas with low risk include Southern Asia, East, and North Africa, New Zealand, and Australia (Stock and Otto 2005).

1.3.2 Risk factors and prevention

Most gastric cancer cases are a result of a combination of different factors such as the accumulation of genetic alterations and environmental factors. Regardless of the global decreasing trends, the prevention of Gastric cancer should stay a priority. Regular screening for premature detection, healthy diet, chemoprevention, and anti-*Helicobacter pylori* treatments are primary prevention methods (Massarrat and Stolte 2014). Dietary factors are the most important factors in gastric carcinogenesis. Having a healthy diet by increasing the vegetables and fresh fruits intake, low sodium diet, Mediterranean diet, reasonable alcohol drinking, high cured and red meat, maintaining normal body weight can reduce the risk of Gastric cancer (Buckland, Travier et al. 2015).

Many studies confirmed that smoking tobacco is associated with the risk of both noncardiac and cardia subtypes of Gastric cancer. The risk of developing gastric cancer in smokers is 60% in men and 20% in women compared to non-smokers (Ladeiras-Lopes, Pereira et al. 2008). High alcohol consumption has been linked with the risk of gastric cancer (Tramacere, Negri et al. 2012). *Helicobacter pylori* infection is a common source of gastrointestinal complications. 10 to 15 % of infected patients develop peptic ulcer whereas 1 to 3% is subjected to Gastric cancer (Moss 2017). The human Epstein Barr Virus has been reported as well to cause gastric carcinogenesis (Nishikawa, Iizasa et al. 2018). Family history with Gastric

cancer is also associated with the risk of developing gastric cancer with 1.5 to 3-fold (Yaghoobi, Bijarchi et al. 2010). Obesity is also a risk factor for developing gastric carcinomas (Kulig, Sierzega et al. 2010).

1.3.3 Management of gastric cancer

For planning the treatment of gastric cancer, a multidisciplinary tactic is mandatory. The multidisciplinary team must include a surgeon, gastroenterologist, pathologist, radiation and medical oncologists (Borras, Albreht et al. 2014). Surgery remains the only option for curative therapy. Other treatments such as adjuvant and perioperative chemotherapy and chemoradiation improve the outcome for gastric cancer resection with prolonged lymph node dissection (Songun, Putter et al. 2010). The efficacy and treatment strategy depends on the stage of cancer. Patients diagnosed with early stages of gastric cancer undertake radical surgery and chemotherapy and the survival rate after 5-year post-operation is 90%. However, the chance of early detection is very low due to the asymptomatic characteristics of gastric cancer at an early stage; thus almost 70% of patients are diagnosed with an already advanced stage of the disease (Smyth, Verheij et al. 2016). Gastric cancer is sensitive to chemotherapy medicines; Surgery and neoadjuvant chemotherapy are both imperative for the treatment. Neoadjuvant chemotherapy is a newly developed method for advanced stages of gastric cancer. No standards are available when it comes to choosing the agents to use for adjuvant chemotherapy. The decision depends on the results of gastroscopy, computed tomography, barium meal, and stage of the disease (Coccolini, Nardi et al. 2018). The benefit of neoadjuvant chemotherapy is that it can reduce the stage of the tumor, increase surgical rate, and extend patient survival time. In the case of unresectable gastric cancer, using neoadjuvant chemotherapy can make the reoperation possible for a complete resection of the tumor. Another method used to treat gastric cancer is radiotherapy. Radiation therapy has been developed as an adjuvant to neoadjuvant treatment (Verma, Lin et al. 2016). Undefined factors for example change in body position during radiotherapy procedures, gastrointestinal

motility, change in gastric volume, and the influence of diaphragmatic respiratory activities should be well-thought-out for radiotherapy. Normally, radiotherapy is well endured, and it reduces successfully the symptoms such as pain, bleeding, obstruction of patients with advanced-stage gastric cancer. Even though there is an improvement in radiotherapy development, gastric injury induced by radiation is still unfortunately inevitable (Bae, Kim et al. 2017). For the past decades, new targets such as molecular targeted therapies were given huge attention in cancer treatment. These targets help to identify some of the crucial factors that help in tumorigenesis through new pathways (Shen, Li et al. 2015). A number of molecularly targeted agents showed important anticancer activities in different types of tumors such as colorectal cancer, hematologic malignancies, renal carcinoma, breast cancer, and gastrointestinal tumors (Hemmatzadeh, Mohammadi et al. 2016). Some of these molecular pathways include cell growth, apoptosis, cell cycle, invasion, and angiogenesis. The therapeutic approaches involve angiogenesis inhibitors, epidermal growth factor receptor inhibitors, matrix metalloproteinase inhibitors, and cell cycle inhibitors (Moroishi, Hansen et al. 2015); (Neuzillet, Tijeras-Raballand et al. 2015). Lastly, Immunotherapy is another novel method for anticancer therapies that involves the use of antibodies or tumor vaccines to stimulate the body's immune system to fight cancer. This system is used to identify and eliminate tumors through immune observation to prevent tumor growth (Li, Wang et al. 2017).

1.4 Inflammation

Inflammation is the set of reactional defense mechanisms by which the body recognizes, destroys and eliminates all the substances which are foreign to it. The inflammatory reaction sometimes exceeds its objectives, responsible for harmful effects, but this is the price that the organism must sometimes pay to ensure the maintenance of its integrity (Netea, Balkwill et al. 2017). The causes of inflammation are many and varied: infectious agent, inert foreign substance, physical agent, post-traumatic to-tissue injury, etc. Inflammation begins with a "recognition" reaction

involving certain cells in the body (monocytes, macrophages, lymphocytes) or circulating proteins (antibodies, complement proteins, Hageman factor, etc.) (Dai and Medzhitov 2017). The recognition phase is followed by the sequential implementation of a whole set of cells and mediators whose order of intervention is complex and variable (V Stankov 2012). Certain mediators, such as prostaglandins and cytokines, are produced by different cell types, act on several cell types and sometimes control their own production by retroactive regulation (Ricciotti and FitzGerald 2011). That is to say, the complexity of the mechanisms of the inflammatory reaction, preventing the description of an overall scheme and requiring an analytical and individual description of the cells and the mediators that compose it.

1.4.1 Inflammatory cells

The cells involved in the mechanisms of inflammation are both circulating cells that migrate to the interstitial tissue and resident cells of the interstitial tissue (Table 1)

Table 1: the different cells involved in the inflammatory reaction.

Circulating blood cells	Resident tissue cells
Polynuclear neutrophils	Macrophages
Monocytes	Histiocytes
Polymorphonuclear Eosinophils	Mast cells
Basophils	Endothelial cells
Platelets	Fibroblasts
Lymphocytes	
Plasma cells	

Polynuclear neutrophils' production is medullary from pluripotent stem cells. Their maturation and proliferation are mainly controlled by 2 cytokines: GM-CSF and G-CSF. There is a basic production of neutrophils, a production which increases when necessary. The maturation of neutrophils requires about 5 days and the lifespan of a neutrophil is 2 days (McDonald, Pittman et al. 2010). Their action in inflammation is exerted via surface receptors:

-different chemotactic receptors (for LTB4, C5a); The activation of these receptors generates the migration of neutrophils to the site of inflammation, but also the production of oxygenated free radicals and the expression of adhesion molecules (Sreeramkumar, Adrover et al. 2014).

- receptors for opsonins: Fc receptors for the Fc fragment of IgG, receptors for fragments of activated complement.

- receptors for adhesion molecules of endothelial cells.

Once activated, Polynuclear Neutrophils synthesize products first stored in primary (lysosomes) or secondary granules, then released either inside the cell itself and acting on phagocytosed substances, or in the extracellular medium (Sadik, Kim et al. 2011).

Monocytes, circulating macrophages and tissue macrophages constitute the mononuclear phagocyte system. All of these cells are derived from circulating monocytes of bone marrow origin. Monocytes have a short lifespan: around 24 hours. Conversely, tissue macrophages have a long lifespan: 2 to 4 months (Davies, Jenkins et al. 2013). Many situations cause activation of macrophages: encounter with a microorganism, with an inert particle, with a tissue breakdown product or binding with a natural ligand for one of their receptors: antibodies (fixation by their Fc), carbohydrates, thrombin, fibrin, growth factors (CSF, M-CSF, GM-CSF), cytokines (Il1, IL6, IL10, TNF, Interferons), parathormone, calcitonin (Gordon and

Martinez 2010). The activation of macrophages has the following consequences: phagocytosis, which is a much slower process than that of neutrophils. The digestion of phagocytosed material is often incomplete and peptides are learned in phagosomes and phagolysosomes to be subsequently presented to T lymphocytes by HLA class II molecules expressed on the surface of the cell and the release of many secretory products involved in the mechanisms of inflammation: enzymes, cytokines, complement components, coagulation components, free radicals (Gordon and Martinez 2010).

The cells of the endothelium of small and medium-sized vessels play an important active role during inflammation. The state of the junction of cells with each other and with the extracellular matrix controls the passage of liquids and macromolecules from the intravascular space to the interstitial tissues. This state of junction involves many trans-membrane or intracellular proteins: connexins, cadherins, cytoskeleton proteins, surface integrins (Muller 2014). Vascular tone and vasomotricity are provided by the smooth muscle fibers of the vessel wall and are regulated by molecules produced by the endothelial cells themselves. These molecules promote either vasoconstriction (endothelin-1, thromboxane A2) or vasodilation (NO, PGI-2). The production of these vasoactive molecules is itself subject to the action of various inflammatory mediators: thrombin, bradykinin, histamine, eicosanoids, cytokines and growth factors (IL1, TNF, TGFß, PDGF, EGF, etc.) (Pober and Sessa 2007). The endothelial cells express on their surface adhesion molecules that are involved in diapedesis: selectins E and P, ICAM-1, VCAM-1. They participate in post-inflammatory repair phenomena by the production of matrix proteins and different proteases (Xiao, Liu et al. 2013).

Platelets are activated as soon as they pass through vessels located within an inflammatory focus. They then produce mediators with proinflammatory activity: eicosanoids, thromboxane A-2, 12 HETE, PAF. They also participate in repair phenomena through the production of fibronectin, TGFß (Transforming Growth

Factor), EGF (Epidermal Growth Factor) and PDGF (Platelet-Derived Growth Factor) (Thomas and Storey 2015). The fibroblasts of the extracellular matrix of the connective tissue produce, during the inflammatory reaction, enzymes that destroy the matrix: collagenases, gelatinase, stromelysin, cathepsins, a serine protease, etc. They also participate in the healing phenomena by the production of different constituents of the matrix: collagens, proteoglycans, fibronectin, elastin (Van Linthout, Miteva et al. 2014). Polynuclear eosinophils act during allergic phenomena but also during inflammatory processes. Activated then through specific receptors for inflammation mediators, they, in turn, produce different molecules that promote inflammation: eicosanoids, PAF, phospholipase, cytokines (IL1, TNFa, etc.) (Fulkerson and Rothenberg 2013). Basophils, circulating cells, and mast cells, tissue cells, have receptors with high affinity for Fc of IgE on their surface. They are capable of releasing several important mediators of the immuno-allergic and inflammatory reaction: histamine, serotonin, leukotriene, PAF (Geering, Stoeckle et al. 2013). Lymphocytes are mainly involved in the mechanisms of immunity but they participate in the inflammatory reaction by their production of different cytokines (Koyasu and Moro 2012).

1.4.2 Inflammatory mediators

Mediators of inflammation can be described on the one hand as plasma activation systems and on the other hand as cellular mediators (Larsen and Henson 1983). Plasma activation systems are multiprotein systems whose components are produced at a distance from the inflammatory focus. The various components of these systems are present in the circulating blood where they remain in the state of inactive precursors until they are put in the presence of a specific activator (Schmaier 2014). These systems have close functional relationships with each other:

-the contact system: Hageman factor (FH) or factor XII, prekallikrein (PK), High molecular weight Kininogen (HMWK), and factor XI. Activated Hageman factor (FHA) stimulates the aggregation and degranulation of neutrophils. Kallikrein

activates chemotaxis. Most importantly, bradykinin triggers the production by endothelial cells of PGI2, thromboxane A2, tissue plasminogen activator (Colman 1984). It is thus a powerful mediator of the inflammation which causes pain, vasodilation, increased vascular permeability, the margination of leukocytes.

-the coagulation-fibrin formation system: During inflammation, the coagulation system is mainly activated by its extrinsic path, that is to say by the action of tissue thromboplastin expressed by means of a tissue lesion on the surface of monocytes and endothelial cells. Fibrin is involved in the inflammatory reaction by stimulating the activity of polymorphonuclear neutrophils, platelets, and endothelial cells. The coagulation mechanisms are regulated by several inhibitors: especially antithrombin III but also alpha-2-macroglobulin, heparin, alpha-1-antiprotease (Chapin and Hajjar 2015).

-the fibrinolysis system: The fibrinolysis system dissolves intravascular or tissue fibrin deposits. Plasmin results from the activation of plasminogen under the action of tissue plasminogen activator. The activity of plasminogen is expressed as soon as it is attached to fibrin (Dobrovolsky and Titaeva 2002).

-the complement system: The complement system is a multiprotein system made of around thirty proteins or components, involved both in the anti-bacterial defense mechanisms by complementing the action of antibodies and in the mechanisms of inflammation (Sarma and Ward 2011). The components of the complement are articulated according to two ways called classic way (comprising C1, C4, and C2) and alternate way (C3, B, and D) which meet at the level of C3 in a terminal common trunk, the activation of which leads to the formation of the membrane attack complex with cytolytic action. The activation of the complement intervenes in many general inflammatory diseases: glomerulonephritis, diseases with immune complexes, diseases by autoantibodies (Vlaicu, Tatomir et al. 2016).

There are many cellular mediators of inflammation: histamine, serotonin, eicosanoids, oxygen-free radicals and nitric oxide, cytokines, substance P, neurokinin, enzymes of tissue destruction. Histamine is mainly synthesized in basophils and mast cells where it is stored in cytoplasmic granules (Schayer 2013). It is released if the cell is activated by an allergen-IgE complex, an anaphylatoxin or substance P. Histamine can then react via 3 specific types of receptors: H1, H2, and H3. It is through the H1 receptors that histamine is involved in the inflammatory reaction (Ganellin and Parsons 2013).

Eicosanoids are compounds with 20 carbon atoms that are derived from arachidonic acid. Arachidonic acid is released from membrane phospholipids of inflammatory cells under the action of phospholipases A2 (Gilroy 2010). Two major varieties of enzymes are involved in the metabolism of arachidonic acid: lipoxygenases induce the formation of leukotrienes: LTB4, LTC4, LTD4, and LTE4; cyclooxygenases generate the formation of prostaglandins (PGI2 or prostacyclin, PGE2, PGD2) and thromboxanes (TXA2 and TXB2). It has been highlighted the existence of 2 types of cyclooxygenases: COX1 and COX2 (Hla, Bishop-Bailey et al. 1999). COX1 is constitutive. It works continuously and provides physiological functions: platelet aggregability, protection of the gastro-duodenal mucosa, regulation of renal blood flow. Conversely, COX 2 is inducible. It is only active when the phagocytes are exposed to an inflammatory process (Morita 2002). Eicosanoids have many biological properties: PGE2 and PGI2 act on the smooth muscle fibers of the vessels: vasodilation, increased permeability, edema; PGE2 facilitates the action of pain mediators. It inhibits the activity of suppressor T lymphocytes, increases the production of immunoglobulins, decreases the production of IL2 by lymphocytes (Birukova, Zagranichnaya et al. 2007); Leukotriene B4 is the most important of the leukotrienes. It is the most potent polynuclear chemotactic agent. It activates the phagocytes (Yokomizo 2015). Thromboxane A2 causes vasoconstriction and promotes the aggregation of platelets (Armstrong, Dhanji et al. 2010).

The phenomena of phagocytosis by neutrophils induce an increase in the consumption of oxygen by these cells, at the origin of the formation of oxygenated free radicals: superoxides O2-, hydrogen peroxide H_2O_2 and hydroxyl radicals OH-. These free radicals are potentially toxic, capable of disorganizing cell membranes and promoting cytolysis. Nitric oxide (NO) is a free radical produced by the oxidation of arginine under the action of a family of enzymes: NO synthases or NOS (Gupta, Fernie et al. 2011). The activation of certain inducible NOS during inflammation generates significant amounts of NO. NO has inflammatory properties: vasodilation, edema, erythema (Faro, Fox et al. 2014).

Cytokines are soluble glycoproteins that act as intercellular mediators. Synthesized and released by their original cell under the influence of various stimuli, they deliver their messages by reacting with specific membrane receptors present on the surface of target cells (Neurath 2014). The same cytokine can be produced by different cell types and act on a large number of different targets. The multiplicity of source and targets, their numerous interactions between them allow us to speak of a cytokine network. They are involved in the mechanisms of inflammation and immunity (Arango Duque and Descoteaux 2014). At least 40 cytokines have been described to date and the complexity of their actions means that only the main cytokines playing a major role in the inflammatory reaction should be mentioned here, some of them having therapeutic action:

- Interleukin-1 (IL1): exists in two molecular forms, IL1-α and IL1-β, which differ in their amino acid sequence but have the same biological properties. However, IL1-α is thought to act primarily as an intracellular messenger, with IL1-β performing extracellular activities. It is produced mainly by cells of the phagocyte system but also by lymphocytes, endothelial cells, epithelial cells, keratinocytes, fibroblasts (Garlanda, Dinarello et al. 2013). IL1 is the only cytokine to be synthesized and then stored in the cell as an inactive precursor. The action of IL1-β -convertase, or ICE,

is necessary for the formation of active IL1-β, which acts on the target cells via a specific IL1-R receptor (Weber, Wasiliew et al. 2010).

- Tumor necrosis factors: TNF-α and TNF-β: TNF also exists in two forms: TNF-α and TNF-β. TNF-β is produced exclusively by lymphocytes. Conversely, TNF-α is produced by many cells: macrophages, monocytes, T and B lymphocytes, keratinocytes, mesangial, epithelial, endothelial cells, basophils and mast cells, neutrophils, eosinophils, fibroblasts (Porter 1990). It plays a major role in inflammation and acts through a specific membrane receptor of which there are two forms: TNF-R1 and TNF-R2. The presence of a soluble form of TNF-R constitutes an important regulatory system, the synthesis of TNF-R being under the control of TNF itself (Al-Meghaiseeb, Al-Robayan et al. 2016).

- Interleukin-6 (IL6): IL6 is produced by most cells (monocytes, fibroblasts, synoviocytes, osteoblasts, etc.) in response to various stimuli, including certain infectious agents or their components. The IL6 receptor is expressed on many lymphoid and non-lymphoid cells (Wolf, Rose-John et al. 2014). IL6 intervenes by stimulating the hepatocyte production of proteins in the acute phase of inflammation: CRP, SAA, haptoglobin, C3, fibrinogen, a1-antitrypsin, a2-macroglobulin. However, IL6 decreases the production of IL1 and stimulates the production of molecules involved in tissue repair processes: collagenase inhibitors, IL1-Ra. IL6 also intervenes in the mechanisms of immunity by promoting the differentiation of B lymphocytes into plasma cells, by stimulating the proliferation of T lymphocytes in association with IL2 and by promoting the generation of cytotoxic T lymphocytes (Schaper and Rose-John 2015).

- Interleukin-8 (IL8): IL 8 is a chemokine whose main property is to attract circulating leukocytes to an inflammatory focus. Many cells stimulated by IL1 or its TNFs (monocytes, macrophages, fibroblasts, endothelial cells, hepatocytes, etc.) produce IL8. Polynuclear neutrophils are the preferred target of IL 8 via a specific receptor: IL8-R. IL 8 induces chemotaxis and activation of polymorphonuclear cells

with induction of cyclooxygenase, lipoxygenase and NO synthase (Shahzad, Knapp et al. 2010).

- Interleukin-10 (IL10): IL 10 is a regulatory cytokine produced by both Th2-type T cells and monocytes. It inhibits the presentation of the antigen by the antigen-presenting cells (macrophages, monocytes) and slows down the production of different cytokines: TNF, IL1, IL6, IL8 (Sabat, Grütz et al. 2010).

1.4.3 Targeting inflammation in diseases

The adrenal glands secrete hydro - cortisol which contributes to the return to calm at the end of the response to normal inflammation. In the case of a chronic inflammatory reaction, different types of anti-inflammatory drugs are used: corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs).

- corticosteroids: The anti-inflammatory action of glucocorticoids was first demonstrated and used in 1948 in the treatment of rheumatoid arthritis by R.S. Hench, which earned him, along with his hospital biochemist, E.C. Kendall, the Nobel Prize in medicine (Bijlsma, Jacobs et al. 2015). Corticotherapy is based today on the use of synthetic hormone derivatives, which increase anti-inflammatory action and reduce metabolic actions (Prigent, Maxime et al. 2003). They freely cross cell membranes. In the cytoplasm, they attach themselves to a specific receptor that belongs to the nuclear steroid receptor superfamily. Glucocorticoid receptors exist in the cell's cytoplasm as a heterooligomeric complex. After fixing the glucocorticoid, the Glucocorticoid-receptor complex migrates towards the nucleus and will act directly on the DNA by fixing itself on specific sequences, called GRE (Glucocorticoid Response Element), thus intervening in the regulation (activation or inhibition) of transcription of target genes. The glucocorticoid receptor GC complex also works by inhibiting the action of certain nuclear transactivating proteins: NF-kB and AP-1 in particular. It is by this latter mechanism that GCs would inhibit the

production of phospholipase A2 and many pro-inflammatory cytokines: IL-1, IL-6, IL-2, TNF-α or interferon-γ (Vandevyver, Dejager et al. 2013).

- Non-steroidal anti-inflammatory drugs: The existence of an anti-inflammatory substance has been known for a very long time since it was at the end of the first century AD that a Greek doctor discovered the pain-relieving properties of willow leaves. It was in 1860 that salicylic acid was synthesized and in 1875 used for the first time in the treatment of rheumatic fever (Norn, Permin et al. 2009). It was in 1946 that the anti-inflammatory powers of phenylbutazone were discovered, those of indomethacin dating from 1963. These molecules opened the way to the therapeutic class of nonsteroidal anti-inflammatory drugs (NSAIDs), so-called by their anti-inflammatory properties without having the action of steroids, unlike glucocorticoids. NSAIDs all work by inhibiting a membrane enzyme, cyclooxygenase (COX) (Rao and Knaus 2008). The inhibition of this enzyme by NSAIDs is responsible for a decrease in the production of prostaglandins E2 and I2, important mediators of inflammatory phenomena. But the inhibition of these prostaglandins, as well as thromboxane A2 in all tissues, accounts for the potential adverse effects of most NSAIDs: decrease in the protection of the gastrointestinal mucosa (responsible for peptic ulcers) and decrease in platelet aggregability (responsible for an increased risk of bleeding) (Næsdal and Brown 2006).

- Pro-inflammatory cytokine inhibitors: They are directly derived from advances in knowledge regarding the role of various pro-inflammatory cytokines, in particular TNF-α and IL-1. The molecules useful for blocking these cytokines are essentially represented by monoclonal antibodies or recombinant proteins. Chimeric monoclonal antibody (human-mouse) anti-TNF-α or infliximab (Remicade®). It is a monoclonal antibody capable of binding free TNF-α as well as TNF-α engaged on its receptor on the surface of cells. It is administered as an infusion (Dekker, Armbrust et al. 2004); TNF-α or Etanercept (Enbrel®) soluble receptor. It is a fusion protein of the TNF p75 receptor or type II receptor. Produced by genetic engineering,

it is a chimeric protein combining two domains of extracellular binding of the human receptor II to TNF and a CF domain of human IgG1. It is able to fix TNF-α and TNF-β. It is administered subcutaneously (Goffe and Cather 2003); Recombinant Il1-Ra. This IL1 inhibitor is marketed under the name of Anakinra®. Its daily subcutaneous injection contributes to the increase in serum levels of IL1-Ra, a natural IL-lβ inhibitor (Mertens and Singh 2009).

1.5 Aim of the thesis

In this thesis, we investigated:

- The potential use of novel green extraction techniques in comparison with conventional organic extraction techniques on the recovery of phytochemicals from *Aspilia africana C.D. Adams*.
- Phytochemical characterization of obtained extracts, their potential antioxidant activities and in vitro cytotoxicity.

- The anticancer effect of Aqueous Extract of *Aspilia africana C.D. Adams* (AAE) in gastric adenocarcinoma AGS cells and the mechanisms of AGS cell death associated with AAE treatment.

- The anti-inflammatory potential of AAE in macrophages RAW264.7 cells stimulated with lipopolysaccharide and the pathways involved.

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CHAPTER TWO

Characterization and *in vitro* cytotoxicity of phytochemicals from *Aspilia africana C.D. Adams* obtained using green extraction techniques.

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2.1 Abstract

In this study, we investigated the effect of different extraction techniques for the recovery of bioactive secondary metabolites from *Aspilia africana* (*A. africana*). Subcritical water extraction (SWE) at different temperatures (SWE140, SWE160, SWE180), and hot water extraction (HWE) were compared with conventional solvent extraction using ethanol (80%, soxhlet). The extracts were characterized for the total phenolic contents (TPC), Total flavonoid contents (TFC), and phenolic compounds using HPLC-UV-VIS. The antioxidant activity by DPPH assay and anticancer activity against AGS, A549, and HeLa cell lines were also analyzed by MTT assay. Results showed that HWE extracts contained the highest concentration of gallic acid, TPC, TFC and exhibited the highest antioxidant activity with an IC_{50} nine times lower than that of ethanol extracts, followed by SWE. MTT assay showed that AGS cells were more sensitive to *A.africana* extracts (AAE), followed by A549 while HeLa showed little response to the treatments. HWE exhibited the highest antiproliferative activity followed by SWE160. DAPI staining revealed that HWE extracts induced a remarkable DNA breakage and nuclear condensation in treated cancer cells. This study revealed that HWE and SWE are efficient techniques to recover extracts with antioxidant and anticancer potential from *A. africana*.

2.2 Introduction

There is a growing interest in the development of pharmaceutical products from botanical extracts attributed to the plant's secondary metabolites, synthesized during plants adaptation to stress and diseases (viral and bacterial) (Akula & Ravishankar, 2011); (Jimenez-Garcia et al., 2013). The well-known secondary metabolites in plants are phytochemicals like phenolic acids, flavonoids, alkaloids, saponins which have been reported to exhibit antioxidant activities, anticancer, anti-inflammatory activities, antimicrobial activities, and many other health-related benefits (Wink, 2012); (Kabera, Semana, Mussa, & He, 2014); (Fresco, Borges, Marques, & Diniz, 2010). The tropical plants have been demonstrated to synthesize a wide range of secondary metabolites due to their prolonged exposure, and adaptation on the tropical environment (Bennett & Wallsgrove, 1994); (Balandrin, Klocke, Wurtele, & Bollinger, 1985); (Engel, Puglisi, Jensen, & Fenical, 2006).

A. africana is an indigenous tropical plant from East Africa that is used as a traditional herbal medicine due to its secondary metabolites' composition (Okwu & Josiah, 2006). In many parts of Africa, these plant species are used in the treatment of wounds due to the antimicrobial activities of extracts, and as anti-malaria medicine, it is also endorsed for the anti-inflammatory activity (Okoli, Akah, & Okoli, 2007); (Waako, Katuura, Smith, & Folb, 2007)). Methanol extracts of *A. africana* leaves showed a high antioxidant potential (Johnson, EI, & Archibong, 2017), and Okoli et al, reported anti-inflammatory activity against edema from hexane extracts of *A. africana* (Okoli, Akah, Nwafor, et al., 2007).

The extraction of targeted bioactive compounds from natural products and the formulation of pharmaceutical drugs using conventional volatile organic solvents is a burden to both environment and economy due to the waste disposal, toxicity of solvents and pollution (Beh, Mammucari, & Foster, 2016). Hence, there is a huge interest in the use of greener solvents for extraction of bioactive compounds and preparation of pharmaceuticals, and nutraceuticals. The use of water in the near-

critical state known as subcritical water is one of the versatile technologies that is highly recommended for the recovery of herbal extracts and nutraceuticals (Mustafa & Turner, 2011). The operation conditions and optimization of organic compounds extraction have also been described (Plaza & Turner, 2015).

Subcritical water extraction is an extraction technique that uses liquid water as an extraction solvent at temperatures above the atmospheric boiling point of water but below the critical point of water, and at a pressure high enough to maintain water in the liquid state (100–374 °C; 1–22 MPa) (Cvetanović et al., 2018). These conditions dramatically change the solvent properties of water, which are very sensitive near the critical point, and the thermodynamic properties of water can be tuned slightly to optimize the extraction of targeted solutes (Plaza & Turner, 2015). The subcritical state of water reduces the dielectric constant to levels comparable to the dielectric constants of organic solvents (like methanol and ethanol at ambient conditions) used conventionally for the extraction of organic compounds (Carr, Mammucari, & Foster, 2011). The change in the dielectric constant of water together with the decrease of the hydrogen bond strength at high temperature and pressure reduces water polarity. In addition, the density of liquid water at elevated temperature and pressure significantly decreases within the subcritical water ranges (100 - 374°C), under these thermodynamic conditions there is a significant change in the solubility of water due to the decreased density and hydrogen bonding as a result of increased temperature. These properties make water an ideal solvent for the extraction of both polar and non-polar organic compounds, and an efficient solvent for the recovery of bioactive compounds from botanical resources (Plaza & Turner, 2017), (Mustafa & Turner, 2011).

The number of studies on the potential of AAE for biological activities has increased enormously in the past years. Although the results are thrilling, there is limited information on the extraction technologies. Existing studies applied conventional extraction techniques like methanol and ethanol for the study of *A.*

africana characterization and biological activities. However, due to the toxicity and cost of conventional organic solvents used in the extraction, there is a limited application of the extracts. Our study explored the potential of novel extraction techniques that use green solvents to recover non-toxic and ready to use extracts. Subcritical water, hot water extraction methods were performed, and ethanol was used as a conventional solvent for comparison. The extracts were characterized for their phenolic contents using both chromatographic (HPLC) and spectrophotometric methods. To this end, we studied the potential antioxidant and anticancer activities of the AAE.

2.3 Materials and Methods

2.3.1 Reagents and chemicals

Phenolic acid standards, flavonoids (gallic acid, chlorogenic acid, syringic acid, ferulic acid, and quercetin) and DPPH standards were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). Solvents used for analysis (methanol, water, and acetonitrile) were HPLC-grade and obtained from Honeywell Burdick & Jackson, (Ulsan, Republic of Korea). 99.5% glacial Acetic acid was obtained from Samchun Pure Chemical Co., Ltd (Pyeongtaek-Si, Republic of Korea). Syringe filters and Thimble were purchased from Advantec Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). Other standards and reagents (unless mentioned above) applied in this study were of analytical grade and obtained from Sigma-Aldrich Co., (St. Louis, MO, USA).

2. 3.2 Sample preparation

A. africana leaves were harvested from Burundi, and directly sun-dried for 3 days, 8 hours every day. Dried leaves were packaged in airtight plastic bags and sent by post to Pukyong National University, Busan South Korea for further treatments. The leaves were pulverized using a laboratory blender, the particles were screened in a 450 µm mesh sieve to obtain uniform particles. Powdered particles were kept in a refrigerator at -4°C during the experiment period.

2.3.3 Extraction procedures

2.3.3.1 Ethanol extraction

Soxhlet extraction of *A. africana* bioactive metabolites was carried out using ethanol as a solvent. Briefly, 2g of powdered *A. africana* particles were added into the extraction section of a Soxhlet apparatus and extracted (80% ethanol (99% purity), 150 mL, and 7 h). After extraction, the sample was filtered using Whatman number 1 filter papers and kept at 4°C until needed for further analysis.

2.3.3.2 Hot water extraction (HWE)

Hot water extraction (HWE) was carried out by using a WiseStir (MSH-30D) premium hot plate stirrer equipped with a thermometer for temperature control. After adding 2 g of *A. africana* powder in 150 ml flask, 50 mL of distilled water was poured in the flask and a magnetic octahedral stir bar was added in the flask, the heating temperature was then set at 80°C for 30 min at ambient pressure. After the heating time the extracts were filtered using F1093 chmlab group filter papers using a laboratory vacuum filtering machine and kept at 4°C until needed for further analysis.

2.3.3.3 Subcritical Water Extraction (SWE)

Subcritical water extraction was carried out with a laboratory scale subcritical water extractor described in details by D Nkurunziza, et al. (Nkurunziza, Pendleton et al. 2019). The instrument is composed of a high-pressure reactor equipped with a double impeller stiller and an electric heating system, a control panel to monitor the operating conditions and safety. The reactor size is one liter in which 600 mL of solvent was used, and hydrolysates were fully withdrawn at the end of each experimental time, and another batch was started after cooling the reactor up to the ambient temperature to maintain the same rate of heat transfer within the reactor at different hydrolysis time. The operation conditions for temperature levels were 140,160 and 180°C at a fixed pressure set at 5 MPa and fixed solid/liquid (powder/water) mixing ratio (40 mg/ml). The extraction of bioactive secondary metabolites from *A. africana* leaves powdered sample was carried for 30 min extraction time. The extracts were freeze-dried at -110 °C for 96 hours, using the laboratory scale HyperCool (HC4110) freeze drier from Qyrozen Co., Ltd. Daejeon, 34187, Korea.

2.3.3.4 Total phenolic and flavonoids composition (TPC and TFC)

The phenolic contents Folin-Ciocalteu assay following the protocol of Ainswoth et, al (Ainsworth & Gillespie, 2007), with minor modifications. Briefly, different concentrations of standard solutions from 31.25, 62.5, 125, 250, 500 and 1,000 mg/L (ppm), made out of Gallic acid stock solution in methanol were prepared. A 1mL aliquot of extracts or a standard was put in a 12 mL flask with 4.5 mL of distilled deionized water. After, 0.5mL Folin-Ciocalteau's phenol reagent was then added to the mixture and carefully shaken. After 5 min, Na_2CO_3 solution (5mL) was also added to the mixture which was then diluted to the volume by adding distilled deionized water, and thoroughly mixed. The solution was incubated in dark for 90 min at room temperature. After incubation, 300 µL of the solution was read using a 96-well microplate reader at 750 nm. TPC of *A. africana* extracts was expressed as mg Gallic acid equivalent per gram of dry powder (mg GAE/g).

Total flavonoid was determined by using the aluminum chloride colorimetric method (Casazza, Aliakbarian, Sannita, & Perego, 2012) with some modifications. Briefly, 0.15 ml of the extracts and standard quercetin were diluted at a ratio of 1:19 and mixed with 1.85 ml of distilled water and subsequently with 0.075 ml of 5% (w/v) sodium nitrite solution and was allowed to react for 5 min. then, 0.15 ml of 10% aluminum chloride was added and allowed to further react for 6 min before adding 0.5 ml of 1 M sodium hydroxide. Distilled water was added to bring the final volume of the mixture to 3 ml. The aliquot of 300 µl from the mixture was taken for analysis in the 96-well plate reader at the absorbance of 510 nm. The calibration curve was plotted using the standard solution of quercetin in the range of 0.01to 1mg/mL, resulting in a linear equation ($R^2 = 0.9999$). The total flavonoids contents of AAE was expressed as mg QE/g of powdered *A. africana* sample.

2.3.3.5 Antioxidant activities determinations

The radical scavenging activities of AAE was determined by DPPH assay carried out according to the method of (Rodríguez-Meizoso et al., 2006), with some modifications. The DPPH radical solutions were prepared as follow: 72 mg of DPPH were dissolved in 300 ml of methanol, after the solution was diluted 10 times with methanol and measured for absorbance at 517 nm (using a Synergy HTX 96 well plate reader spectrophotometer) to set the initial absorbance of the DPPH radical solution. Different concentrations of the extracts were tested by mixing an aliquot of 0.15 ml from each prepared concentrations of the extracts with 2.85 ml of DPPH radical solution in the test tubes and incubated for 30 min in the dark at room temperature. Methanol was used to adjust the absorbance at zero and the DPPH solution was used to get the initial absorbance before the reaction of the DPPH radicals with the extracts. The DPPH absorption percentages (AA %) after the reaction was calculated by the following formula:

$$AA\% = \frac{(AC-AS)}{AC} \times 100$$

Where: *AC* is the absorbance of the control or initial DPPH radical solution used, and *AS* is the absorbance after the reaction of DPPH radicals with the sample. The IC_{50} of the extracts was determined as the amount of extracts required for 50% inhibition of DPPH radicals. The values were obtained by plotting the percentage inhibition against the linear calibration curve (n=5, r=0.995) from different concentrations of extracts (mg/ml). The lower the $IC_{50,}$ the higher the antioxidant activity.

2.3.3.6 High-performance liquid chromatography analysis of phenolic compounds

The analysis of single phenolic compounds from *A. africana* hydrolysates was achieved by using a reverse phase HPLC system (Hitachi Model-2000 Series, Japan), according to the method used previously by Saravana et al. (Saravana et al., 2016)

with some modifications. The unit is equipped with L-2420 UV-VIS Detector, a Hitachi pump L-2130 and, an L-2300 Column oven holding an Agilent Eclipse plus C_{18} Column (5.0 µm particle size, 4.6 × 250 mm ID) as stationary phase for phenolic compounds separation. The analysis of the phenolic compounds was performed according to the method mentioned in the above reference. The detection wavelength was set at 280 nm for phenolic compounds determination. The mobile phase was made of water with 0.1% glacial acetic acid as solvent A, and acetonitrile with 0.1% glacial acetic acid was used as the solvent B. The mobile phase gradient of the two eluents was made as follows: solvent A (92%), solvent B (8%) (0-2 min); solvent A (90%), solvent B (10%) (2-25 min); 70% solvent A, 30% solvent B, (25- 50 min). The total elution time for each trigger was 50min with the mobile-phase flow rate set at 1 mL/min. A comparison of spectrum and retention time with reference phenolic standards was used for the detection of phenolic compounds. The quantification of every single phenolic compound was determined by using the corresponding linear calibration curves (with $r^2 \geq 0.998$), and results were expressed in mg/g DW.

2.3.3.7 Cell culture and cell viability

The powder obtained after freeze-drying of AAE was used for cell viability experiment. 100 mg of powdered sample was diluted in 1mL of distilled water and used as a stock solution for the preparation of further dilutions, and it was kept at 4°C during the experiment. Prior to treatment, a working solution of 10 mg/mL was prepared from the stock solution using the specific culture media. To evaluate the cytotoxicity of obtained extracts, we used five cell lines HacaT (human normal Keratinocytes), HEK-293(human normal embryonic kidney), HeLa (cervix adenocarcinoma), A549 (lung carcinoma) and AGS (Gastric adenocarcinoma). HEK-293, HeLa, A549, and AGS were purchased from American Tissue Culture Collection (Manassas, VA, USA) and HaCaT cells were bought from AddexBio (San Diego, CA, USA). Cells were maintained in specific media (Dulbecco's Modified Eagle's Medium (DMEM) for HacaT, Roswell Park Memorial Institute 1640 (RPMI-1640) for AGS and A549 and modified eagle's medium (MEM) for (HEK-293 and HeLa) obtained from Hyclone Laboratories (UT, USA). All media contained heat-inactivated Fetal Bovine Serum (10%) (Hyclone Laboratories) and a mixture of 100 Uml^{-1} penicillin-10μgml^{-1}streptomycins (1%) (GmbH PAA Laboratories, Austria). All Cells were kept at 37 ºC and 5% CO_2 in an incubator and sub-cultured until they were ready for use. The viability assay was conducted as follows: a density of 10^4 from each cell line was seeded in triplicate in 96 well plates and incubated at 37 ºC and 5% CO_2 for 24h. After 24 h of incubation, cells were treated with different concentrations of each extract separately and incubated at 37 ºC and 5% CO_2 for 24h. After 24h of treatment, media were removed from cells and replaced with fresh media containing 10 µl of WST-1® solution obtained from Daeil Lab Service (Seoul, Korea) and the cells were again incubated for 3 h at 37 ºC and 5% CO_2. Then the viability was measured by reading the absorbance at 460 nm using ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3.3.8 DAPI Staining

AGS, A549, and HeLa cells were seeded on cover glass dishes and incubated at 37 ºC and 5% CO_2 for 24h. After 24h, cells were treated or not with 200 µg/ml of each extract (Ethanol, HWE, SWE180, SWE160, and SWE140) and incubated for 24h. Then the treated cells were twice washed in PBS (Phosphate Buffered Saline), stained with 1µg ml^{-1} DAPI diluted in methanol (Roche Applied Science, IN, USA) and incubated for 20 min in dark at 37 ºC. After incubation, cells were washed twice in PBS, fixed in 4 % formaldehyde for 15 min and then mounted in Prolong Gold antifade solution on microscopic slides. Cells were then observed with a Laser LSM 700 Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

2.3.3.9 Statistical analysis

The IBM SPSS software version 20 (Chicago, USA) was used to analyze the data statistically. Turkey HSD ($p < 0.05$) was used to identify significant differences within groups.

2.4 Results and discussion

2.4.1 Effect of extraction techniques of *A. africana* on TPC and TFC

The total phenolic and flavonoid content of AAE obtained using different extraction techniques are depicted in Fig. 2.1 and 2.2, and there was a significant difference ($p < 0.05$) in the concentrations of TPC, and TFC from different extraction techniques. As seen in the Fig.2.1, the highest and lowest concentrations of TPC were from hot water extraction technique (HWE) and ethanol extraction, with 76.61±3.90 and 7.91±1.76 mgGAE/g, respectively ($p < 0.05$). The TPC increased slightly when temperatures were augmented from 140 to 160 °C from 60.05±1.07 to 63.91±0.43 mgGAE/g, and followed by a slight decline at 180°C to become 61.36±2.71 mgGAE/g (Fig. 2.1). The TFC of different AAE is displayed in Fig. 2.2 and they expressed a similar trend seen with TPC, the highest and lowest TFC contents were found from HWE and ethanol extraction techniques with 62.71±2.10 and 20.12±0.62 mgQE/g, respectively. The values of TFC increased slightly when temperature conditions were increased from 140 to 160°C, from 51.52±2.41 to 53.72±0.87 mgQE/g, and followed by a substantial decrease at 180°C to become 42.12±2.67 mg QE/g.

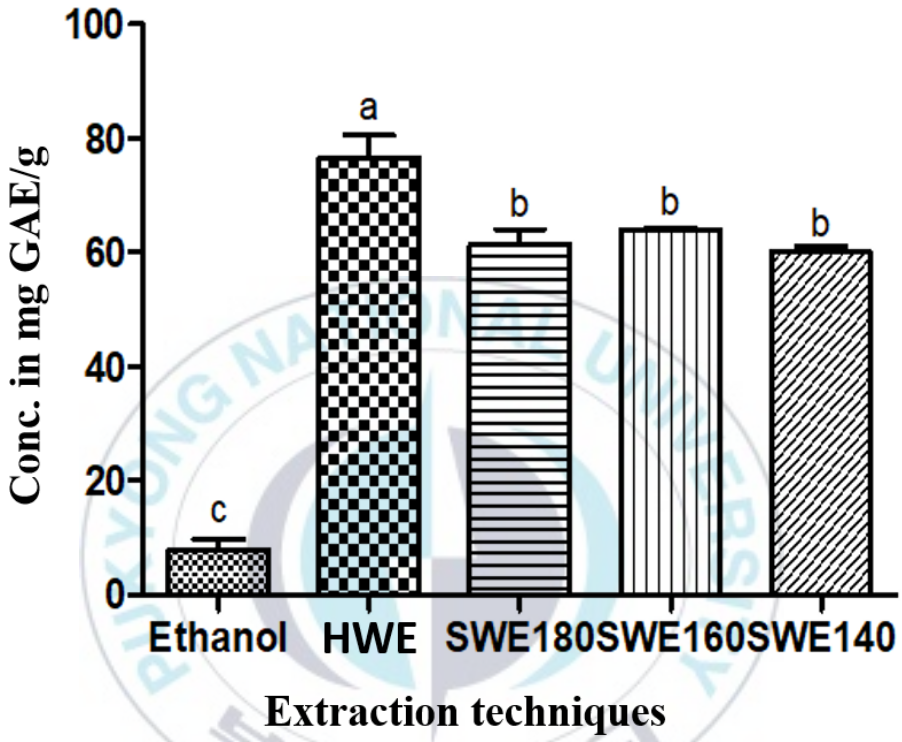

Figure 2. 1: The concentrations of total phenolic contents (TPC) of *A. africana* from different extraction techniques in mg GAE/g (mg Gallic acid equivalent per gram of *A. africana* dry powder sample). Values are presented as ± SD (n=3). The different letters on the histogram imply statistical differences ($p \leq 0.05$) between different extraction techniques

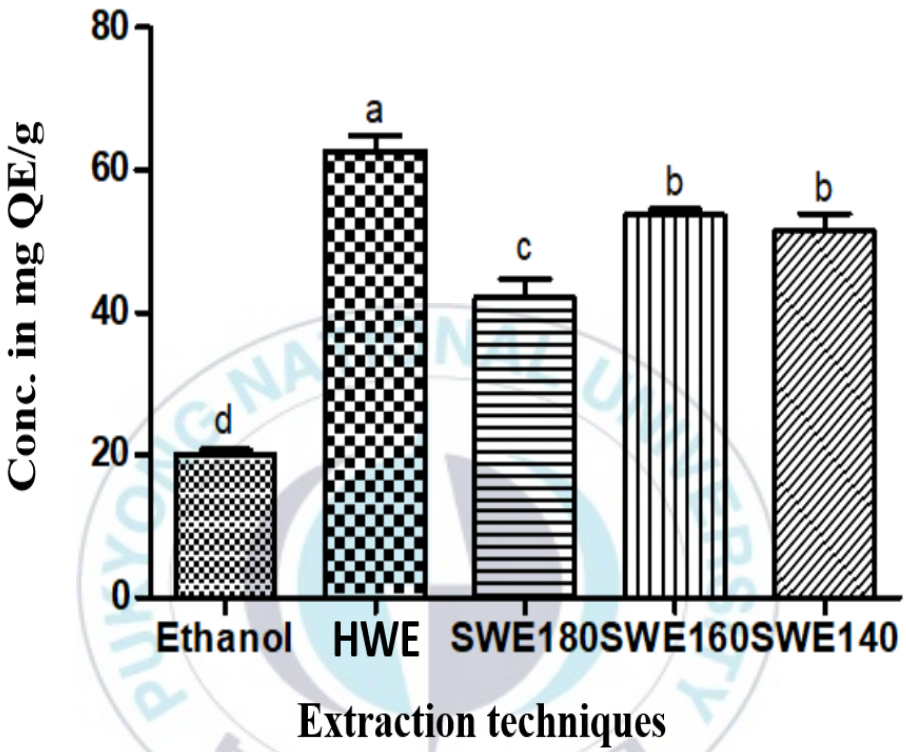

Figure 2. 2: The concentrations of total flavonoid contents (TFC) of *A. africana* from different extraction techniques in mg QE/g (mg Quercetin equivalent per gram of *A. africana* dry powder sample). Values are presented as ± SD (n=3). The different letters on the histogram imply statistical differences ($p \leq 0.05$) between different extraction techniques

These results were consistent with previously reported findings, during the extraction of green tea leaves metabolites, hot water extraction (heating and agitation) increased the polyphenols contents by three-fold (Das & Eun, 2018), and also the best extraction temperature for total phenolics from *Teucrium montanum L.* using subcritical water was found to be 160°C (Nastić et al., 2018). The increase in TPC and TFC during subcritical water extraction at 160°C, and during hot water extraction could be caused by the improved solubility, mass transfer, and reduced water dielectric constant (Armenta, Esteve-Turrillas, Garrigues, & de la Guardia, 2017). Additionally, another understandable factor contributing to the increase in phenolic compounds is the release of phenolics bound to the cell wall of the plant. The extraction of phenolics bound to the plant cell wall and cell wall intramolecular compounds such as hemicellulose, structural protein, and pectin requires the conditions capable of triggering the release of these compounds by cleaving the plant's cell wall structure and hydrolyzing molecules on which they are bound (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). It is seen that HWE was found to be the best condition for extracting both TPC and TFC, followed by subcritical water at 160°C. The decreased TPC and TFC at 180°C could result from the degradation of heat sensitive phenolic compounds at an elevated temperature.

2.4.2 Effect of extraction techniques of *A. africana* on phenolic compounds

The chromatographic analysis of phenolic and flavonoid compounds is shown in Table 2.1. Gallic acid (GA) was the most predominant phenolic acid observed in all extracts, and it was followed by chlorogenic acid (CHL.A) and trace amounts of syringic acid (S.A), ferulic acid (F.A) and quercetin. The concentration of GA in HWE acquired extracts were almost two times higher than that of SWE140, and SWE160, and more than three and five times higher than the concentrations of GA in SWE180 and ethanol obtained extracts, respectively (Table 2.1). There was no CHL. A detected at SWE160, and quercetin was not detected with any subcritical water extracts while S.A was detected in SWE140. The high concentrations of GA

in HWE as compared to SWE would also be interpreted by the high concentration of solutes in HWE, on the other hand, both HWE and SWE performed better than the organic solvent, confirming that water was a better solvent in both conditions as compared with the organic solvent (Ethanol).

Table 2.1. Concentrations of individual phenolic compounds in (mg/g DW) obtained by HPLC analysis of *Aspilia africana* from different extraction techniques. Where; GA: Gallic acid, CHL.A: Chlorogenic acid, SA: Syringic acid, FA: Ferulic acid, QUER; Quercetin

ND: Not Detected (The different superscripts letters are statistical differences ($p \leq 0.05$) between different extraction techniques)

Conditions	GA	CHL.A	SA	FA	QUER
Ethanol	10.3 ± 0.64^{d}	4.2 ± 0.2^{b}	0.2 ± 0.06^{b}	1.6 ± 0.2^{b}	0.7 ± 0.05^{b}
HWE	51.6 ± 1.9^{a}	10.7 ± 1.1^{a}	1.6 ± 0.3^{a}	2.5 ± 0.3^{a}	1.8 ± 0.07^{a}
SWE180	15.9 ± 0.9^{c}	1.4 ± 0.6^{c}	ND	0.6 ± 0.1^{d}	ND
SWE160	26.7 ± 1.4^{b}	ND	ND	0.8 ± 0.02^{cd}	ND
SWE140	28.08 ± 0.8^{b}	1.5 ± 0.01^{c}	1.7 ± 0.1^{a}	1.04 ± 0.02^{c}	ND

2.4.3 Effect of extraction techniques of *A. africana* on the antioxidant activities

DPPH assay was used to study the antioxidant activities of different extracts from *A. africana*. The results were expressed as the minimum concentration of extracts required for 50% inhibition (IC_{50} value) of DPPH radicals, and the results are displayed in Fig 2.2. The highest IC_{50} value was found from ethanol extracts (3±0.03 mg/ml). This value was almost nine times larger than the lowest IC_{50} obtained from HWE extracts (0.42 ±0.004 mg/ml). There was no significant difference in the IC50 values obtained at different subcritical water conditions, however, it was notable that the IC_{50} from all the three subcritical water conditions were two times higher than the lowest IC_{50} value gotten from HWE. We can conclude that HWE was the best extraction technique for recovering the bioactive compounds with the highest antioxidant activities, followed by the subcritical water extraction technique.

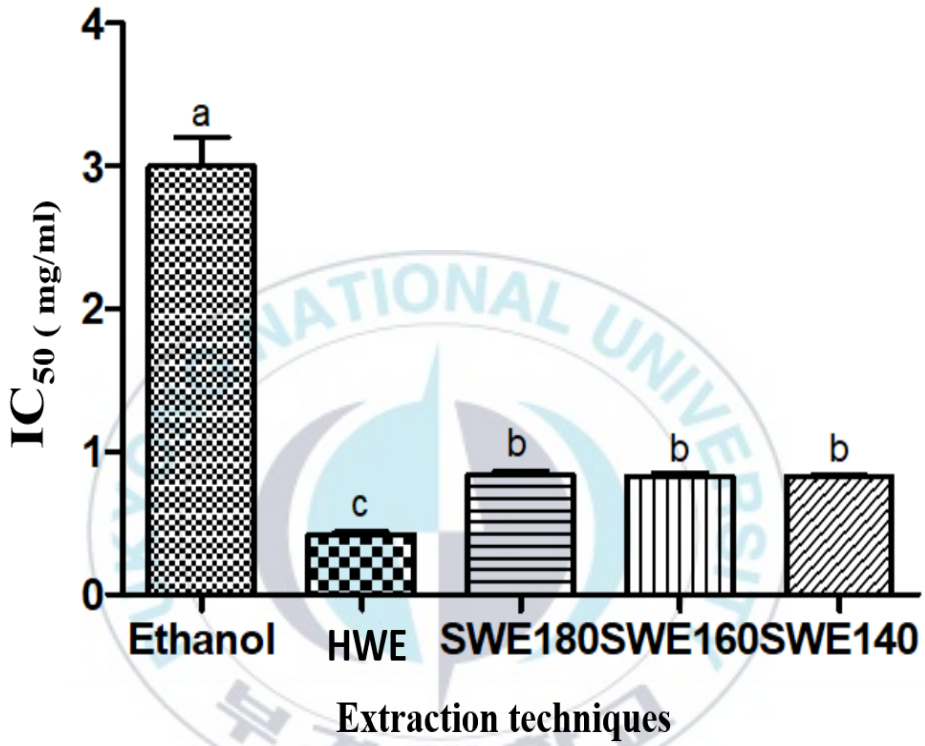

Figure 2. 3: Antioxidant activities of *A. africana* from different extraction techniques expressed as IC_{50} values. The different letters on the histogram imply statistical differences ($p \leq 0.05$) between different extraction techniques

These results could be interpreted by the high concentration of TPC and TFC in HWE, also Gallic acid present in HWE at the highest concentration as compared to other extraction techniques applied is a well-known antioxidant phenolic acid. These results were consistent with previous findings, during the extraction of bioactive compounds from green tea, agitation showed the best recovery of phenolic antioxidants(Das & Eun, 2018), also subcritical water showed the highest efficacy in the recovery of phenolics from potato peels, and antioxidants from winery wastes (Singh & Saldaña, 2011) ; (Aliakbarian, Fathi, Perego, & Dehghani, 2012).

2.4.4 Effect of extractions techniques of *A. africana* on Cell proliferation

HaCaT, HEK-293, HeLa, AGS and A549 cells were treated separately with various concentrations (0, 50, 100, 200, 300, 400, 500 µg/ml) of AAE in order to evaluate the cytotoxicity of *A. africana*. Results obtained by MTT assay revealed that all extract concentrations investigated did not show a significant effect between different concentrations and control on normal HaCaT cell line ($p<0.05$). The viability was above 90 % even on the highest concentration (500 µg/ml) as demonstrated on the mean plots of cell viability (Fig. 2.3). On the other hand, a slight difference was shown on the normal HEK-293 cell line at the highest concentrations (Fig. 2.4). The viability was below 90% at 500 µg/ml for Ethanol, HWE, and SWE140 with 89.9, 88.8, and 86.5 % respectively. Overall, we can infer that there was no significant effect of *A. africana* extracts on normal cells tested for positive control; however, caution should be taken in the choice of concentrations; Concentrations from 200 µg/ml and below maintained the viability of normal cells above 90%.

Among symptoms of cervical cancer, bleeding predominates during menopause, between periods, and after intercourse. Since *A. africana* is used to stop bleeding (Okoli, Akah, & Okoli, 2007), we investigated the effect of AAE on the proliferation of cervical cancer cells (HeLa), (Fig.2.5). All extraction techniques did not show a

significant effect against HeLa cells proliferation. The lowest viability was 76.4% at 500 µg/ml from HWE treatment. Ethanol extract showed a viability above 90% even for the highest concentration of 500 µg/ml. The viability was 79.3%, 86.9%, and 86.9% at 500 µg/ml for SWE180, SWE160, and SWE140 respectively. SWE160 and SWE140 showed no significant difference on HeLa cells.

Different extracts of *A. africana* leaves are used traditionally as cough remedies in different African countries (Okoli, Akah, Nwafor, et al., 2007), so we investigated the potential effect of AAE on the proliferation of lung cancer cell lines (A549). The effect of AAE on lung cancer cell lines (A549) studied based on the 5 extraction techniques showed that lower concentrations (200 and 100 µg/ml) of HWE exhibited an effect comparable to the highest concentrations (500 and 400 µg/ml) for ethanol, SWE180 and SWE 140 (Fig. 2.6). Both HWE and SWE160 showed a remarkable anti-proliferative effect on A549 at 200 µg/ml with 73.7 and 77.6 % of cell viability. The concentration as low as 100 µg/ml of HWE also exhibited a remarkable effect with the viability of 75.5 %. Although, all extracts exhibited a significant effect on A549 cells, the requirement of high concentrations (above 200 µg/ml) from ethanol, SWE180, and SWE140 favor HWE and SWE160 because they demonstrated the similar effect on lower concentrations.

A. africana is also used traditionally for the treatment of digestive disorders. Gastric adenocarcinoma AGS cell lines were the most sensitive to AAE for all the extraction techniques applied. The lowest cell viability count was recorded from HA at 500 µg/ml with 31.9% (Fig.2.7) All concentrations were significantly different ($p<0.05$) from each other for HWE and the concentration of 200 µg/ml showed a viability count below 60 %. In comparison with SWE, this concentration had the highest effect even when compared with 500 µg/ml. It is also noticed that the concentrations as low as 100 µg/ml from HWE had a higher effect than the maximum concentration for SWE180, while 50 µg/ml showed higher effect than 400 µg/ml from ethanol, and comparable with 200 µg/ml from SWE180. Although the best

extraction method was HWE, we found out that both SWE160, 140 extracts could also have the potential to inhibit the proliferation of AGS cells, while ethanol performed poorly.

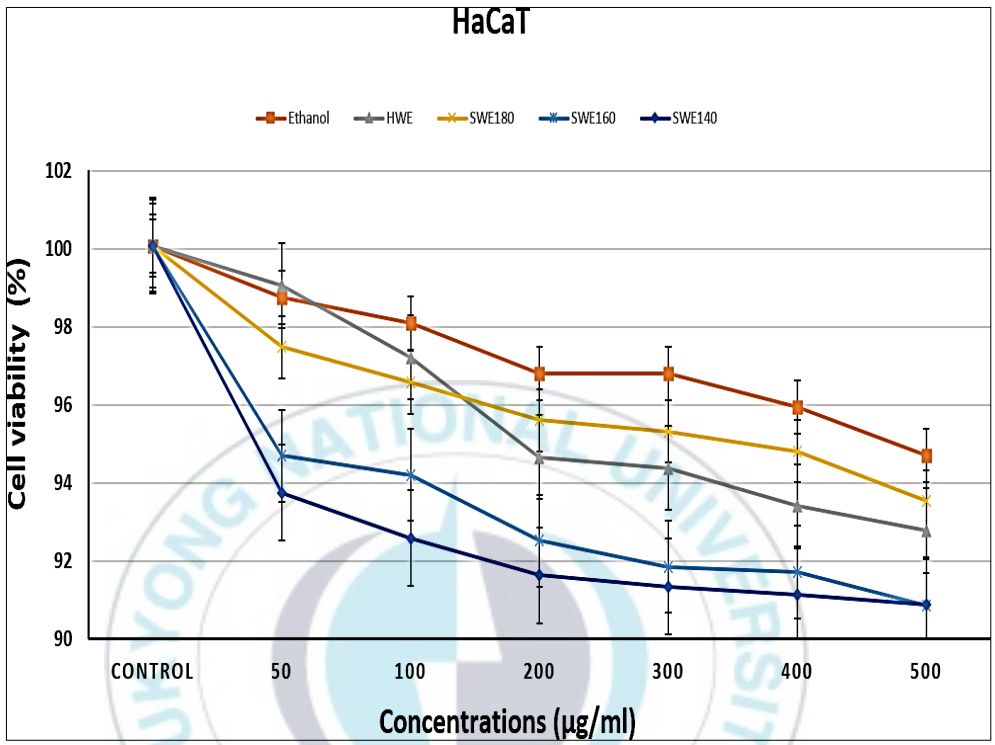

Figure 2. 4: Anti-proliferative effects of *A. africana* extracts (AAE) obtained from different extraction techniques on HaCaT cell line. Cells were treated separately with 0 – 500 µg/mL of each extracts for 24 h and the cell viability was calculated based on the percentage of cell count by MTT assay using 96 well microplate reader

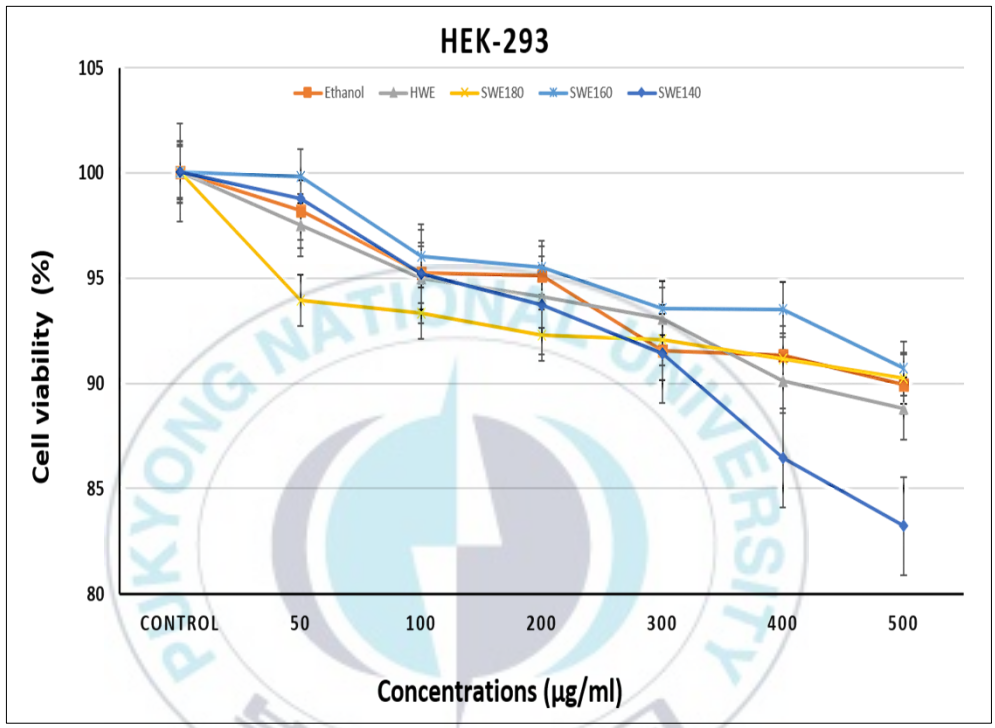

Figure 2. 5: Anti-proliferative effects of *A. africana* extracts (AAE) obtained from different extraction techniques on HEK-293 cell line. Cells were treated separately with 0 – 500 µg/mL of each extracts for 24 h and the cell viability was calculated based on the percentage of cell count by MTT assay using 96 well microplate reader

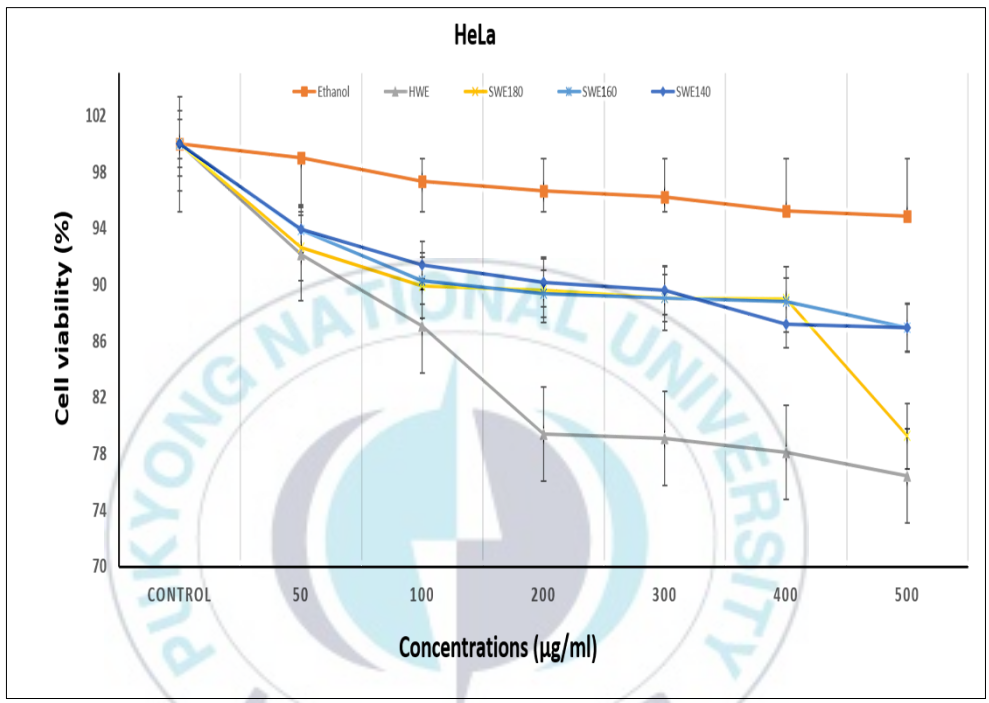

Figure 2. 6: Anti-proliferative effects of *A. africana* extracts (AAE) obtained from different extraction techniques on HeLa cell line. Cells were treated separately with 0 – 500 µg/mL of each extracts for 24 h and the cell viability was calculated based on the percentage of cell count by MTT assay using 96 well microplate reader

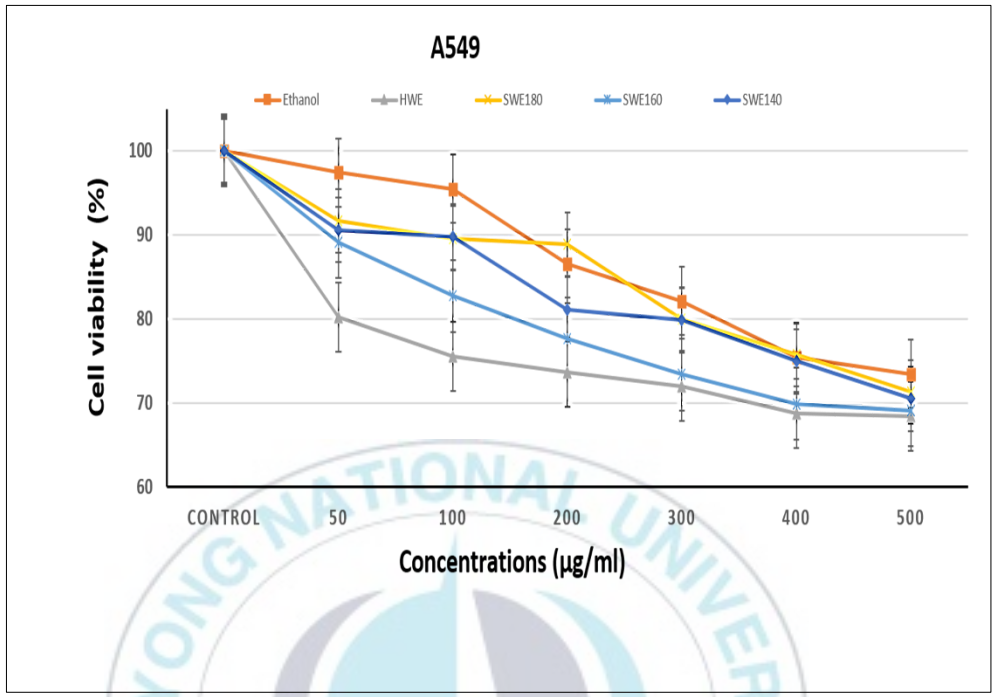

Figure 2. 7: Anti-proliferative effects of *A. africana* extracts (AAE) obtained from different extraction techniques on A549 cell line. Cells were treated separately with 0 – 500 µg/mL of each extracts for 24 h and the cell viability was calculated based on the percentage of cell count by MTT assay using 96 well microplate reader

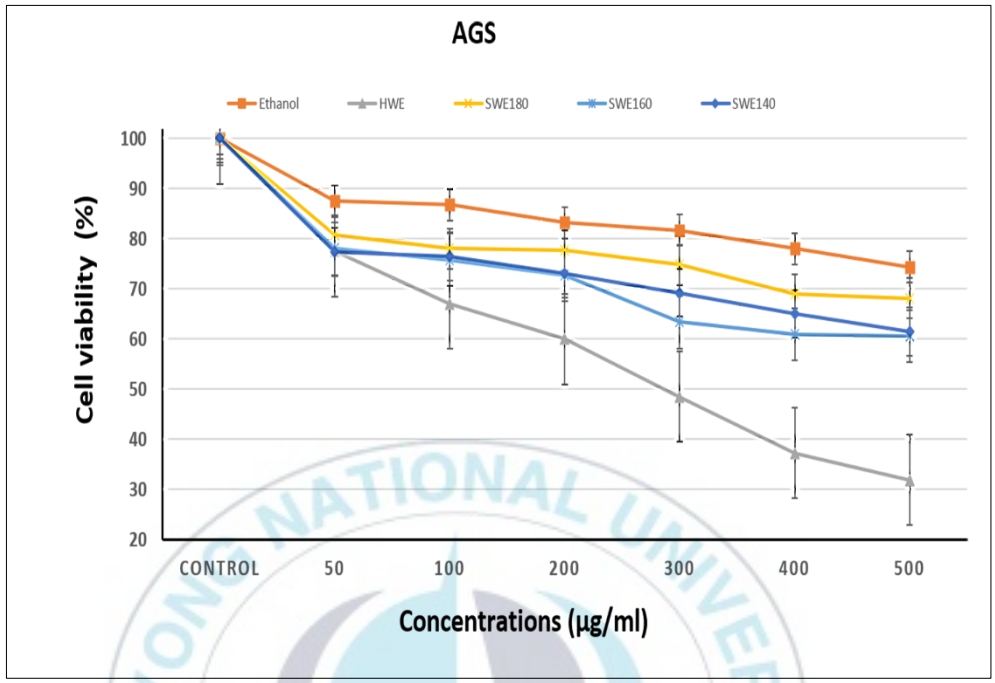

Figure 2. 8: Anti-proliferative effects of *A. africana* extracts (AAE) obtained from different extraction techniques on AGS cell line. Cells were treated separately with 0 – 500 µg/mL of each extracts for 24 h and the cell viability was calculated based on the percentage of cell count by MTT assay using 96 well microplate reader

2.4.5 Effect of extractions techniques of *A. africana* on the induction of apoptosis

In order to understand whether the anti-proliferative effect of AAE was apoptosis-related, we stained A549, HeLa and AGS cancer cells treated with 200 μg/ml of each extract with DAPI solution to analyze the nuclear morphology. Treatment of these cancer cells with the indicated dose of AAE induced morphology change followed by cells shrinkage (Fig. 2.8). The change of morphology in treated cancer cells was observed using an inverted microscope (Olympus CKX41) with a magnification of x100. DAPI results also showed that AAE induced nuclear morphology change in treated cancer cells followed by the formation of apoptotic bodies which are known as a hallmark of apoptosis for dying cells (Fig. 2.9).

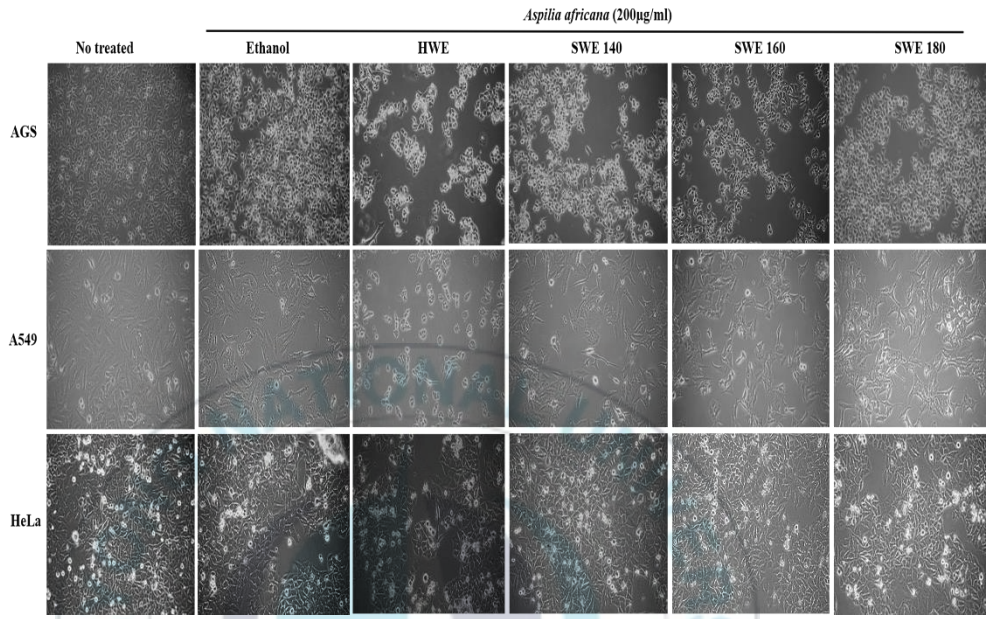

Figure 2. 9: Effect of *A. africana* extracts from different extraction techniques on the morphology of treated cancer cells. AGS, A549, and HeLa cells were treated separately with 200 µg/mL of each extract for 24 h. The morphology of treated cells was observed after 24 h of treatment under an inverted microscope (magnification, X100)

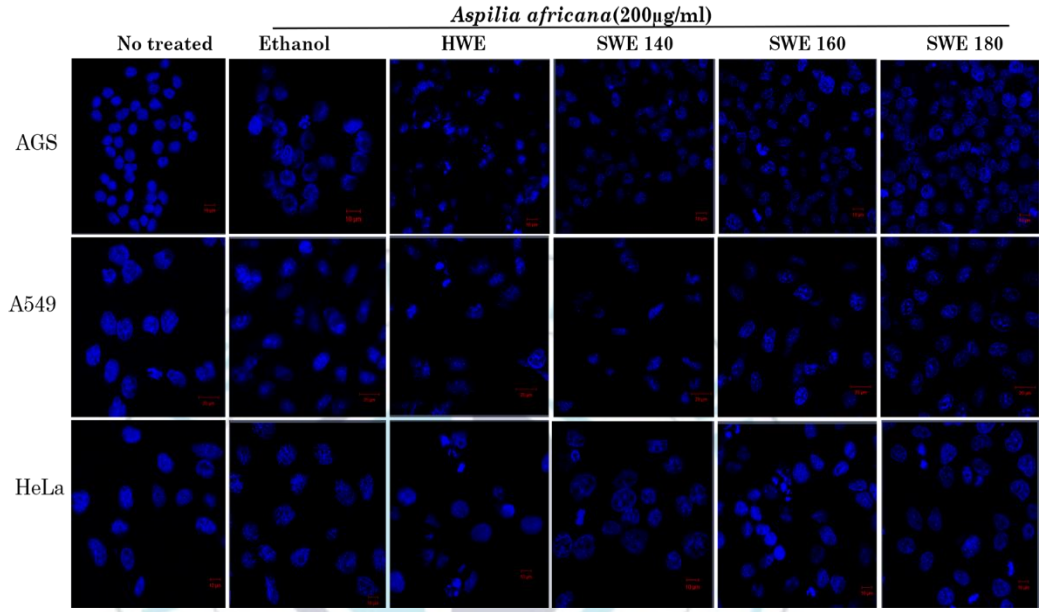

Figure 2. 10: Effect of *A. africana* extracts from different extraction techniques on the nuclear morphology of treated cancer cells. AGS, A549, and HeLa cells were treated separately with 200 μg/mL of each extract for 24 h and then stained with DAPI solution (1 μg/mL). The nuclear contents of treated cells were observed under a fluorescent confocal microscope

The cause of cancer in humans has been correlated with the DNA damages from cellular oxidative damages due to the increasing number of free radicals also known as reactive oxygen compounds, (Jackson & Bartek, 2009). Damaged cells are removed from the organism by the natural mechanism of programmed cell death known as apoptosis (Jorgensen, Rayamajhi, & Miao, 2017). During apoptosis, a serial number of signals are responsible for distinct morphological alterations resulting in cell death. The morphological changes include membrane blebbing due to the degradation of cytoskeleton components, destruction of the nuclear lamina which results in nuclear envelope destruction, DNA fragmentation and finally chromatin condensation followed by the formation of apoptotic bodies (Saraste & Pulkki, 2000); (Ngabire et al., 2018). However, cancer cells escape the anti-proliferative signaling by evading growth suppressors, enabling replicative immortality, therefore resisting cell death, which results in angiogenesis, invasion, and cancer metastasis (Hanahan & Weinberg, 2011). Hence, there is an enormous necessity in the search of novel natural drugs that have the ability to control the proliferation of cancer cells. Overall, Gallic acid, different phenolic acids and flavonoids have been previously endorsed to arrest the proliferation of numerous cancer cell lines by multiple mechanisms, including induction of apoptosis (Subramanian et al., 2015), (Gutiérrez-Uribe, Romo-Lopez, & Serna-Saldívar, 2011), (Lutterodt, Slavin, Whent, Turner, & Yu, 2011), and in our study, HWE was shown to cause more formation of apoptotic bodies in AGS and A549, respectively (Fig. 2.9). It is followed by SWE 160, and these results were consistent with MTT results which could be explained by the high content of TPC, TFC, and GA extractable in these conditions.

2.5 Conclusion

In conclusion, the findings of this work suggested that hot water and subcritical water would be the best extraction techniques for the recovery of bioactive compounds, such as phenolic antioxidants from *A. africana*, as compared with the conventional organic solvent (ethanol). It demonstrated by means of HPLC that the predominant active phenolic compound was gallic acid, which may be responsible for the biological activity of *A. africana*, and the hot water extraction technique showed the highest recovery of this active compound. Amongst the extraction techniques studied, Hot water extraction, and subcritical water at 160°C showed the best anti-proliferative activity against AGS (Gastric adenocarcinoma cells) and A459 (lung adenocarcinoma cells). AGS was the most sensitive to the extracts of *A. africana*, especially from hot water extracts. These findings suggest that *A. africana* possess anticancer activity by inducing apoptosis with high effect on AGS gastric adenocarcinoma, as demonstrated by DAPI staining results. The use of green technologies to enhance the recovery of bioactive compounds from *A. africana* was applied for the first time, and we showed that this was the best approach compared to conventional methods that use organic solvents. Further studies should be conducted to further understand the molecular pathways involved in the anticancer activities of *A. africana* extracts.

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CHAPTER THREE

Anticancer effects of *Aspilia africana C.D. Adams* in gastric adenocarcinoma AGS cells.

CHAPTER 3

Anticancer activity of phytochemicals from *Aspilia africana C.D. Adams* against gastric adenocarcinoma AGS cells.

3.1 Abstract

Aspilia africana C.D. Adams (*A. africana*) is used traditionally for the treatment of several ailments such as cough, wound healing, and digestive disorders. In this study, we examined the potential anticancer effect of *A. Africana* aqueous extracts (AAE) obtained using green extraction techniques in human adenocarcinoma AGS cells. The cytotoxicity of AAE was evaluated using normal cell lines (HaCaT: Keratinocytes and HEK-293: embryonic kidney) and AGS cells by MTT assay. Western blot, DAPI staining, and immunofluorescence staining were also performed to analyze molecular pathways behind AAE activities. Concentrations between 100-200 µg/ml of AAE arrested the proliferation of AGS cells without affecting normal cells investigated. The results showed that AAE treatment induced morphological change in AGS cells followed by nuclear condensation which led to the formation of apoptotic bodies and accumulation of AGS cells in the G1 phase. Western blot of apoptotic-related proteins showed that AAE increased the expression levels of pro-apoptotic proteins such as tBid, Bad, Bak, Bax, Cytochrome c, FLIP, cleaved form of caspases 3,8 &9 and cleaved Parp while anti-apoptotic proteins such as Bcl-2, Bcl-xL were decreased. Cyclins such as cyclin E1&D1 and CDK2,4&6 that play a major role in G1/S phase evolution were down-regulated where the expression of CDK inhibitors (p21, p27, p18, and p15) was up-regulated by AAE treatment. In conclusion, our results demonstrated that *A. Africana* induces apoptosis in AGS cells via caspase-dependent mitochondrial-mediated pathways and cell cycle arrest.

3.2 Introduction

Gastric cancer, being one of the most common malignant tumors in humans, occupies the third position in the structure of mortality in men and women worldwide (Bray, Ferlay et al. 2018). More than 1 million gastric cancer cases are diagnosed every year where the incidence of developing gastric cancer is 2.3 times more in men than in women at an age between 0-74 worldwide (Rawla and Barsouk 2019). Depending on the stage of the disease, surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy are suggested treatments for stomach cancer; however, despite all the existing treatment options, recurrence of cancer is observed after initial treatment (Smyth, Verheij et al. 2016). Available chemical anticancer drugs present lethal side effects in patients and due to this matter, researchers started to focus on finding new drugs that are less toxic from natural products including traditional medicinal plants (Ngabire, Seong et al. 2018).

The use of traditional medicines and the practice of Phytotherapy are now receiving scientific attention with the aim to develop effective, non-toxic, and inexpensive new drugs that are needed especially in developing countries (Atanasov, Waltenberger et al. 2015);(David, Wolfender et al. 2015). Most of the population in developing countries rely on traditional medicines to relieve their pain due to the high cost of western drugs (Taylor, Rabe et al. 2001). Natural products especially medicinal plants are known for containing beneficial bioactive compounds such as phenolic and flavonoids responsible for their biological activities (Ghasemzadeh and Ghasemzadeh 2011). These active compounds are known as secondary metabolites present in plants as a result of abiotic and biotic stress features. Over the past two decades, numerous studies have been conducted in order to find new drugs with potential anticancer effects from natural and edible resources such as vegetables, fruits, and traditional medicinal plants (Jo, Kim et al. 2012).

The family of Asteraceae plants is widely used for various useful purposes due to their availability in most parts of the world (Kenny, Smyth et al. 2014). *A. africana*

belongs to this family and is native to Africa where it grows in many African countries all the way through the tropical regions (Komakech, Matsabisa et al. 2019). *A. africana* is an important plant in the African folk medicine where it is mostly used as a wound healing remedy (Agyare, Boakye et al. 2016) and many of other ailments such as headache, cough, rheumatic pains, gonorrhea, and digestive disorders (Komakech, Matsabisa et al. 2019). *A. africana* has been reported to possess numerous biological activities such as anti-inflammatory, antimicrobial, and antimalarial activities (Okoli, Akah et al. 2007),(Christian, Mfon et al. 2012).

To the extent of our knowledge, no studies have been yet reported on the possible potential anticancer effect of *A. africana*. In our previous study, we reported the potential anticancer activities of AAE against different cancer cell lines where the aqueous extract obtained by hot water extraction (heating and agitation) technique showed a remarkable antiproliferative effect on AGS cells (Niyonizigiye, Nkurunziza et al. 2020). In the present study, we investigated possible molecular pathways involved in the anticancer activities of aqueous extract of *A. africana* against gastric adenocarcinoma AGS cells, supporting a favorable anticancer agent for gastric cancer.

3.3 Materials and Methods

3.3.1 Sample preparation

Aspilia africana leaves (traditional name: Icumwa/Icyumwa co ku musozi) were collected in Bujumbura city in the garden of a traditional healer located in Gihosha rural and were then identified at the Herbarium of the National Institute of the Environment and Nature Conservation (INECN) PN Ruvubu where a specimen from NDIKUBWAYO Ernest's collection (n°6082) is deposited. Further procedures used for the treatment of *A. africana* leaves including the extraction techniques applied are described in our previous report (Niyonizigiye, Nkurunziza et al. 2020). Aqueous extract obtained from the hot water extraction technique was used for this study. The stock solution used was obtained by diluting 100 mg of *A. africana* powder in 1mL of distilled water. A working solution of 10 mg/mL was made from the stock solution (100 mg/mL) before cell treatment.

3.3.2 Reagents and cell culture

AGS and HEK-293 cells were bought from ATCC (Manassas, VA, USA). HaCaT cells were from Addexbio (SanDiego, CA, USA). Culture media: DMEM, MEM, and RPMI-1640 were obtained from Hyclone (Logan, UT, USA). FBS ((10%); Hyclone) and 1% of antibiotic mixture ((10 µg/ml of streptomycin + 100 U/ml of penicillin); PAA Laboratories, Austria) were added to each media. Z-VADfmk caspase inhibitor was gotten from Sigma-Aldrich (MO, USA). WST-1 solution (Daeil lab service, Seoul, South Korea) DAPI staining solution was from ThermoFisher Scientific (MA, USA). Antibodies: cleaved caspases 9,8 &3 , cleaved parp, FLIP, cytochrome c, Bcl-2, Bcl-CL, Bad, Bak, tBid, cyclinD1,cyclinE1, phospho-p53,pRb,E2F-1, Cdc25A, p21,p27,p18,p15,pChk2,CDK2,CDK4,CDK6, GAPDH,β-actin were purchased from cell signaling technology(Danvers, USA). Cells were maintained in their specific media, DMEM for HaCaT, MEM for HEK-

293, and RPMI-1640 for AGS, and kept in an incubator at 37°C and 5% CO_2 and sub-cultured until they were ready for use.

3.3.3 Cell viability assay and zVAD-fmk, caspase inhibitor treatment

For assessing the cytotoxicity of AAE, HaCaT, HEK-293, and AGS cells (10^4 cells/well) were seeded in 96 well-plate for 24 h and then treated with different concentrations of AAE (0-200 µg/ml) and incubated again for 24 h. Then WST-1 (10 µl) with fresh media were added to all wells and again incubated protected from light for more 3 h at 37°C. The cell viability was then measured by evaluation of the absorbance on a 96-microplate reader (Molecular Devices, CA, USA) at 460 nm. In order to investigate whether the AAE antiproliferative activity in AGS was caspase-related, AGS cells (10^4 cells/well) were seeded in 96 well-plate in 4 groups for 24 h. First group was control, second group of cells was incubated 1h with zVADfmk (50 µm), caspase inhibitor without AAE, third group of cells was treated with 200 µg/ml of AAE (for 24 h) without zVADfmk, and the last group of cells was first incubated with 50µM of zVADfmk for 1h then the media was removed and the cells were therefore treated with 200 µg/ml of AAE for 24 h. After the treatment time (24 h), the media was changed and replaced with new fresh media containing WST-1 (10 µl) solution and cells were further incubated for 3 h at 37°C. The cell viability was then determined by calculating the absorbance at 460 nm using 96 well microplate reader.

3.3.4 DAPI staining

DAPI staining was used to analyze the effect of AAE treatment on AGS cells' nuclear morphology. AGS cells were seeded on cover glass dishes and incubated for 24 h at 37°C and 5% CO_2. Seeded cells were then treated separately with 100, 150, and 200 µg/ml of AAE for 24 h. After treatment time, cells were washed with PBS and stained in DAPI solution (1 µg/ml) for 20 min in dark. After 20 min, DAPI was removed and cells were washed again with 1x PBS before being mounted on

microscopic slides using prolong gold antifade reagent. Cells were then observed with a ZEISS LMS 710confocal microscope (Carl Zeiss, Germany).

3.3.5 Immunofluorescence staining

Immunofluorescence staining was performed to analyze the presence of cleaved caspase 3 which is known as an executioner of apoptosis, in AGS cells after treatment with AAE. AGS cells were seeded on cover glass dishes for 24 h and then treated with 200 µg/ml of AAE for 24 h. After that, cells were stained with DAPI (1 µg/ml) for 20 min in dark then fixed for 15 min in 4% formaldehyde. Fixed cells were then blocked in a blocking solution containing 5% of mouse and rabbit sera (Santa Cruz, CA, USA) and 0.3% of Triton X-100 for 1 h at room temperature. Cells were then washed with PBS and incubated with anti-cleaved caspase-3, a primary antibody overnight at 4°C. The next day, cells were first washed in PBS 3 times, and again incubated with secondary antibody IG anti-mouse conjugated with red Alexa Fluor 555 (Cell Signaling Technology) for 1h at room temperature followed by a 3-time wash of cells in PBS and then mounted on microscopic slides using prolong gold antifade reagent and then visualized with LMS 710 confocal microscope.

3.3.6 Western blot analysis

For western blot analysis, AGS cells were cultivated in culture dishes (100 mm) for 24 h and were then treated with 100, 150, and 200 µg/ml concentrations of AAE for 24 h. Then proteins were extracted from treated cells using cell lysis buffer at 4°C; Cells were quantified by Bradford protein assay before being separated by electrophoresis in 12% SDS-PAGE. After electrophoresis, blots were transferred on nitrocellulose membrane and then blocked in 5% skim milk diluted in 1xPBST (2.7 mM potassium chloride, 135 mM sodium chloride, 1.4 mM potassium dihydrogen phosphate, 4.3 mM sodium phosphate, and 0.5% Tween-20) for 1 h at room temperature. After blocking, blots were washed 3 times in 1x PBST and incubated with primary antibodies: anti-cleaved caspases3,8&9, anti-cleaved PARP, anti-FLIP,

anti-Bcl-2, anti- Bcl-xL, anti-cytochrome c, anti-Bax , anti- Bad, anti-Bak, anti-tBid, anti-β-actin, anti- phospho-p53, anti-pRb, anti-cyclin E1&D1, anti-E2F-1, anti-cdc25A, anti-p21, anti- p27, anti-p18, anti- p15, anti- pchk2, anti- cdk2,4&6, and anti-GAPDH at 4°C overnight. After overnight incubation, blots were washed 3 times in 1xPBST and incubated with HRP conjugated secondary antibodies (anti-rabbit, anti-mouse) for 1 h at room temperature. After being washed 3 times in 1x PBST, blots were detected in ECL solution under iBright CL1000 (Thermo Fisher, MA, USA).

3.3.7 Flow cytometry analysis

Flow cytometry analysis was conducted in order to analyze the effect of AAE treatment of the AGS cell cycle. For that, AGS cells were seeded in 100 mm culture dishes for 24 h. Cells were then treated with 100, 150, and 200 µg/ml concentrations of AAE for 24 h and were then collected by trypsinization and were after fixed at 4°C overnight in 70% ethanol. After that, Cells were resuspended with PBS solution having 0.2 $\mu g ml^{-1}$ RNase and incubated for 30 min at 37°C. After incubation cells were then stained at room temperature in 40 µg/ml PI for 30 min protected from light. Then, the cell cycle distribution was analyzed via flow cytometer BD FACs (Becton Dickinson, CA, USA).

3.3.8 Statistical analysis

All the results presented are the mean value (±) of standard deviation from three separate experiments. GraphPad Prism 7 (San Diego, CA, USA) was used for statistical analysis using Bonferroni post-hoc analysis where the mean value of the control group was compared with the mean value of treated groups. Statistical differences were set at ** $p < 0.01$; *** $p < 0.001$

3.4 Results

3.4.1 AAE effect on AGS cells proliferation

For assessing the cytotoxicity of AAE, we treated AGS cells, HaCaT cells, and HEK-293 cells with different concentrations of AAE. Results obtained showed that AAE treatment for indicated concentrations repressed the proliferation of AGS cells without affecting the proliferation of investigated normal cells (HaCaT, and HEK-293 cells) demonstrating that concentrations of AAE up to 200 µg/ml are safe for normal cells (Fig.3.1a). The more the concentration of AAE increased the less viability of AGS cells was observed. From this, we concluded that the effect of AAE on AGS cells was dose-dependent (Fig.3.1a). Treatment of AAE also affected the morphology of AGS cells where the cell shrinkage was observed in treated groups compared to the non-treated group (Fig.3.1c). The morphology of cells was observed via an inverted Olympus microscope (CKX41; magnification, x 100).

3.4.2 AAE treatment induces apoptosis in AGS cells

We further analyzed whether the effect of AAE on AGS cells was caspase-dependent by incubating AGS cells 1 h with 50 µM of zVADfmk, a caspase inhibitor prior to the treatment with AAE (200µg/ml). Results obtained by WST-1 assay showed that incubation with caspase inhibitor increased the proliferation of AGS cells (Fig.3.1b) revealing that cell death induced effect of AAE in AGS cells is related to the caspase pathway. We also analyzed the nuclear morphology of AAE treated AGS cells by staining the treated cells with DAPI (1 µg/ml). Results demonstrated that the condensation of the nucleus followed by the formation of apoptotic bodies, the hallmarks of apoptosis were observed in treated cells in a dose-dependent way (Fig.3. 1d).

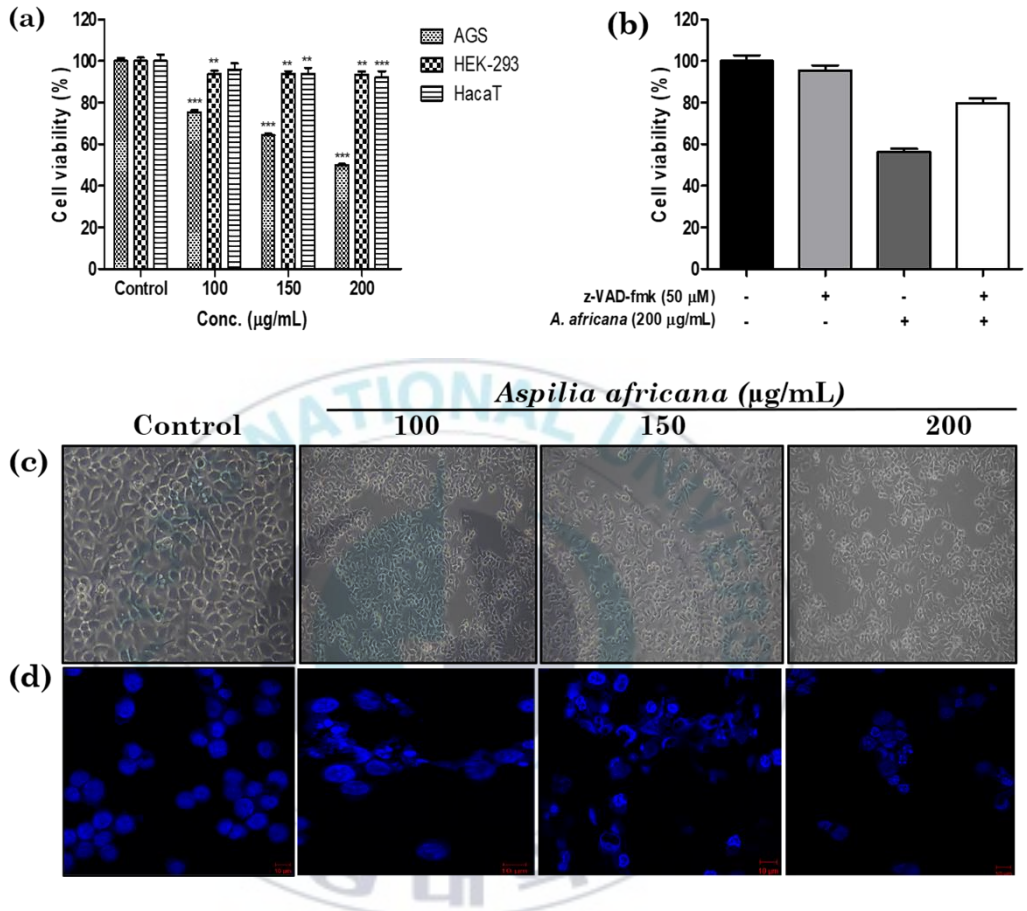

Figure 3. 1: Effect of *A. africana* extract (AAE) on cell lines proliferation; (a) Cell viability of AGS, HEK-293, and HaCaT cell lines treated with AAE for 24 h; (b) Cell viability of AGS cells incubated with z-VADfmk, caspase inhibitor for 1 h prior to the treatment with AAE; (c) Morphology of AGS cells after 24 h treatment with AAE. Images were observed under a CKX41 Olympus inverted microscope (magnification, x 100); (d) DAPI staining of AGS cells treated with indicated concentrations of AAE for 24h. Statistical differences between control and treated group was set at **P<0.01, ***P<0.001

3.4.3 Effect of AAE on the expression of apoptosis-related proteins in AGS cells

To confirm our findings, a western blot analysis of pro- and anti-apoptotic related proteins was performed to investigate whether their expressions were affected or no by AAE treatment in AGS cells. Both pathways (intrinsic, and extrinsic) were analyzed. The results showed that expression of pro-apoptotic proteins (Cleaved caspases3,8&9, cleaved PARP, Bad, Bak, Bax, cytochrome c, and tBid) increased in a dose-dependent way in AGS cells treated with AAE as compared to control group whereas the expression of anti-apoptotic proteins (FLIP, Bcl-xL, and Bcl-2) decreased (Fig.3.2a). An immunofluorescence staining of AAE treated AGS cells was also conducted to analyze the presence of cleaved-caspase 3 using a caspase3 antibody. Results showed that the cleaved form of caspase3 was greatly expressed in AAE treated AGS cells (Fig. 3.2b). These findings confirm that AAE induces apoptosis in AGS cells.

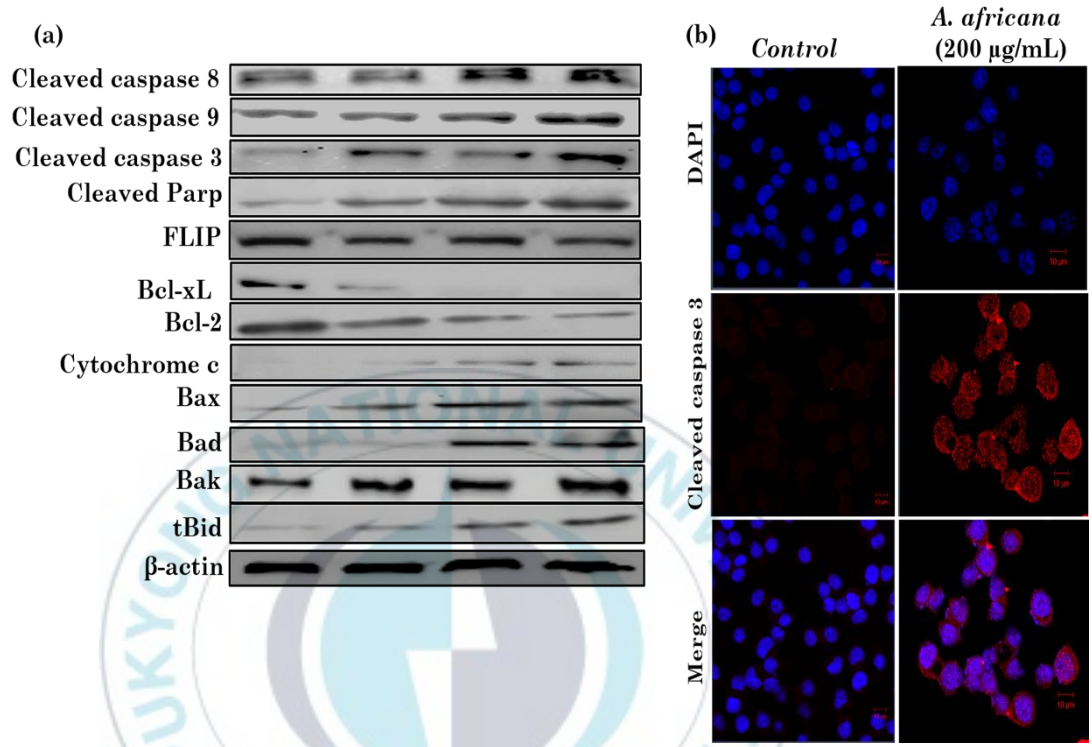

Figure 3. 2: Effect of *A. africana* extract (AAE) on the induction of Apoptosis in AGS cells. (a) Western blot analysis of apoptosis-related proteins in AGS cells treated with indicated concentrations of AAE for 24 h. (b) Immunofluorescence staining of cleaved caspase 3 in AGS cells treated with 200 μg/ml of AAE for 24 h. Immunofluorescent stained cells were analyzed under a confocal microscope

3.4.4 Effect of AAE on AGS cell cycle progress

The effect of AAE treatment on the AGS cell cycle was investigated by using a flow cytometer. AGS cells were first treated with indicated concentrations of AAE for 24 h and then stained with PI. Obtained results showed that treatment of AAE caused an accumulation of cells in G0/G1 phase with a percentage of 28.31% in no treated cells while the percentage in treated cells were 35.92, 64.41%, and 71.55% for 100, 150, and 200 µg/ml of AAE, respectively (Fig.3.3a). Further, a western blot analysis of CDKs, cyclins, and CDKs inhibitors implicated in G1 phase evolution was performed. Results demonstrated that Cyclins such as cyclin E1&D1, and CDK2,4&6 that play a major role in G1/S phase evolution were down-regulated where the expression of CDK inhibitors (p21, p27, p18, and p15) was up-regulated by AAE treatment (Fig.3.3b). Overall, from these findings, we can conclude that AAE induces cell cycle arrest in AGS cells by blocking the G1/S phase progression.

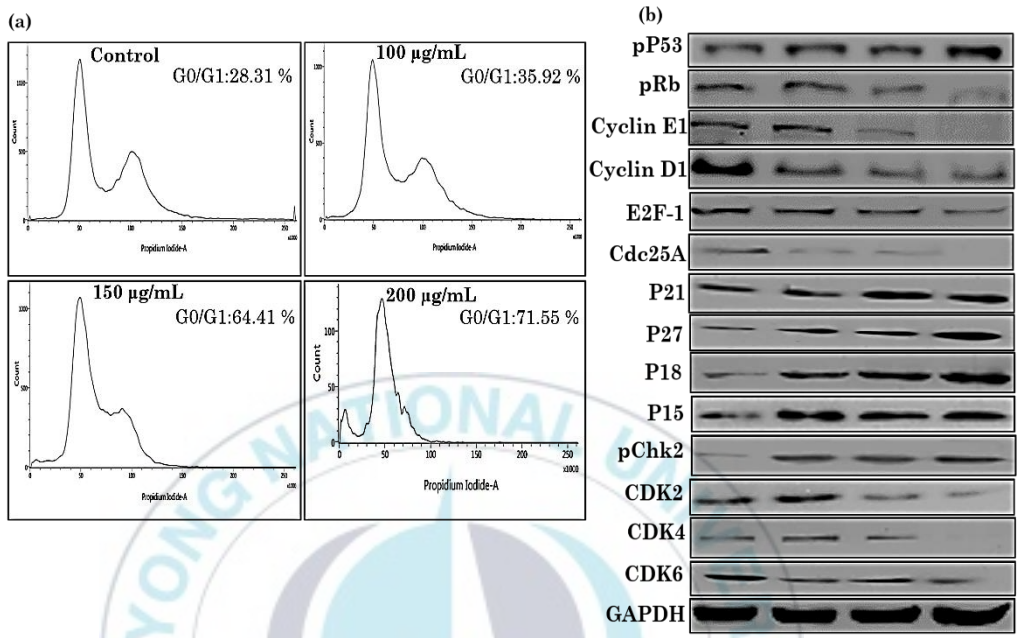

Figure 3. 3: Effect of *A. africana* extract (AAE) on AGS cell cycle progression. (a) Flow cytometry analysis of AGS cell cycle phase distribution after treatment with indicated concentrations of AAE for 24 h. (b) Westernblot analysis of G1 phase-related proteins

3.5 Discussion

Various studies reported that *A. africana* contains a large number of secondary bioactive metabolites such as flavonoids, tannins, terpenoids, saponins, glycosides, and alkaloids (Etiosa, Akeem et al. 2017); (Okoli, Akah et al. 2007); (Oko, Agiang et al. 2014). In our preview work on the characterization of bioactive compounds recovered from *A. africana* using green extraction techniques, we found that extract from the hot water extraction technique contained the highest amount of Gallic acid compared to other investigated extracts (Niyonizigiye, Nkurunziza et al. 2020). Gallic acid was endorsed to possess anticancer effects against numerous cancer cell lines (Subramanian, John et al. 2015). In this study, we demonstrated that the AAE treatment inhibited the proliferation of AGS cells (Fig.3.1a) which may be due to the high content of Gallic acid in AAE.

One of the fundamental features of cancer cells is their ability to sustain prolonged proliferation resulting in the resistance of cell death. In normal conditions, the normal tissue architecture and function are maintained as well as the homeostasis of the number of normal healthy cells by the simple fact that normal tissues are able to control the production, and release of growth-inducing signals that regulate their growth and division (Hanahan and Weinberg 2011). A number of pathological conditions can irreversibly damage and disrupt the function of cells resulting in unhealthy and damaged cells that are then eliminated from the body by apoptosis, a mechanism of programmed cell death which is required for tissue homeostasis and development (Niyonizigiye, Ngabire et al. 2019); however cancer cells have developed a way of deregulating these growth-inducing signals making them rulers of their destinies (Hanahan and Weinberg 2011). The objective of anticancer agents is then to restore the deregulated apoptosis system in cancer cells triggering cancer cells death (Khan, Saeedi et al. 2019). Here in our study, we examined the potential anticancer activity of AAE against gastric adenocarcinoma AGS cells. Obtained

results showed that AAE reduced the viability of AGS cells treated with AAE (Fig.3.1a).

Apoptosis is a multiphase process. The apoptosis process is characterized by certain morphological features - the nucleus and cytoplasm decrease in size, condense, fragment, the cell breaks up into several parts (apoptotic bodies) containing elements of the nucleus and intact organelles (Jin and El-Deiry 2005). The nucleus undergoes destruction through the formation of large fragments with their subsequent internucleosomal degradation. The plasma membrane of the cell undergoes a number of changes that make it recognizable for phagocytes, as a result of which apoptotic bodies are rapidly absorbed by macrophages, as well as often by surrounding cells that do not specialize in phagocytosis(Brunelle and Letai 2009) (Elmore 2007). In our study, we showed that AAE induced the morphological changes in AGS cells after treatment (Fig.3.1c) as well as nuclear condensation and formation of apoptotic bodies in AGS cells treated with AAE (Fig.1d).

We then conducted further experiments to explore mechanisms of AAE action in AGS cells on molecular levels. Our results demonstrated that AAE affected the expression of apoptosis-related proteins by downregulation of antiapoptotic proteins (FLIP, Bcl-xL, Bcl-2) (Fig.3.2a) and upregulation of proapoptotic proteins (cleaved forms of caspase3,8, &9; cleaved PARP, cytochrome c, Bak, Bax; Bad; and tBid) (Fig.3.2a). Further Immunofluorescence staining of cleaved caspase 3 known as an activator of apoptosis demonstrated that AAE treatment increased the expression of this protein in AGS treated cells as compared to no treated group (Fig.3.2b). Apoptosis can be activated via an extrinsic pathway through activation of caspase 8 which in return initiates the cleavage of effector caspases or the cleavage of Bid which becomes tBid or via intrinsic pathway where the Bcl-2 family proteins are crucial (Kantari and Walczak 2011) (Brunelle and Letai 2009). The BH-3 members of Bcl-2 proteins family are responsible for screening cellular processes and transduce death signals to Bcl-2 proteins family located at the outer mitochondrial

membrane in order to control the permeability of the mitochondrial outer membrane. The rise in mitochondrial outer membrane permeability causes the release of cytochrome c which migrates to the cytosol where it associates with Apaf-1 resulting in the cleavage of procaspase9 into cleaved caspase9 which activates caspase3 (Ow, Green et al. 2008); (Jiang and Wang 2004). In our study, we found that the treatment of AGS cells with AAE altered the expression of both caspase 8 and caspase 9 (Fig.3.2a) suggesting that AAE Induces Apoptosis in AGS cells by activating both intrinsic and extrinsic pathways.

In addition, we analyzed the effect of AAE treatment on the AGS cell cycle and found that AAE treatment caused AGS cell accumulation in G0/G1phase (Fig.3.3a) and altered the expression of numerous proteins responsible for G1/S phase transition (Fig.3.3b). The cell cycle arrest was related to the blockage of Cyclins-CDKs, Rb, and E2F signaling pathways. Rb is responsible for the G1/S phase transition in mammalian cells. On the other hand, CyclinD/CDK4&6 complex together with CyclinE/CDK2 complex work cooperatively to phosphorylate and inactivate Rb (Giacinti and Giordano 2006);(Dick and Rubin 2013). Phosphorylated Rb causes E2F protein activation which is a crucial for cell cycle progression into the G1/S phase (Sun, Bagella et al. 2007). P53, an important tumor suppressor also plays a crucial role in the G1/S phase transition where it causes G1 phase arrest by activating CDK-inhibitors in response to DNA damage (Reinhardt and Schumacher 2012). Another protein that is activated in response to DNA damage is Chk2. Activation of Chk2 inactivates Cdc25A resulting in the elevation level of inactive cdk2, therefore, stopping the progression of the cell cycle into the S phase(Yun, Hyun et al. 2012) (Agarwal, Tyagi et al. 2006). In this study, we showed that AAE treatment blocked the AGS cell cycle progression by downregulating Cdc25A, Cyclin E1, Cyclin D1, E2F-1, and CDK2,4&6 while the phosphorylated p53, pChk2, and CDK inhibitors such p15, p18, p21, and p27 were upregulated (Fig.3.3b).

In conclusion, we demonstrated here that AAE exhibited an antitumor effect in gastric adenocarcinoma AGS cells by inducing apoptosis and altering the G1/S cell cycle phase progression. Our findings suggest *Aspilia africana C.D. Adams* as a promising anticancer agent against gastric cancer. Further investigations are necessary to better understand the mechanisms of antitumor actions *in vivo* studies.

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CHAPTER FOUR

Anti-inflammatory effects of *Aspilia africana C. D. Adams* in murine macrophages RAW 264.7 cells stimulated with LPS

CHAPTER 4

A. africana inhibits the production of lipopolysaccharide-induced inflammatory mediators in murine macrophage RAW264.7 cells by suppressing the NF-kB and PI3K / Akt pathways

4.1 Abstract

Aspilia africana (*A. Africana*) is used in African folk medicine as a wound healing remedy. In this study, the potential anti-inflammatory effect of *A. africana* was investigated in lipopolysaccharide stimulated (LPS-stimulated) RAW264.7 macrophages cells. The cytotoxicity of *A. africana* was assessed in RAW 264.7, HEK-293, and HaCaT cells using the WST-1 assay. The results indicated that concentrations up to 200 μg / ml were safe for all studied cell lines. Further analyses, such as nitric oxide (NO), RT-PCR, and Western blotting, were performed to evaluate the anti-inflammatory potential of *A. africana*. The results of NO analysis showed that *A. africana* inhibited NO production, while results of Western blot analysis showed that the expression of important inflammatory enzymes such as iNOS and COX-2, pro-inflammatory cytokines (tumor necrosis factor (TNF) –α, interleukin (IL) - 6, and IL-1β was significantly suppressed by *A. africana*. Results demonstrated also that *A. africana* prevented the activation of NF-*k*B and PI3K/Akt pathways by inhibiting phospho-p65 NF-*k*B, p-Akt, p-I*k*Bα, and pPI3K phosphorylation. RT-PCR analysis revealed that *A. africana* affected the expression of pro-inflammatory cytokines at the gene level. The results of this study showed that *A. africana* prevents the production of inflammatory mediators in RAW264.7

cells stimulated by LPS by regulating the signaling pathways of NF-kB and PI3K / Akt.

4.2 Introduction

Inflammation is a protective and adaptive local reaction of the body to the action of various damaging factors and one of the most frequent forms of the body's response to pathogenic stimuli (Arulselvan, Fard et al. 2016). Various reports have reported the involvement of inflammation in the evolution of a number of diseases such as autoimmune diseases, diabetes, infections, cardiovascular and inflammatory disorders, and cancer (Crusz and Balkwill 2015). During the process of inflammation, several immune cells are activated, especially macrophages, which have been found to play a central role in the immune response against inflammation. (Oh, Cho et al. 2013).

Lipopolysaccharide (LPS) is one of the factors that can stimulate macrophage activation, which in turn secrete various inflammatory cytokines (IL-1β, TNF-α, IL-6, etc.) and inflammatory mediators such as prostaglandin (PG) E2, NO produced by their specific proteins, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively (Li, Fu et al. 2015),(Becker, Mundandhara et al. 2005); (Lee, Lee et al. 2017). These cytokines and inflammatory mediators are necessary for the repair and restoration of tissue injury, as well as for the survival of the host after infections (Becker, Mundandhara et al. 2005); however, overexpression of inflammatory cytokines is harmful to organisms (Philippou, Maridaki et al. 2012). Imbalance in pro- and anti-inflammatory cytokines is observed in patients with inflammatory-related diseases (Kim, Jung et al. 2007). A number of investigations demonstrated the crucial role of NF-*k*B and PI3K/Akt pathways in the regulation and production of proinflammatory cytokines and other immune-related cytotoxic factors making these pathways important targets for anti-inflammatory therapy (Xu, Liu et al. 2010; Qi, Xin et al. 2012; Kim and Cho 2008).

A. africana was reported to possess anti-inflammatory activity (Okoli, Akah et al. 2007) but no report has been yet reported on the molecular pathways involved in the anti-inflammatory activity of *A. Africana.* In our previous study, we compared

the use of green extraction techniques with conventional organic techniques for the recovery of phytochemicals from *A. africana* (Niyonizigiye, Nkurunziza et al. 2020). Characterization of the extracts showed that *A. africana* contained a remarkable amount of Gallic acid which has been reported to exhibit anti-inflammatory activity (Kroes, Van den Berg et al. 1992);(Kim, Jun et al. 2005). In this study, we studied the effect of *A. africana* on the production of inflammatory cytokines and inflammation-related pathways in RAW 264.7 cells stimulated by LPS. This is the first report showing that *A. africana* attenuates LPS-induced NO production and inflammatory cytokines such as IL-1β, IL-6, and TNF-α by inhibiting NF-kB and PI3K / Akt pathways.

4.3 Material and methods

4.3.1 Sample preparation

Aspilia africana leaves (local name: Icumwa co ku musozi) were collected in Bujumbura city in the garden of a traditional healer located in Gihosha rural and were then identified at the Herbarium of the National Institute of the Environment and Nature Conservation (INECN) PN Ruvubu by comparing with already existing specimen (PN6080) collected at RG1, MASHENYO locality at an altitude of 1427m, on 11th June 2013 and deposited by NDIKUBWAYO Ernest. Further procedures used for the treatment of *A. africana* leaves including the extraction techniques applied are described in our previous report (Niyonizigiye, Nkurunziza et al. 2020). Aqueous extract obtained from the hot water extraction (boiling and agitation) technique was used for this study. The stock solution used was obtained by diluting 100 mg of *A. africana* powder in 1mL of distilled water. A working solution of 10 mg/mL was made from the stock solution (100 mg/mL) before cell treatment.

4.3.2 Cell lines and reagents

Human embryonic kidney cells (HEK-293) and murine macrophages (RAW264.7) were gotten from ATCC (Manassas, VA, USA); Human keratinocytes cells (HaCaT) were purchased from Addexbio (San Diego, CA, USA). Culture media (DMEM, MEM) were from Hyclone Laboratories Inc (San Angelo, Texas, USA); antibiotics (streptomycin, penicillin) and Fetal Bovine Serum (FBS) were bought from cellgro® (Manassas, VA, USA). Cell Lysis Buffer (Intron biotechnology, Gyeonggi, Korea); Reagent WST-1 was obtained from the laboratory Daeil (Seoul, Korea); LPS (Sigma, MO, USA); Griess Reagent (Sigma, MO, USA); Antibodies: anti-iNOS, anti-Cox-2, anti-TNF-α, anti-pIkBα, anti-phospho-p65NFkB, anti-NFkB, anti-pAkt, anti-IkBα, anti-Akt, anti-PI3K, anti-pPI3K, anti-GAPDH and peroxidase-conjugated secondary antibodies (anti-mouse IgG, anti-rabbit IgG) were purchased from Cell Signaling Technology (CST) (Danvers, MA, USA);4% formaldehyde (Sigma, MO, USA); Chemiluminescence detection solution (ECL) (Rockford, IL, USA).

4.3.3 Cell culture and cytotoxicity analysis.

The studied cell lines were cultured in specific media: Dulbecco's modified Eagle's medium (DMEM) for RAW264.7 cells and HaCaT, and in the Eagle's modified medium (MEM) for HEK-293 cells. Each medium was supplemented with FBS (10%) and penicillin-streptomycin antibiotic mixture (1%). Cells were kept in an incubator at 37 °C and 5% CO_2 and subcultured until they were ready for use. For cytotoxicity studies of *A. africana*, HaCaT, HEK-293, RAW 264.7 (10^4 cells/well) were seeded separately in a 96-well plate for 24 h. After 24 h, cells were then treated with 0-500 µg/ml concentrations of *A. africana* aqueous extract (AAE) and incubated for 24 h 37ºC and 5% CO_2. After 24 h of treatment, the media were removed from cells and replaced with fresh containing WST-1 solution (10 µl) and further incubated at 37ºC and 5% CO_2 for 3 h protected from the light. Cell viability

was then calculated as a result of absorbance read at 460 nm using a 96-well plate reader (Molecular Devices, CA, USA).

4.3.4 Nitric Oxide (NO) assay

The effect of *A. africana* on NO production was evaluated by performing NO assay as follows: RAW264.7 cells (5 x 10^4) were seeded in a 24 well-plate and incubated in an incubator at 37ºC and 5% CO_2 for 24 h. After 24 h, RAW 264.7 cells were then treated for 4 h with 100 or 200 µg / ml AAE and then challenged with LPS (1 µg / ml) in the presence or absence of AAE for an additional 24 h. A ration volume of 1:1 culture supernatant and Griess reagent were used to quantify the NO produced at 540 nm using a microplate reader.

4.3.5 Western blot analysis

RAW264.7 cells were seeded in culture dishes (100 mm) and incubated at 37ºC and 5% CO_2 for 24 h. Then cells were treated for 4 h with 100 or 200 µg/ml AAE before the 18 h or 30 min LPS (1 µg/ml) stimulation time. Proteins were then extracted from the cells using cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl, 1 mM DTT, 1% Triton X-100, 0.5% NP-40, 0.1% SDS, and 1% Deoxycholate) for 30 min at 4ºC. The lysates were quantified using the Bradford protein quantification assay (CBB solution) and separated on 12% SDS-polyacrylamide gel by electrophoresis. After the separation of lysates by electrophoresis, proteins were transferred onto a nitrocellulose membrane at 50 volts for 2 h 30 min and later blocked in 5% dry non-fat milk in 1XPBST for 1 h on a shaker (5 rpm) at room temperature. After blocking, the membranes were washed 3 times every 10 min with 1X PBST and incubated at 4°C with primary antibodies overnight. The next day, the membranes were again washed 3 times in 1X PBST and incubated for 1h with secondary antibodies conjugated with peroxidase. The blots were then analyzed in an ECL detection solution using the iBright CL1000 machine (Thermo Fisher, MA, USA).

4.3.6 Immunofluorescence staining

$2X10^5$ RAW264.7 cells were seeded in confocal dishes at 37ºC and 5% CO_2 for 24 h. After 24 h, the cells were then treated with 200 μg / ml AAE for 4h, followed by an LPS stimulation (1 μg / ml) for 30 min. After treatment and stimulation, cells were washed in PBS and stained in 1 μg / ml DAPI solution (Thermo scientific, RO, IL) and incubated at 37ºC in dark for 20 min and then fixed in 4% formaldehyde for 15 min. Fixed cells were then blocked for 1h in skim milk (5%) followed with incubation with anti-phospho-p65 NF-kB primary antibody overnight. The next day, the cells were washed in PBS and again incubated for 1h with Alexa-Fluor 488 conjugated secondary antibodies, then washed again in PBS and mounted in Antifade Prolong Gold reagent on microscopic slides, and visualized using confocal microscopy (Zeiss, Jena, Germany).

4.3.7 Reverse Transcriptional Polymerase Chain Reaction (RT-PCR) Assay

Macrophages (RAW264.7) cells were seeded in culture dishes (100 mm) for 24 h. The cells were then treated for 4h with 100 or 200 μg/ml of AAE and then stimulated for 6 h with 1 μg/ml LPS. The QIAGEN RNeasy Mini kit was then used to isolate total RNA from the treated cells by following the manufacturer's instructions (QIAGEN GmbH, HI, Germany). Total RNA (1 mg) was reverse transcribed using AccuPower RT premix containing M-MLV reverse transcriptase (Bioneer, Daejon, Korea). Resulted DNA was then amplified using general PCR reactions.

Table 4.1: The primer sequences of inflammatory markers used:

Primers	Sequences	
	Sense	Antisense
TNF-α	5′-ATGAGCACAGAAAGCATGATC-3′	5′-TACAGGCTTGTCACTCGAATT-3′
IL-6	5′-AGTTGCCTTCTTGGGACTGA-3′	5′-CAGAATTGCCATTGCACAAC-3′
IL-1β	5′-ATGGCAACTGTTCCTGAACTCAACT-3′	5′-TTTCCTTTTCTTAGATATGGACAGGAC-3′
GAPDH	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′	5′-CATGTAGGCCATGAGGTCCACCAC-3′

4.3.8 Statistical analysis

Data are presented as mean values (±) standard deviation from three separate experiments. Obtained data were analyzed statistically using GraphPad prism7 (San, Diego, CA, USA). A p-value (p <0.05) was set as statistically significant.

4.4 Results

4.4.1 Cytotoxicity of *A. africana* on RAW264.7 cells viability

Before evaluating the potential anti-inflammatory effect of *A. africana*, the cytotoxicity assay was conducted on HaCaT, HEK-293, RAW264.7 cells using WST-1 assay. The cells were treated with concentrations of *A. africana* from 0 to 500 µg/ml for 24 h. The results showed that *A. africana* was not toxic on investigated cells up to 500 µg/ml as shown in Fig. 4.1. However, lower concentrations up to 200 µg/ml were applied for further investigations.

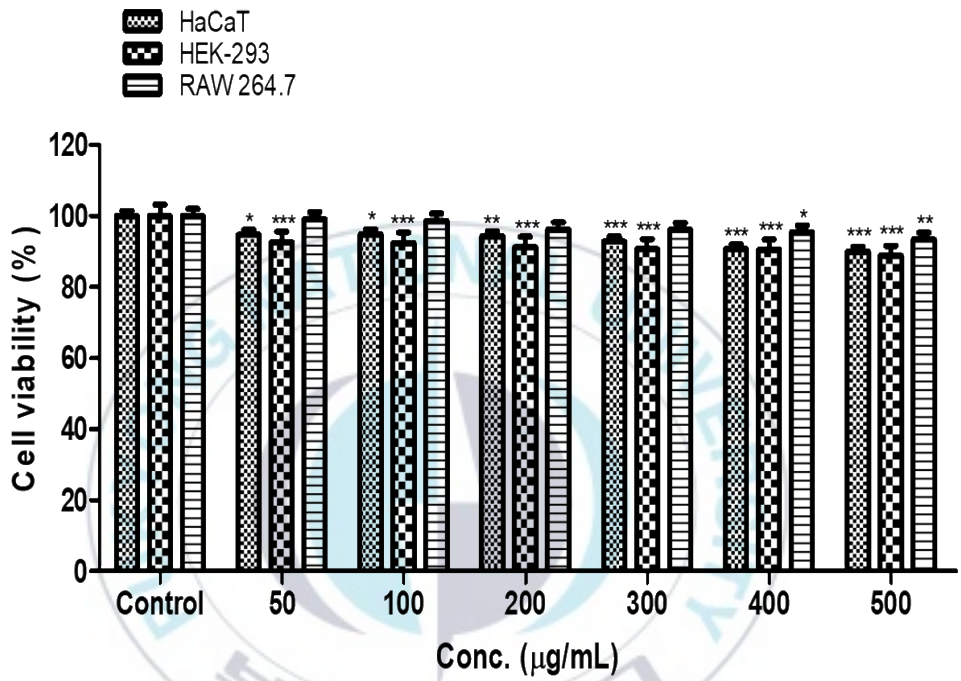

Figure 4. 1: Effect of *A. africana* on the viability of HaCaT, HEK-293 and RAW 264.7 cells treated with various concentrations of *A. africana* for 24 h. Results were obtained byWST-1 assay. Statistical significance differences between control and treated groups were set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4.4.2 *A. africana* inhibits NO production in LPS- stimulated RAW264.7 cells

To evaluate the effects of *A. africana* extract on NO production in macrophages stimulated with LPS, the supernatant media from LPS-stimulated (1 μg/ml) and *A. africana* (100, 200 μg/ml) treated RAW264.7 cells for the indicated time were used to measure the production of NO. RAW264.7 cells stimulated with LPS produced an important amount of NO compared to the control group (Fig. 4.2). *A. africana* considerably inhibited the production of NO in a dose-dependent manner as shown in Fig. 4.2. From these results, we confirmed that *A. africana* inhibits LPS-induced production of NO in RAW264.7 cells.

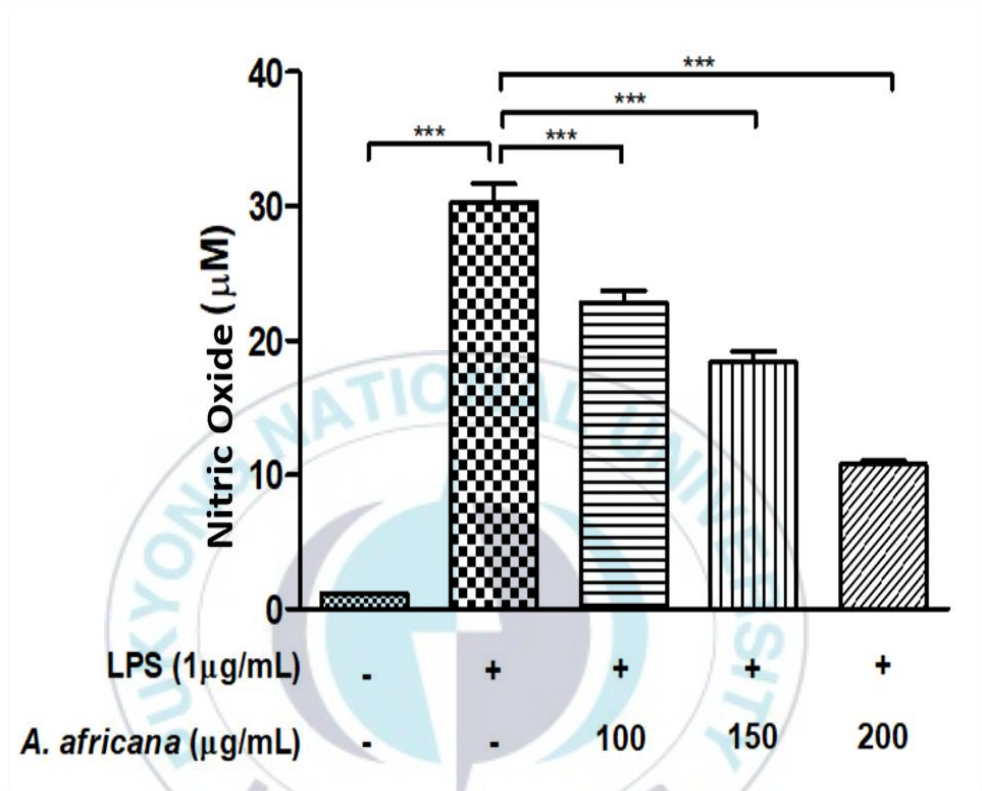

Figure 4. 2: Effect of *A. africana* on nitric oxide (NO) production in RAW 264.7 macrophages cells treated for 4h with indicated concentrations of *A. africana* and then stimulated with 1 μg/mL LPS for further 20 h. Griess reagent was used to determine NO concentrations. LPS-stimulated no treated group was compared with the control group and treated groups and statistically significant differences were set at ** *$p < 0.001$.

4.4.3 A. africana inhibits the expression levels of COX-2, iNOS, and TNF-α in RAW264.7 stimulated with LPS

Western blotting was performed to define the mechanism by which *A. africana* exerted its anti-inflammatory effects on the expression levels of cytokines and inflammatory enzymes (COX-2, and iNOS) in RWA264.7 macrophages cells. Results showed that 20h of LPS stimulation upregulated the expression of iNOS, Cox-2, and TNF-α in RAW264.7 stimulated with LPS while 4h pretreatment of RAW264.7 macrophages with indicated concentrations of *A. africana* significantly reduced their expression in a dose-dependent manner (Fig. 4.3(a)). We further examined the effect of *A. africana* on COX-2; iNOS, IL-1 β, and TNF- α gene expression to investigate if *A. africana* affected their mRNA levels in RAW264.7 macrophages stimulated with LPS using RT-PCR. Expression quantity of TNF-α, IL-1β, IL-6 genes were analyzed by RT-PCR from their cDNA template. Results showed that *A. africana* affected the expression of IL-6, IL-1β, TNF-α, at the mRNA levels (Fig. 4.3(b)). These results suggest *A. africana* as a potential regulator of proinflammatory cytokines at the gene level.

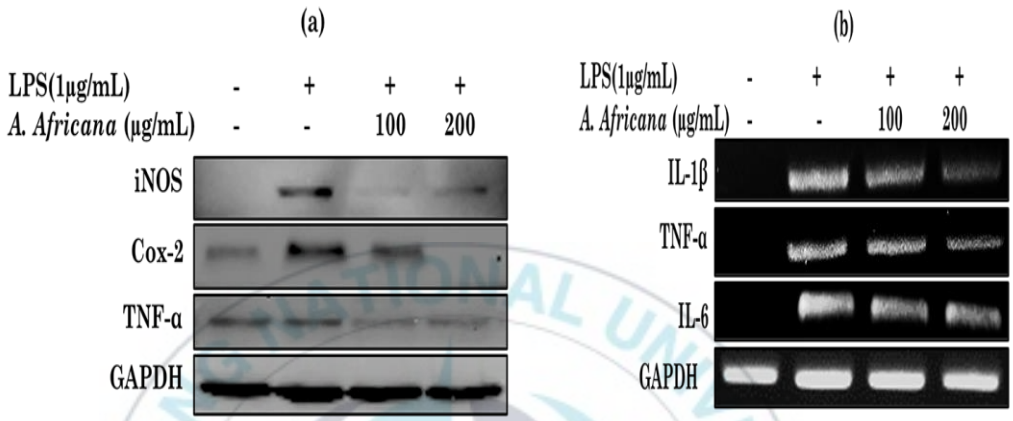

Figure 4. 3: Effect of *A. africana* on the expression of inflammatory cytokines and enzymes. (a) Western blot analysis of iNOS, COX-2, and TNF-α in RAW264.7 macrophages cells treated with *A. africana* and stimulated with LPS. (b) RT-PCR analysis of IL-1β, TNF-α and IL-6 related messenger RNA (mRNA) in RAW264.7 macrophages cells treated with *A. africana* for 4 h and stimulated with LPS for 6 h.

4.4.4 A. africana inhibits the induced LPS activation of the NF-kB signaling pathway in RAW264.7 cells

For evaluating the effect of *A. africana* on the activation of the NF-kB pathway induced by LPS, a westernblot of NF-kB, IkB-α and their phosphorylated form (p65NF-kB, p-IkB-α), as well as immunofluorescent staining of phospho-p65 NF-kB, were performed. Levels of phospho-p65NF-kB and p-IkB-α were significantly downregulated by *A. africana* treatment (Fig. 4.4(a)). Immunofluorescent staining revealed that *A. africana* treatment inhibited the phosphorylated p65NF-kB protein translocation from the cytoplasm to the nucleus (Fig. 4.4(b)). These results confirmed that *A. africana* affects the NF-kB signaling pathway by preventing NF-kB, and IkB-α Phosphorylation.

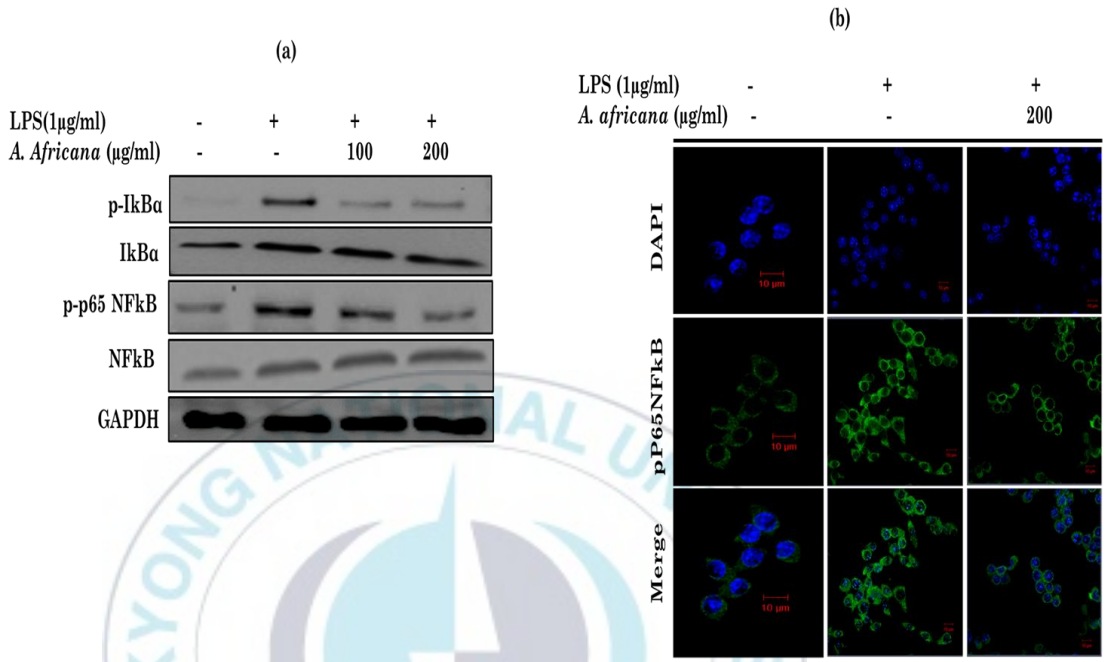

Figure 4. 4: Effect of *A. africana* on the NF-kB pathway. (a) Western blot expression of NF-kB involved proteins. RAW264.7 cells were treated with AAE for 6 h and then stimulated with LPS for 30 min; Proteins were extracted from the cells and analyzed by western blot. (b) Immunofluorescence analysis of phospho-p65NF-kB translocation.

4.4.5 A. africana inhibits the PI3K/Akt pathway activation

The PI3K/Akt signaling pathway plays a significant role in the production of inflammatory mediators. Activation of this pathway is crucial for the initiation and mediation of NF-kB signal transduction. PI3K/Akt pathway was found to be responsible for the expression of pro-inflammatory markers by degrading IkB and activating NF-kB in LPS induced cells. Here we investigated the effect *A. africana* on the activation of the PI3K/Akt pathway in RAW264.7 cells stimulated with LPS. Western blot was conducted to examine the production of PI3K, pPI3K, Akt, and pAkt proteins in RAW264.7 cells treated with *A. africana* and stimulated with LPS. Results obtained showed that *A. africana* was able to inhibit the phosphorylation of PI3K and Akt proteins induced by LPS in a dose-dependent manner (Fig.4.5).

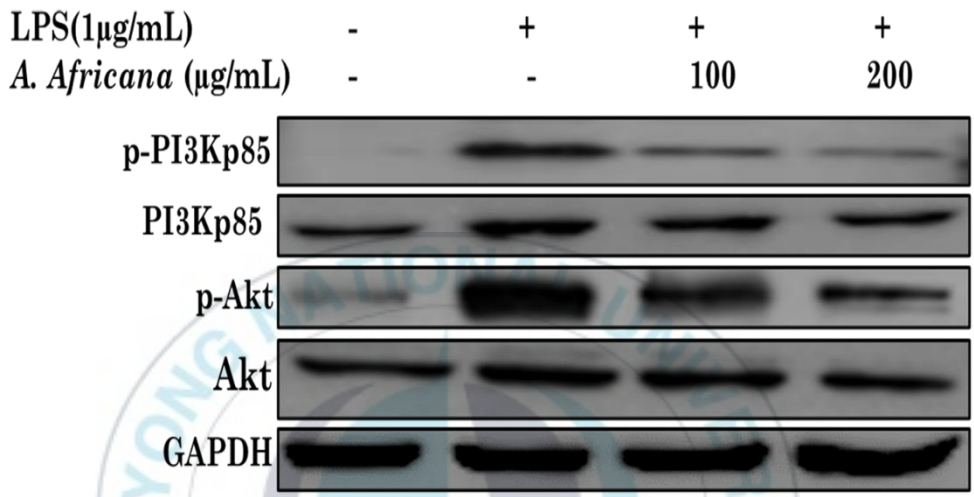

Figure 4. 5: Effects of *Aspilia africana* on the PI3K/Akt pathway. Western blot analysis of PI3K and Akt proteins together with their phosphorylated forms. Macrophages RAW264.7 cells were treated with AAE and then stimulated with LPS. Proteins were then isolated from cells and analyzed by Western blot.

4.5 Discussion

Currently, various investigations using in *vivo* and *vitro* systems are being conducted to find new potential anti-inflammatory agents from natural products including herbal medicine. Even though inflammation is beneficial for the body during infections, the extreme production of inflammatory-mediators can cause tissue injury and metabolic cellular stress (Soufli, Toumi, et al. 2016). Therefore, agents capable of regulating the production of inflammatory cytokines are of necessity. *A. africana* is a promising medicinal herb that possesses various beneficial therapeutic potential due to its numerous secondary active metabolites (Oko and Agiang 2011); (Oko, Agiang, et al. 2014). *A. africana* has been reported to possess anti-inflammatory effects, however molecular pathways involved in the anti-inflammatory effects of *A. africana* are still not well investigated (Okoli, Akah, et al. 2007). In this study, we investigated in vitro the effects of *A. africana* using murine macrophages RAW264.7 cells stimulated with LPS.

Macrophages when stimulated with LPS produce proinflammatory mediators such as NO, TNF-α, IL-6, and IL-1β by triggering signaling pathways such as MAPKs and NF-kB (Haque, Jantan et al. 2018); (Roh, Kim et al. 2016). COX enzyme plays a crucial role in the conversion of arachidonic acid into prostaglandins. Two forms of COX, COX-1, and COX-2 exist. COX-1 ensures the homeostatic role of $PGE_{2,}$ COX-2, is responsible for the excess production of PGE2 at inflammation region which is critical in chronic inflammation diseases (Aluwi, Rullah et al. 2017); (Kiemer, Hartung et al. 2003). The results of our study revealed that *A. africana* inhibited the expression of the COX-2 gene and protein.

Upon the NF-kB activation due to numerous stimuli such as LPS, viral and bacterial components, the iNOS become extremely expressed leading to the high production of NO. Significant evidence has suggested that iNOS-induced NO has a protective role during colitis(Avdagić, Zaćiragić et al. 2013);(Jädert, Phillipson et al. 2014). However, excessive levels of NO have been linked with the progression of a

number of inflammation-related diseases (Barraud, J Kelso et al. 2015);(Yuste, Tarragon et al. 2015). Here in our study, we proved that *A. africana* strongly prevents the production of NO in RAW264.7 cells stimulated with LPS (Fig.4.2). *A. africana* was able to suppress the synthesis of NO by suppressing the expression of iNOS as well as COX-2 (Fig. 4.3).

As already mentioned above, LPS activates macrophages, therefore, inducing the activation of inflammatory cytokines such as TNF-α, IL-6, IL-10, and IL-1β (Charrad, Berraïes, et al. 2016). TNF-α and IL-1β are known to be secreted during chronic inflammation development (Jung, Seo, et al. 2009). Excessive levels of IL-6 and IL-1β have been stated to be present in many malignancies such as lung, colon, breast, colorectal, gastric, head, and neck cancers (Grivennikov and Karin 2011);(Siveen and Kuttan 2009);(Rodrigues, Pietrani et al. 2017). Elevated concentrations of TNF-α have been reported in hidradenitis suppurativa pathogenesis (Van der Zee, de Ruiter et al. 2011). Results of our study showed that *A. africana* significantly inhibits the protein expression of TNF-α and mRNA expression of IL-6, TNF-α, and IL-1β in LPS-stimulated macrophages RAW264.7 cells (Fig.4.3).

For further understanding of the molecular pathways involved in the anti-inflammatory activities of *A. africana,* we investigated two pathways (NF-kB and PI3K/Akt) that are recognized to play a major role in the inflammation process. NF-kB is known to be a key controller of inflammation because it regulates the transcription of numerous key inflammatory-mediators such as IL-1β, COX-2, IL-6, and TNF- α (DiDonato, Mercurio, et al. 2012). In normal physiological conditions, NF-kB remains in association with the IkBs which enclose NF-kB in the cytoplasm. Ubiquitination followed by proteasomal degradation of IkB induces activation of NF-kB which then migrates to the nucleus and stimulates the transcription of directed genes (Hinz and Scheidereit 2014). The results of our experiment demonstrated that *A. africana* was able to inhibit the phosphorylation of IkBα which resulted in the

inhibition of phospho-p65NF-kB, therefore, stopping the NF-kB translocation to the nucleus (Fig.4.4).

Another pathway investigated in this study is the PI3K/Akt pathway. The PI3K/Akt signaling pathway has been stated to regulate a number of cellular processes such as cell survival as well as proliferation (Jason and Cui 2016). PI3K/ Akt pathway is activated in macrophages stimulated with LPS where it controls the inflammatory marker's production via NF-kB activation (Laird, Rhee, et al. 2009). Therefore, the blockage of Akt LPS induced phosphorylation is a crucial target in controlling inflammatory diseases. Kim and his colleagues reported the PI3K/Akt as a negative regulator of extreme innate immune and TLR- mediated proinflammatory responses (Kim, Choi et al. 2005). Various studies have reported the negative regulating role of the PI3K/Akt pathway in LPS-induced inflammatory responses in vivo and in vitro investigations (Schabbauer, Tencati et al. 2004); (Guha and Mackman 2002). Another report reported that PI3K/Akt may be involved in the transactivation of NF-kB but the mechanism remains debatable (Takeshima, Tomimori et al. 2009). Our results demonstrated that *A. africana* inhibits PI3K/Akt pathway activation by preventing the phosphorylation of PI3K and Akt proteins (Fig.4.5).

In conclusion, the results obtained in our study are consistent with other studies that have informed that *A. africana* possesses anti-inflammatory virtues. Here we demonstrated the molecular ways by which *A. africana* exerts its inflammatory effect. By stimulating the RAW264.7 macrophages cells with LPS and then treating with various concentrations of *A. africana*, we were able to demonstrate that *A. africana* attenuates the production of inflammatory cytokines and prevents the activation of PI3K/Akt and NF-kB signaling pathways. The results obtained from this study support the use of *A. africana* as a potential anti-inflammatory agent.

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General conclusion

The number of studies on the potential of *Aspilia africana* extracts (AAE) for biological activities has increased enormously in the past years. Although the results are thrilling, there is limited information on the extraction technologies. Existing studies applied conventional extraction techniques like methanol and ethanol for the study of *A. africana* characterization and biological activities. However, due to the toxicity and cost of conventional organic solvents used in the extraction, there is a limited application of the extracts. Our study explored the potential of novel extraction techniques that use green solvents to recover non-toxic and ready to use extracts. Subcritical water, hot water extraction methods were performed, and ethanol was used as a conventional solvent for comparison. The extracts were characterized for their phenolic contents using both chromatographic (HPLC) and spectrophotometric methods. The potential antioxidant and anticancer activities of the AAE were also studied.

Our results demonstrated by means of HPLC that the predominant active phenolic compound was gallic acid, which may be responsible for the biological activity of *A. africana*, and the hot water extraction technique showed the highest recovery of this active compound. Amongst the extraction techniques studied, Hot water extraction (HWE), and subcritical water (SWE) at 160°C showed the best anti-proliferative activity against AGS (Gastric adenocarcinoma cells) and A459 (lung adenocarcinoma cells). AGS was the most sensitive to the extracts of *A. africana*, especially from hot water extracts. Both HWE and SWE performed better than Ethanol extraction, confirming that water was a better solvent in both conditions as compared with the organic solvent (Ethanol). These findings suggested that hot water and subcritical water would be the best extraction techniques for the recovery of bioactive compounds, such as phenolic antioxidants from *A. africana*, as compared with the conventional organic solvent (ethanol).

Further investigations were conducted to explore molecular pathways behind the anticancer effects of AAE against gastric adenocarcinoma AGS cells. As demonstrated by the results, AAE exhibited an antitumor effect in gastric adenocarcinoma AGS cells by inducing apoptosis and altering the G1/S cell cycle phase progression. Our findings suggest Aspilia *africana C.D. Adams* as a promising anticancer agent against gastric cancer. Further investigations are necessary to better understand the mechanisms of antitumor actions *in vivo* studies.

Additionally, we studied the effect of *A. africana* on the production of inflammatory cytokines and inflammation-related pathways in RAW 264.7 cells stimulated by lipopolysaccharide (LPS). The results obtained in our study are consistent with other studies that have informed that *A. africana* possesses anti-inflammatory virtues. We demonstrated the molecular ways by which *A. africana* exerts its inflammatory effect. By stimulating the RAW264.7 macrophages cells with LPS and then treating with various concentrations of *A. africana,* we were able to demonstrate that *A. africana* attenuates the production of inflammatory cytokines and prevents the activation of PI3K/Akt and NF-kB signaling pathways. The results obtained from this study support the use of *A. africana* as a potential anti-inflammatory agent.

국문요약

암은 세계적으로 대표적인 사망 원인 중 하나이며, 2018 년 사망자 960 만명 중 78 만 3 천명이 위암으로 사망했다. 암은 세포의 통제할 수 없는 성장으로 정의되며, 종양 형성과 면역체계 손상을 초래한다. 항암연구는 주로 세포사멸의 활성화를 통한 암세포의 사멸을 유도하거나 세포주기 억제를 유도하여 암의 진행을 조절하는 데 초점을 맞추고 있다. 염증은 암의 진행 및 발달과 관련이 있다. 만성 염증은 심혈관 질환, 관절염, 당뇨병, 폐질환, 알츠하이머병, 자가면역질환 등 광범위한 질병과도 관련이 있다. 대식세포는 여러 종류의 사이토카인과 성장인자의 생성을 통해 염증 시 면역조절에 중요한 역할을 한다. 과거부터 건강에 좋은 약용식물에 대한 관심이 높아지고 있다. 약용식물에는 생물학적 활동과 관련된 생리활성 화합물의 함유량이 높은 것으로 알려져 있다. 아스필리아 아프리카나 C.D. 아담스(*A. africana*)는 아프리카 동부의 열대 아프리카에서 자란다. *A. africana* 는 아프리카의 민간요법에서 선호되는 약초로서 항균, 항염증, 항산화제 등 수많은 생리적 효과가 있으며 여러 종류의 중요한 생리활성 화합물을 보유하고 있다. 본 논문에서는 *A. africana* 의 생리활성 화합물 회수를 위한 친환경적 추출법의 사용 이점을 기존의 추출법과 비교하여 조사하였으며, 인간의 AGS 위암세포에 대한 *A. africana* 의 항암 효과와 마우스 RAW264.7 대식세포에 대한 *A. africana* 의 항염증 효과를 연구하였다. 이 논문에 제시된

결과는 기존의 유기 용제(에탄올) 추출을 열수 추출(HWE)과 비교하였다. 그리고 초임계 물 추출법(SWE)이 *A. africana* 페놀성 항산화 물질과 같은 생리활성 화합물의 회수에 최적의 추출법이 될 것이라는 것을 보여준다. HWE 추출물에서 갈릭산, 총 페놀 성분(TPC), 총 플라보노이드 성분(TFC)의 농도가 가장 높았으며 에탄올 추출물보다 IC_{50} 값이 9 배 낮은 가장 높은 항산화능을 나타냈고, SWE 가 그 뒤를 이었다. 또한 *A. africana* 는 G1 세포주기 진행을 억제하고 세포사멸을 유도함으로써 AGS 세포의 증식을 억제하였다. G1/S 진행에 중요한 역할을 하는 사이클린 E1 & D1, CDK2, 4 & 6 들은 CDK 억제제(p21, p27, p18, p15)의 발현이 상향된 곳에서 하향 조절되었다. AGS 세포에 *A. africana* 를 처리하였을 때 tBid, Bad, Bak, Bax, Cytochrome C, FLIP, cleaved Caspases 3, 8&9, cleaved Parp 와 같은 pro-apoptotic 단백질들의 발현은 감소한 반면 Bcl-2, Bcl-xL 와 같은 anti-apoptotic 단백질들의 발현은 증가하였다. LPS 로 염증을 유도한 RAW264.7 대식세포에 *A. africana* 를 처리하였을 때에는, 질소산화물(NO)의 생산을 억제하고 iNOS, COX-2, 프로-염증성 사이토카인(TNF–α, 인터루킨(IL)-6, IL-1β)들과 같은 중요한 염증성 효소들의 발현을 억제하여 PI3K/Akt 및 NF-kB 신호 경로들의 활성화를 억제시켰다.

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