Addis Ababa University

College of Health Sciences

School of Pharmacy

Department of Pharmacology and Clinical Pharmacy

Chemopreventive Effect of 80% Methanol Leaf Extract of *Vernonia auriculifera* Hiern (Asteraceae) against Dimethylhydrazine-Induced Colorectal Carcinogenesis in Rats

By

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A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the Master of Science Degree in Pharmacology.

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This is to certify that the thesis prepared by Yohannes Tsegyie, entitled: Chemopreventive Effect of 80% Methanol Leaf Extract of *Vernonia auriculifera* Hiern (Asteraceae) against Dimethylhydrazine-Induced Colorectal Carcinogenesis in Rats and submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abstract

Chemopreventive Effect of 80% Methanol Leaf Extract of *Vernonia auriculifera* Hiern (Asteraceae) *against* Dimethylhydrazine (DMH)-Induced Colorectal Carcinogenesis in Rats

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Addis Ababa University, 2019

Colorectal cancer (CRC) is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract. Researchers revealed that herbal sources can be useful for the successful management of CRC. *Vernonia auriculifera* is among the Ethiopian folk medicine claimed to be used for different types of cancer and other diseases. Nevertheless, *in vivo* pharmacological investigations have not been performed to substantiate its chemopreventive and curative activities. To this effect, this study aimed at investigating the chemopreventive potential of hydroalcoholic leaf extract of *V. auriculifera* in dimethylhydrazine (DMH)-induced CRC in rats through a two-phase study (pre-initiation and post-initiation). The crude extract was administered at doses of 100, 200 and 400 mg/kg. Parameters including percent weight increment, growth rate, average tumor number, size, progression and incidence as well as biochemical markers such as total protein, total cholesterol, and triglyceride were then determined. The extract exerted significant chemoprevention in terms of increasing percent body weight increment, reduction in average tumor number and size compared to untreated rats ($p < 0.05$). The extract also decreased tumor incidence. High dose of the extract in the pre-initiation phase had reduced tumor incidence by 50%. However, the lowest tumor incidence (33%) was obtained by treatment with aspirin (60 mg/kg). Furthermore, pre-initiation regimens showed greater reduction in the number of invasive adenocarcinomas compared to post-initiation regimens. The extract inhibited CRC associated serum total cholesterol and triglyceride increment ($p < 0.05$), but didn`t produce significant effect on total protein level. In conclusion, the findings of the present study showed that the leaf of *V. auriculifera* possesses chemopreventive activity which supports the traditional claim and is a potential source for developing more effective and safer anticancer drugs following in-depth investigations on the plant.

Key words: Colorectal cancer, *Vernonia auriculifera*, chemoprevention, pre and post-initiation

List of Abbreviations/Acronyms

ACF	Aberrant Crypt Foci
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
CIMP	CpG island methylator phenotype
CIN	Chromosomal Instability Pathway
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer
DFMO	D,L-alpha-difluoromethylornithine
DMH	1,2-dimethylhydrazine
EGFR	Epidermal Growth Factor Receptor
FAP	Familial adenomatous polyposis
KRAS	Kirsten Rat Sarcoma
MMR	Mismatch repair
MSI	Microsatellite Instability Pathway
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
ODC	Ornithine Decarboxylase
PGE2	Prostaglandin E2

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1. Introduction

1.1. Overview of colorectal cancer

Cancer is a disease characterized by uncontrolled proliferation of slightly modified normal human cells (Akindele *et al.*, 2015a). It represents a major public health burden and the leading cause of death all over the world (Park and National, 2012). In addition, cancer is the principal cause of death in economically developed countries and the second leading cause of death in developing countries (WHO, 2004). Meanwhile, the burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and "westernized" diets (Ahmedin and Forman, 2011). Studies estimated that the total number of new cases of cancer will rise from 10 million in 2000 by approximately 25% in each decade, reaching 24 million new cases per year in the year 2050. Moreover, the total number of deaths will rise from 6 million in 2000 to 10 million in 2020 to over 16 million in the year 2050. In the year 2050, there will be 17 million new cases of cancer in less developed countries, while only 7 million new cases of cancer will occur in the more developed countries *(*Murray and Lopez, 2014).

Colorectal cancer (CRC) or bowel cancer is a malignancy originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract (Rehemtulla, 2010). It was first diagnosed in an ancient Egyptian mummy and also, recorded in 2 Chronicles 21, Biblical king Jehoram with fatal disease of the bowel carcinoma (Rehemtulla, 2010). Clinical symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time (Donada *et al.*, 2013). The most common colon cancer cell is adenocarcinoma, which accounts for 98% of cases. Other, less common types include lymphoma and squamous cell (Matthew *et al*., 2012).

The colon is an ideal target organ to develop chemopreventive interventions for a number of reasons. First, it is estimated that CRCs develop over a period of 10–20 years, providing a large frame of opportunity for therapeutic intervention. Second, the relative sequence of genetic events required for tumor formation has been investigated most extensively in the colon (Fearon and Vogelstein, 1990). Third, the established histopathological progression of normal tissue to an intermediate adenoma and, ultimately, invasive cancer presents milestones with respect to where a particular lesion is in the carcinogenic sequence, both in the presence and absence of

chemopreventive agent exposure. Fourth, the adenomatous polyp serves as a preneoplastic marker of CRC risk, aiding in the identification of the subpopulation of individuals who would benefit most from chemopreventive therapy. Finally, unique insight can be gained from evaluating chemopreventive response in individuals known to carry germline mutations that predispose them to such familial colorectal syndromes as familial adenomatous polyposis and hereditary nonpolyposis CRC (Kinzler and Vogelstein, 1996)

1.2. Epidemiology

Globally, CRC is the second most commonly diagnosed cancer in females and the third in males, with 1.8 million new cases and almost 861,000 deaths in 2018 according to the World Health Organization (Cronin *et al.*, 2018). Incidence rates are substantially higher in males than in females and the regional incidence of CRC varies over 10-fold. The highest incidence rates are in Australia, New Zealand, Europe, and North America, and the lowest rates are found in Africa and South-Central Asia (Haggar and Boushey, 2009). These geographic differences appear to be attributable to differences in dietary and environmental exposures that are imposed upon a background of genetically determined susceptibility (Kinzler and Vogelstein, 1996).

In the United States, both the incidence and mortality have been slowly but steadily decreasing (Siegel *et al*., 2019). Annually, approximately 145,600 new cases of large bowel cancer are diagnosed, of which 101,420 are colon and the remainder are rectal cancers. An estimated 50,630 Americans die of CRC, accounting for approximately 8 percent of all cancer deaths in a year decreasing. However, reliable statistics on deaths from colon and rectal cancers separately are not available because almost 40% of deaths from rectal cancer are misclassified as colon cancer on death certificates (Siegel *et al*., 2019).

The incidence rates continue to decline in people 50 years and older, dropping by 32% just since 2000. This trend is thought to be largely a result of screening, which can prevent CRC by detecting and removing precancerous polyps. Incidence drops with the fastest rate in people with age 65 and older and for tumors located in the distal colon, while the drop is slowest for people with age 50 to 64 and for rectal tumors (Siegel *et al.*, 2017). For example, there was only a 9% decline in the incidence of rectal tumors in men 50 to 64 year and no decline in women in that age range compared to 38% and 41% declines in men and women, respectively, of age 65 and older.

In contrast to the CRC incidence trends in those 50 and older, incidence rates among people younger than 50 continue to rise, increasing by 22% from 2000 to 2013. Similar to incidence patterns, CRC death rates decreased by 34% in people with age 50 and above during 2000-2014 but increased by 13% in those under 50. National Health Interview Survey data indicated that from 2013 to 2015, screening with any guideline-recommended test increased from 53% to 58% in those aged 50 to 64, from 65% to 68% in those 65 and older, and from 59% to 63% in both age groups combined (Siegel *et al.*, 2017).

CRC occur at a much younger age in Ethiopia than in the developed world. More than half of the cases were in the rectum. And also, the shift of CRC to the right colon reported elsewhere was not observed. The clinician should expect CRC also in young patients, and most of these carcinomas are still detectable by proctosigmoidoscopy (Ashenafi, 2000).

1.3. Etiology of colorectal cancer

Genetic and environmental factors play an important role in the etiology of CRC. Other risk factors include older age, male gender, high intake of fat, inherited genetic disorders, consumption of alcohol and red meat, obesity, smoking, and lack of physical activity (Wei *et al*, 2004). Approximately, 10% of the cases are linked to insufficient activity. The risk for alcohol appears to increase at greater than one drink per day. The majority of CRCs are sporadic; approximately three-quarters of patients have a negative family history (Wei *et al*, 2004). In addition, a number of chemical carcinogens such as 1, 2-dimethylhydrazine (DMH), azoxymethane (AOM), 2-amino-3-methyl imidazole (4, 5f) quinoline, 2-amino-1-methyl-6-phenylimidazo [4, 5-6] pyridine, methyl-nitrosurea, N-methyl-N-nitro-N-nitrosoguanidine are known to cause benign and malignant neoplasm in colon of the rodents (Rosenberg, *et al*., 2009). These agents found in some natural products with minor amount may cause CRC in human even though there is no sufficient data (Gordon and David, 2013). .

1.4. Pathophysiology

CRC can be classified in three major forms: inherited, sporadic, and familial. Although the mechanisms underlying familial CRC are poorly understood, a large body of evidence suggests that inherited and sporadic CRC are caused by sequential genetic and molecular events. CRC pathogenesis has three distinct pathways which are; chromosomal instability pathway (CIN), microsatellite instability pathway (MSI), and serrated pathway. Mostly, CRCs arise from the CIN pathway, which is characterized by defects in chromosomal segregation, telomere stability,

and DNA damage response. MSI originates from the loss of DNA mismatch repair (MMR) and is found in about 15 % of all CRCs, 3 % of which are associated with Lynch syndrome. The serrated pathway describes the progression of serrated polyps to CRC (Král *et al.*, 2016).

1.4.1. Chromosomal instability pathway

The first step in CRC carcinogenesis is supposed to be the formation of aberrant crypt foci (ACF). Activation of the Wnt pathway occurs during this step as a result of inactivating mutations in the adenomatous polyposis coli (APC) gene. Advancement to adenoma and carcinoma is usually mediated by activating mutations in K-RAS (Kristen Rat Sarcoma) and loss of tumor protein-53 expression, respectively. A subset of advanced adenomas may progress due to mutations in Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha and loss of 18q (Král *et al.*, 2016).

1.4.2. Microsatellite instability pathway

Approximately 25 % of MSI-associated CRC arises from the Lynch syndrome, in which inactivating germline mutations in MMR genes are inherited in an autosomal dominant pattern. Additional second hit to the wild type copy of the gene (inherited from the unaffected parent) in the form of somatic mutations, deletions, and methylation leads to MSI. About 75 % of MSI-associated CRC is sporadic. These cancers are associated with CpG island methylator phenotype (CIMP) and undergo MMR gene inactivation via hypermethylation of the Mut L homolog 1 promoter. Both Lynch syndrome and sporadic CIMP positive-associated defects in MMR lead to MSI and rapid accumulation of somatic mutations in genes with coding “microsatellite repeats.” Many of these microsatellite repeats may not contribute to carcinogenesis but provide a signature that can be used for identification of MSI. Lynch-associated CRCs often harbor K-RAS mutations, whereas sporadic CIMP-associated tumors are often B-Raf mutant (Nojadeh *et al.*, 2018).

1.4.3. The serrated pathway

In this pathway, activation of B-Raf proto-oncogene (a serine/threonine kinase) induces the formation of ACF with serrated features and microvesicular hyperplastic polyp. Further cell proliferation is controlled by cell senescence, which is mediated by a family of cyclin-dependent kinase inhibitors, p16INK4a, expression and insulin-like growth factor-binding protein 7 secretions. Methylation-induced silencing of p16INK4a or loss of p53 function allows early

polyps to escape the senescent state and develop into sessile serrated adenomas (Yamane *et al.*, 2014)

1.5. Diagnosis

Diagnosis of CRC is via sampling of areas of the colon suspicious for possible tumor development typically done during colonoscopy or sigmoidoscopy, depending on the location of the lesion. The extent of the disease is then usually determined by a computed tomography scan of the chest, abdomen, and pelvis. There are other potential imaging tests such as positron emission tomography and magnetic resonance imaging, which may be used in certain cases. Colon cancer staging is done next and based on the TNM (T. primary tumor, N. regional lymph nodes, and M. distant metastasis) system which is determined by how much the initial tumor has spread, where lymph nodes are involved, and what is the extent of disease metastasis (Bruening *et al.*, 2014).

The microscopic cellular characteristics of the tumor are usually reported from the analysis of tissue taken from a biopsy or surgery. A report from pathology usually contains a description of cell type and grade (Bruening *et al.*, 2014).

1.6. Management of colorectal cancer

1.6.1. Chemopreventive agents

Since the burden of CRC on society with respect to mortality, morbidity, and costs is enormous, the prevention of CRC is an important public health objective. For instance, dietary interventions are supposed to prevent 70–80% of CRC, because diet may carry chemopreventive agents that could reduce cancer risk. In addition, more than 300 agents have been tested in rodents; most of these agents were fed to rats during or after the injection of a colon carcinogen (Brown *et al.*, 2009). These agents were inhibiting the initiation of preneoplastic lesions by carcinogens or reverse their progression to invasive cancers. In randomized controlled trials, a number of pharmacological interventions have been investigated as chemopreventive agents for CRC in persons at average risk (those without personal or family history of colorectal neoplasia or conditions such as inflammatory bowel disease or hereditary CRC syndrome) with variable results (Veettil *et al.*, 2018).

Considerable efforts are in progress for prevention of early stages or recurrence of CRC or new polyp formation by chemoprevention strategies. Of the several drugs tested in the past for CRC

prevention, significant evidence for the anti-cancer effects in *in vitro*, *in vivo*, epidemiological and clinical studies exist for; non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin, naproxen, sulindac, celecoxib, licofelone), statins and other natural agents both individually, and in combinations (Mohammed *et al.*, 2018).

i. Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the most commonly studied agents for slowing the progressions of CRC both preclinically and clinically. Aspirin, naproxen, sulindac, and celecoxib are extensively studied for chemoprevention of CRC at the laboratory, preclinically and clinically. Epidemiological studies also clearly indicated the chemopreventive effects of NSAIDs against CRC. Aspirin (acetylsalicylic acid) exhibits anti-inflammatory and chemopreventive effect against several cancers including CRC. A large number of clinical trials on aspirin for CRC have shown a promising chemopreventive effect against CRC. Aspirin shows its effects by inhibiting elevated cyclooxygenase (COX-1&2) enzymes activity and by modulating Wnt/β-catenin signaling pathway. Recently, Kagbo-Kue *et al*. (2018) reported that aspirin inhibited the metastasis of CRC cells by suppressing the expression of toll-like receptors.

Previous studies in animal models also revealed a clear CRC chemoprevention efficacy of aspirin both at initiation and progression stages. The carcinogen, DMH/ AOM-induced rat CRC precursor lesions, ACF, formation and progression were significantly lowered by aspirin. Aspirin was also found to significantly reduce multi-crypt aberrant foci in rats (Alfonso *et al.*, 2014). Pronounced aspirin effects at 60 mg/kg body weight were seen in the inhibition of distal ACF in DMH induced rat colon carcinogenesis. Dietary daily oral administration of 200 and 400 ppm of aspirin significantly inhibited the incidence and multiplicity of carcinogen-induced invasive adenocarcinomas of the colon as well as the size of adenocarcinomas in rats along with the reduction of colonic mucosal and tumor prostaglandin E2 (PGE2) levels (Alfonso *et al.*, 2014).

Naproxen also exerts its protective effects as anti-inflammatory agents and lately as anti-cancerous agent due to the similar molecular pathways that exist in inflammatory diseases and cancer. H2S-releasing naproxen derivative, ATB-346, reduced expression of c-Myc and β-catenin, thereby exhibits chemopreventive potentials in intestinal tumors of *Apc*Min/+ mice than naproxen (Paul-Clark *et al.*, 2016). In a preclinical study, naproxen treatment twice-daily for four weeks showed a dose-dependent preventive effect on AOM-induced ACF in the colon of mice (Elsheikh *et al.*, 2014). The study showed that chronic dietary administration of 150 ppm of

naproxen significantly inhibited AOM-induced colon adenocarcinoma multiplicity in rats (Mohammed *et al.*, 2018). In rat colon carcinogenesis, on the other hand, sulindac induced apoptosis during the initiation and promotion stages (Samaha *et al.*, 1997). Singh and Reddy (2006) studied the inhibition of expression of ras-p21 and p53 by sulindac during AOM-induced colon carcinogenesis. Other studies also reported inhibition of both carcinoma induction as well as progression by sulindac in DMH-induced mouse colon carcinogenesis (Mohammed *et al.*, 2018).

Celecoxib exhibits chemopreventive potentials against CRC by down-regulating expressions of COX-2, vascular endothelial growth factor, and nuclear factor kappa-light-chain-enhancer of activated B cells, as well as by increasing caspases-3 activity in HT-29 human CRC cells along with imatinib (Atari-Hajipirloo *et al.*, 2016). It also inhibits Wnt/β-catenin signaling pathway and its genes products survivin and cyclin (Egashira *et al.*, 2017). In pre-clinical animal models of colon carcinogenesis, dietary administration of celecoxib at different doses showed inhibition of total ACF induction and crypt multiplicity that strengthened the hypothesis of selective COX-2 inhibition for chemoprevention of colon carcinogenesis (Gonzalez-angulo and Fuloria, 2002).

Single laboratory study also showed that oral administration of celecoxib inhibited both incidence and multiplicity of colon tumors by about 93% and 97%, respectively. Administration of celecoxib to rats during the initiation and post-initiation stages significantly inhibited tumor volume ($p < 0.0002$ to $p < 0.001$) and the incidence as well as the multiplicity of colon adenocarcinomas ($p < 0.01$ to $p < 0.0001$) in a dose-dependent manner (Jang *et al.*, 2002). Twice daily administrations of celecoxib (200 or 400 mg) were found to be very effective for the prevention of colon adenomas in randomized clinical trials but caused potential cardiovascular events particularly for patients with preexisting atherosclerotic heart disease thus limiting its advancement (Bertagnolli *et al.*, 2009).

ii. Statins

Results from a population-based case control study in Northern Israel indicated that at least 5 year of statin use reduces the relative risk of CRC compared to controls (Lochhead, 2006). This study supports further investigation into the appropriate dose, the optimal length of treatment, and the most active type of statin. The potential benefit of statins in CRC with less toxicity profile make to use them as a chemopreventive agent (Lochhead, 2006).

iii. Eflornithine

Difluoromethylornithine or eflornithine (DFMO) irreversibly inhibits ornithine decarboxylase (ODC), which is the first and rate-limiting step in polyamine synthesis. ODC and polyamines are both elevated in CRC and adenomatous polyps (Urban, 2010). A study on patients with a history of resected colon polyps found that three doses of eflornithine (0.2 g/m2 /d) suppressed polyamine levels in rectal mucosa with minimal side effects. Because eflornithine does not completely suppress tumorigenesis, there is ongoing interest in combining it with other agents such as NSAIDs (Urban, 2010)

iv. Combinational interventions

Combination intervention is used to increase effectiveness and reduce the doses of individual drugs to overcome drug toxicities and drug resistance (Mohammed *et al.*, 2018). Several preclinical studies have been carried out using many combination agents for CRC prevention in mice and rat models. Randomized, placebo-controlled, double-blind, 2 × 2 biomarker trial of a combination of aspirin and metformin showed better efficacy as compared to each agent given alone in diabetes mellitus and CRC patients (Higurashi and Nakajima, 2018). In addition, several researchers have been exploring a combination of aspirin and calcium as one of the cost-effective and low-risk strategies with colonoscopy for primary prevention of CRC (Pence *et al*., 2013).

In a double-blind, placebo-controlled, randomized trial (phase II) by Samadder *et al*. (2016), combination treatment with sulindac and erlotinib significantly reduced colorectal polyp burden after 6 months of treatment as compared with placebo. A preclinical study by Chang *et al*. (2018) demonstrated that treatment with a combination of sulindac and atorvastatin decreased the multiplicity of colon adenomas in ApcMin/+-FCCC mice already with adenomas. Sulindac along with DFMO also showed significant CRC prevention activity. A successful combination chemoprevention drugs DFMO at the dose of 500 mg and sulindac at the dose of 150 mg once daily was significantly effective for prevention of adenomas in 3 years randomized placebo-controlled clinical trial (Sporn and Hong, 2008).

v. Chemopreventive agents under investigation

a. Curcumin

Curcumin (diferuloylmethane) is a low molecular weight polyphenol that is a major component of the yellow spice turmeric. It is thought to prevent cancer by suppressing COX-2 as well as glutathione-S- transferase activity (Patel *et al.*, 2010). A single pilot study in humans found that oral curcumin was rapidly degraded to metabolites that exhibit less COX-2 inhibitory potential. Nevertheless, dose-dependent reductions of COX-2 activity and PGE2 levels were reported (Garcea *et al.*, 2005). Another study of patients with CRC showed that although peripheral blood levels may be low, high levels of curcumin glucuronide and curcumin sulfate were found in the rectal mucosa. However, this study did not show an increase in COX-2 levels (Patel *et al.*, 2010).

b. Nitric oxide-releasing non-steroidal anti-inflammatory drugs

Nitric oxide-releasing NSAIDs (NO-NSAIDs) are a class of anti-inflammatory agents that contain an NSAID molecule linked to a NO-donating group. In vitro studies indicate that NO-acetylsalicylic acid, NO-sulindac, and NO-ibuprofen reduce CRC cell growth more effectively than their corresponding NSAIDs, making these potential chemopreventive agents worthy of further study (Williams *et al.*, 2001). NO-aspirin, an NO-releasing pro-drug of aspirin, inhibits the catalytic activity of nitric oxide synthase 2, along with two COX-2, as well as down-regulated expression of β-catenin and proliferating cell nuclear antigen in colon tumors induced by AOM (Williams *et al.*, 2001).

c. Inulin Derivatives

Inulin-type fructans (beta (2, 1) fructans) extracted from chicory roots are prebiotic food ingredients, which in the gut lumen are fermented to lactic acid. Research in experimental animal models revealed that inulin-type fructans have anticarcinogenic properties (Pool-Zobel, 2005). A number of studies report the effects of inulin-type fructans on chemically induced pre-neoplastic lesions ACF or tumors in the colon of rats and mice. In twelve studies, there were twenty-nine individual treatment groups of which twenty-four measured ACF and five measured tumors. There was a significant reduction of ACF in twenty-one of the twenty-four treatment groups and of tumor incidence in five of the five treatment groups. Higher beneficial effects were achieved by synbiotics (mixtures of probiotics and prebiotics), long-chain inulin-type fructans compared

to short-chain derivatives and feeding high-fat Western-style diets (Markowiak and Ślizewska, 2017).

d. Epidermal growth factor receptor inhibitors

Inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase are thought to have chemopreventive effects through transforming growth factor which increases as neoplasms progress from adenomas to *in situ* disease to invasive cancer (Gadgeel *et al.*, 2009). Nearly half of the Min mice (mice which had a point mutation at the Apc gene) treated with a combination of sulindac and EKI-569, an EGFR inhibitor, showed reduced polyp formation (Gadgeel *et al.*, 2009). When the Min mice were crossed with mice significantly reduced levels of EGFR (the EGFRWA model), intestinal polyps were reduced by 90% as compared to those with the wild-type allele. Both findings support the role of EGFR in intestinal polyp formation and carcinogenesis (Hawk *et al*., 2004).

1.6.2. Surgery, radio and chemotherapeutic interventions

The management of CRC should be a multi-modal approach according to tumor localization, extent and biology, and patient factors. Hence, treatment approaches may include a combination of surgery, radiation therapy, chemotherapy, and targeted therapy. Although optimal surgical resection is still the mainstay of curative treatment, optimal multi-modal treatments could maintain long-term survival, quality of life and even cure in selected patients. Treatment and management decisions regarding CRC must be on evidence-based guidelines, including the National Comprehensive Cancer Network and the European Society for Medical Oncology guidelines (Nakayama *et al.*, 2014). If the cancer is found at a very early stage, it may be removed during a colonoscopy.

For patients with localized cancer, the preferred treatment is complete removal with adequate margins, with the attempt of achieving a cure. This can be done by an open laparotomy or sometimes laparoscopically and then the colon may be reconnected or a person may have a colostomy. If there are only a few metastases in the liver or lungs they may be removed. Sometimes chemotherapy is used before surgery to shrink cancer before attempting to remove it. The two most common sites of recurrence of CRC are the liver and lungs. In stage I CRC, no chemotherapy is offered, and surgery is definitive treatment. Use of chemotherapy in stage II CRC is controversial, and usually not offered unless risk factors such as T4 tumor or inadequate

lymph node sampling is identified. It is also known that the patients who carry abnormalities of the mismatched repair genes do not benefit from chemotherapy (Ong and Schofield, 2016)

Chemotherapy is an integral part of treatment for stage III and stage IV CRC, if cancer spreads to the lymph nodes or distant organs, which is the case with stage III and stage IV CRC, respectively. Adding chemotherapy agent such as; fluorouracil, capecitabine or oxaliplatin increases life expectancy. If the lymph nodes do not contain cancer, the benefits of chemotherapy are controversial. If the cancer is wide metastatic or unresectable, treatment is then palliative and a number of different chemotherapy medications may be used. Chemotherapy for this condition may include capecitabine, fluorouracil, irinotecan, oxaliplatin and UFT (Tegafur+5-fluorouracil) (Morse, 2005). While a combination of radiation and chemotherapy may be useful for rectal cancer, its use in CRC is not routine due to the sensitivity of the bowels to radiation. Just as for chemotherapy, radiotherapy can be used in the neoadjuvant and adjuvant setting for some stages of rectal cancer (Morse, 2005).

1.7. Medicinal plants in colorectal cancer

Mankind has used plants to cure disease in all times and cultures, so it is not unexpected that 60% of the current drugs used in the treatment of cancer are derived from sources found in nature (Gordaliza, 2007). Indeed, ethnopharmacological studies have helped in the construction of databases of medicinal plants that are used as a source of molecules with antineoplastic activity. For instance, the well-known alkaloids vinblastine and vincristine (Srivastava *et al.*, 2005), paclitaxel, podophyllotoxin precursor of the drug etoposide, or camptothecin are derived from plants. All of the above are remarkable examples of successful findings in natural source for cancer treatment, thus justifying the search of new molecules in nature (Jacobo-Herrera *et al.*, 2016).

More specifically, different studies revealed that many plants have shown activity against CRC such as; *Atractylodes japonica*, *Poncirus trifoliate*, *Aronia melanocarpa* (Chokeberry), *Peucedanum japonicum* (Japanese common name: botan-bofu), *Carum carvi*, Willow bark (Salix species), Brassicaceae, *Withania somnifera*, *Ilex paraguariensis* (Mate tea), *Centaurea montana* (Asteraceae), and *Cynodon dactylon* (L.) Pers. In addition, there are CRC natural chemopreventive agents that inhibit the transformation of normal cells to premalignant cells or the progression of premalignant cells to malignant cells by modulating processes associated with xenobiotic biotransformation, along with the protection of cellular elements from oxidative

damage (Ansil *et al.*, 2014). For example, *Rheum ribes, Linum usitatissimum, Punica granatum, Cornus mas,* and *Vaccinium myrtillus* were found to be protective against CRC in particular (Akay and Yılmaz, 2018)

Likewise, *Khaya senegalensis* bark extract (KSBE) was hypothesized to contain inhibitors of the cyclooxygenase-2 (COX-2) gene and to be useful in the prevention and treatment of CRC. But according to a single study in the Republic of Guinea, KSBE suppressed the growth of human colon cancer (HCT-15) cells, suggesting that KSBE may exert some of its effects through COX-independent mechanisms. The growth inhibition effect of KSBE was associated with an up-regulation of peroxisome proliferator-activated receptor gamma and decreased expression of cyclin D1 (Androulakis *et al.*, 2006).

1.8. Overview of the experimental plant

Vernonia is a genus of about 1000 species in the family Asteraceae growing all over the world (Oketch-Rabah *et al.*, 1997). The genus is named after an English botanist William Vernon. The genus has some edible species and others are of 23 ecological value. *Vernonia auriculifera* Hiern (Asteraceae) (Figure 1) is commonly known with the vernacular name of "Reji" in Afan Oromo and "Aegidim" in Guragigna. It is a small tree or woody herb that grows between 1 and 7.5 meters high which is easily recognizable by its purple flowers and distributes/ed from Nigeria and Cameroon eastward to Ethiopia and southward to Angola and Tanzania. It occurs at 750-3000 m altitude in open grassland or at the margin of forests in mountainous areas. *V auriculifera* has a diversity of applications in traditional medicine. Meinit people in Ethiopia apply the root topically to treat toothache and also ethnobotanical study in Ethiopia revealed, leaves of the plant have nonspecific anti-cancer activity (Mirutse *et al.*, 2009; Esubalew *et al.*, 2017).

In Kenya, *V. auriculifera* is used in similar manners as *V. amygdalina* Delile (Hindmarsh, 1982). Traditionally, the Kikuyu people of central Kenya use the leaves of this plant as a wrap for pouched material used as a poultice (Kokwaro, 1976). In Tanzania, the root soaked in water was as purgative for children (Beentje, 2000). In Democratic Republic of Congo, a drop of juice from the crushed stem bark is instilled in each nostril to treat headache (Chifundera, 2001). Besides, leaf preparations are used in various countries against dysentery and stomachache. In Uganda, an infusion of the leaves is taken against worms and a leaf decoction is drunk for the treatment of malaria (Katende *et al*., 1995). Another study revealed that leaf extracts are drunk as

oxytocic and abortifacient and against post-partum pains (Hamill *et al*., 2000). Pulverized leaves are used against impetigo, and in Congo, the dried and pounded leaves are applied on wounds. While in Cameroon, the leaf juice is used as eye drops for the treatment of cataract. Moreover, triterpenoids isolated from *V. auriculifera* exhibit antimicrobial activity (Kiplimo *et al*., 2011).

Figure 1: Photograph of *Vernonia auriculifera* (Tarwish, 2015)

1.9. Rationale for the study

CRC is one of the most common cancers in the world, with over 1.2 million new cases diagnosed each year. Despite improvements in screening for early diagnosis, CRC remains one of the biggest cancer killers in the world and is responsible for over 600,000 deaths each year. Over the years, different approaches have been employed and are still in use, individually or in combination, in the treatment of cancer. Chemotherapeutic agents are cytotoxic and, apart from affecting tumor cells, these active principles also deleteriously impact on rapidly proliferating normal cells, including those localized in the gastrointestinal tract, hair, and bone marrow, thus eliciting gastrointestinal side effects like nausea and vomiting, alopecia, and myelosuppression. Although many side effects that occur during cancer treatment are temporary, some persist after treatment has ended (long-term effects) and others do not arise until several years later (late effects). For example, surgical patients and those treated with radiation are at increased risk of future bowel obstruction (Wang *et al.*, 2013)

The efficacy of cancer drugs is often limited by their insolubility and instability, the low rate at which the tissue absorbs them, and tumor's drug resistance. Antitumor drugs have also been

associated with the development of secondary malignancy (Akindele *et al.*, 2015b). All of the drawbacks presently associated with available chemotherapeutic agents are the impetus for the search for newer, more efficacious, and better-tolerated drugs. Plants have a long history of use in the prevention and treatment of cancer and the interest in nature as a source of potential chemotherapeutic agents continues (Henrique *et al.,* 2015). Accordingly, researches were focusing on the discovery of new chemopreventive and antineoplastic agents from natural products have been sustained by improvement in the science and technology of anticancer drug discovery. Once promising chemopreventive agents have been identified through observational and *in vivo* efficacy studies or have exhibited evidence of *in vitro* molecular targeting, these agents are then subjected to examination in clinical trials (Brown *et al.,* 2009).

Several epidemiological, clinical and preclinical studies to date have supported the chemopreventive potentials of many targeted drug classes including NSAID, statins and other natural agents both individually, and in combinations (Mohammed *et al.*, 2018). However, there are limitations for these agents that hinder their approval by the food and drug administration for chemoprevention use in high-risk individuals and in patients with early stages of CRC. The aim of this animal study was to evaluate chemopreventive effects of leaf extract of *V. auriculifera* Hiern (Asteraceae) which might eventually be given to humans to reduce their risk of CRC. This is due to evidence that the experimental plant showed ethnobotanical claim for different types of cancer (Esubalew *et al.*, 2017). Therefore, this study attempted to explore the cancerous cell proliferation suppressive activity of *V. auriculifera* leaf extract.

2. Objectives

2.1. General objective

- To evaluate the chemopreventive potential of the 80% leaf extract of *Vernonia auriculifera* in rat model of dimethylhydrazine-induced colorectal carcinogenesis.

2.2. Specific objectives

- To conduct an acute toxicity study of the extract.
- To assess the effect of the extract on body weight and growth rate of rats.
- To determine the effect of the extract on the number, size, and incidence of adenoma and adenocarcinoma produced by dimethylhydrazine.
- To evaluate histological variations of the colon tumor produced by dimethylhydrazine among the treatment groups of rats.
- To assess the effect of the extract on serum level of total protein, triglyceride, and total cholesterol in dimethylhydrazine-treated rats.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and reagents

Chemicals, drugs, and solvents used in this study included; 1, 2-Dimethylhydrazine dihydrochloride (TCI Chemicals, India), sodium hydroxide (Merck KGaA), distilled water, formalin (10%, Reagent chemical services limited)), diethyl ether (Pubchem, USA), methanol (Carlo Erba, France), normal isotonic saline (Aculife Healthcare, India) and, aspirin (Bayer Pharma, Germany).

3.1.2. Plant material

Vernonia auriculifera leaves were collected in April 2018 from Gilgel Gibe River area in western Oromia Region, approximately 450 km south of Addis Ababa, Ethiopia. The plant material was identified and authenticated by a taxonomist and a voucher specimen (Y001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia for future reference.

3.1.3. Experimental animals

Male Wistar Albino rats (6-7 weeks old, weighing 115-200 gm.) obtained from the animal house of School of Pharmacy, College of Health Sciences, Addis Ababa University were used. Sixty-three male Wistar rats were randomly kept in a group of 9 (7 rats of each group) in clean polypropylene cages with a wire mesh top and a hygienic bed of sawdust (regularly changed every 3 days), and maintained in a room with ambient temperature at 12 h light/dark cycle. The rats were acclimatized with the environment for one week before initiation of the experiment. All animal procedures were conducted in accordance with the standard guidelines for care and use of laboratory animals (Albus, 2012).

3.2. Methods

3.2.1. Extract preparation

The leaves of *V. auriculifera* were dried under shade and then, pulverized using a mortar and pestle to get a coarse powder used for the extraction. A portion of *V. auriculifera* leaf powder sample was weighed (1.5kg) and macerated for three consecutive days at room temperature in 80% methanol. The extraction procedure was repeated twice by adding another fresh solvent to

the marcs. The mixture was then filtered through Whatman no 1 filter paper (Whatman Ltd., England) & concentrated using Rotavapor (Buchilabortechnik AG, Switzerland) at 40°C under vacuum and the collected concentrated extract was freeze-dried using a lyophilizer (operon, Korea). The resulting extract was then transferred into a vial and kept in a desiccator layered with $CaCl_2$ during usage until the end of the experiment.

3.2.2. Acute toxicity study

The acute toxicity of the 80% methanol extract of *V. auriculifera* was performed using standard conventional procedures as described by OECD/OCDE 425 guideline (OECD, 2008). Fasted (over-night) female rats of 6-8 weeks age were used for the toxicity study. First, a sighting study was performed to determine the starting dose. For this, a single female rat was given 2000 mg/kg of the extract as a single dose by oral gavage. The rat was observed continuously for the first 30 min after administration of the test sample; intermittently every 4 h, over a period of 24 h. Death was not recorded with or without toxicity in the first 24 h, as a result, another 4 female rats were given similar doses and were observed for onset, duration, and severity of toxic signs like changes in physical appearance and behavior, lacrimation, loss of appetite, hair erection, salivation, diarrhea, motor and feeding activities, and other signs of acute toxicity and mortality within 14 days.

3.2.3. Preparation of carcinogen

DMH (2 mg/mL) was dissolved in normal saline solution (0.9% NaCl). The pH of the solution was adjusted to 7.0 with 1 mM NaOH to ensure the pH suitability and stability of the chemical. The final solution of the carcinogen was used immediately after preparation. DMH was injected intraperitoneal (i.p) at a dose of 20 mg/kg/body weight once a week for fifteen consecutive weeks on the ventral front of the animal (Kanna *et al*., 2003)

3.2.4. Grouping and dosing

After one week of acclimatization, sixty-three rats were randomly assigned into nine groups. DMH (20 mg/kg) was administered for 15 weeks (Kanna *et al*., 2003). The extract was administered at different doses prior (pre-initiation group) or following (post-initiation group) DMH administration. Group I rats served as normal control and received only distilled water (1 ml/100 g). Group II rats were considered as carcinogen control and received DMH (DMH20) injections i.p once a week for 15 consecutive weeks and followed for 25 weeks (Ravnik-Glavač *et al*., 2003). Group III, IV, and V rats constituted the pre-initiation group and received DMH

and *V. auriculifera* at a dose of 100 (PIV100), 200 (PIV200), 400 (PIV400) mg/kg/day orally respectively. Administration of *V. auriculifera* was started two weeks before the first DMH injection and continued until the end of the experiment. Group VI, VII, and VIII formed the post-initiation group and received DMH like the above mentioned groups and treated with *V. auriculifera* extract at a dose of 100 (POV100), 200 (POV200), 400 (POV400) mg/kg/day orally respectively, starting from two weeks later after the initiation of DMH injections and continued until the end of the experimental period. Group IX (standard control group) rats received DMH like the above mentioned groups and treated with aspirin at a dose of 60 mg/kg/day orally starting from the first day of DMH administration up to the end of the experiment. The total experimental period was 25 weeks.

The doses of the extract were selected based on the acute oral toxicity study. A middle dose (200 mg), which was one-tenth of the maximum dose during acute toxicity study (2 g/kg); a low dose (100 mg), which is half of the middle dose, and a high dose (400 mg), which was twice of the middle dose were taken.

3.2.5. Biochemical analysis

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anesthesia and blood samples were collected by cardiac puncture. Blood samples were allowed to clot for 30 min at room temperature and then centrifuged (4000 rpm) for 10 min. Serum was collected using micropipette and an aliquot was stored at −20°C using serum vial until the required analysis was performed. Total protein (gm/dl) was estimated by applying the colorimetric assay method (Lovrien and Matulis, 2004). The color intensity is directly proportional to the protein concentration. It was determined by measuring the increase in absorbance at 552 nm. Similarly, total cholesterol and triglyceride (mg/dl) were estimated according to the enzymatic colorimetric method (Biolabs, 2012; Cole *et al.*, 2013). All biochemical analyses were done in the Ethiopian Public Health Institute.

3.2.6. Body weight measurement

The body weight of all rats was measured at the beginning of the experiment, subsequently, once every week and finally before sacrificed (Ghadi, 2009). The respective percent weight increment and growth rate were calculated (see formulas below).

$$\text{Percent (\%) weight increment} = \frac{\text{(Final bodyweight-Initial body weight) x 100}}{\text{Initial body weight}}$$

$$\text{Growth rate} = \frac{\text{(Final bodyweight-Initial body weight)}}{\text{175 days}}$$

3.2.7. Colon tumor enumeration and histopathological examination

At the end of the experiment, the rats were sacrificed and their colons were removed and cut open along the longitudinal axis from the cecum to the end of the rectum and flushed with isotonic normal saline. The respective incidences, numbers, and sizes of tumors were calculated (see formulas below) and recorded. Then, the colons were fixed in 10% neutral buffered formalin and embedded in paraffin (Aranganathan and Nalini, 2009). Later, each polyp (tumor) were taken and stained with hematoxylin and eosin (H&E) for histological analysis (Banchroft *et al*., 2008)). Slides reading and interpretation was performed by a blinded pathologist from the Department of Pathology, School of medicine, Addis Ababa University. Finally, the colon tumors were classified in to tubular, tubulovillous and villous adenoma as well as invasive adenocarcinoma according to morphology, the extent of invasion, and differentiation.

$$\text{Tumor incidence (\%)} = \frac{\text{Number of tumor bearing rats}}{\text{number of tested rats}} \text{ x 100}$$

$$\text{Average tumor number} = \frac{\text{Total number of tumors}}{\text{number of tested rats}}$$

$$\text{Tumor size} = [\text{Maximal length X (perpendicular width) 2/2}]$$

4. Statistical analysis

All statistical analyses were performed using the statistical software for social science (SPSS), version 25 for Windows (SPSS Inc, Chicago, Illinois, USA). The results were expressed as mean ± standard error of the mean (SEM) for each group. Statistical differences between groups were analyzed by One-way analysis of variance (ANOVA) using Tukey as a post hoc test. Statistical significance was declared when $P < 0.05$.

5. Result

5.1. Acute toxicity test

According to the acute toxicity test conducted to determine the safety of the crude extract of *V. auriculifera*, the lethal dose for the crude extract was found to be above 2000 mg/kg. The test substance that was administered orally in a single dose of 2000 mg/kg to the laboratory Wistar Albino rats caused no mortality within the first 24 h as well as for the following 14 days of the observation period. In addition, the gross behavioral and physical observation of the experimental rats revealed that the extract caused no visible signs of acute toxicity such as lacrimation, loss of appetite, hair erection, salivation, and diarrhea as well as reduction in motor and feeding activities.

5.2. Induction of colorectal cancer

Most rats in the experimental groups had tolerated i.p injections of DMH as well as oral administration of *V. auriculifera* and aspirin. However, out of sixty-three rats, nine died due to administration error and injection site infection. As a result, fifty four rats were sacrificed at the end of the experiment to investigate the effects of DMH, extract, and aspirin. The effect of DMH on change in body weight, growth rate, average number and size of tumor is presented in Table 1.

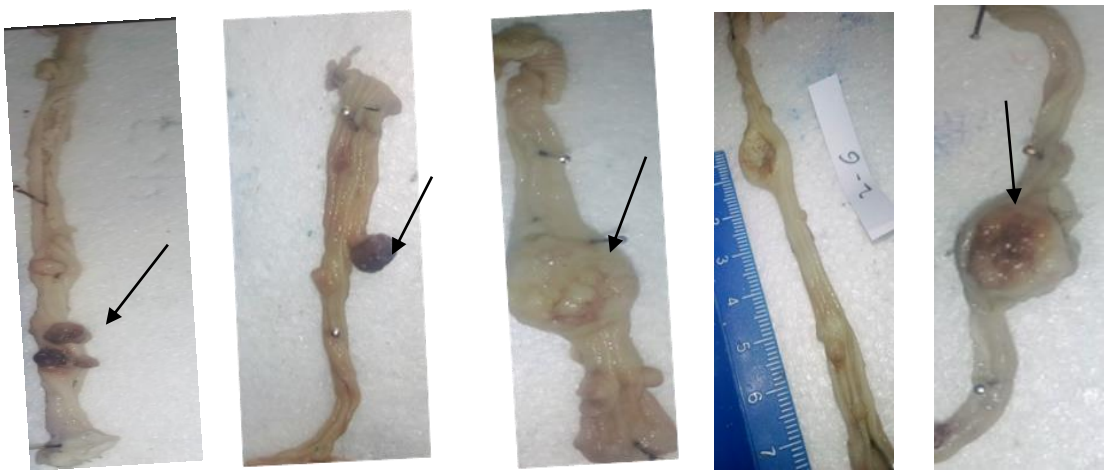
Table 1: Parameters showing induction of colorectal cancer by dimethylhydrazine after 25 weeks

Parameters	Groups	
	Normal Control (n=6)	**DMH20 (n=6)**
% Weight increment	180.62 ± 18.44	91.47 ± 10.84**
Growth rate	1.29 ± 0.13	0.71 ± 0.07**
Average no.of tumor	0.00	4.00 ± 0.52***
Tumor size (mm3)	-	125.00 ± 34.33***
Tumor incidence (%)	0.00	100
TG (mg/dl)	59.82 ± 4.25	89.25 ± 8.0**
TCHOL (mg/dl)	55.57 ± 1.24	71.43 ± 5.84*
TP (mg/dl)	6.54 ± 0.08	5.60 ± 0.14*

Data are expressed as mean ± SEM (n=6); Analysis was performed by one-way ANOVA; NC, normal control without dimethyl hydrazine; DMH20, the carcinogenic group taking dimethyl hydrazine; TG, triglyceride; TCHOL, total cholesterol; TP, total protein. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Body weight increment and growth rate of DMH20 were significantly ($p < 0.01$) lower than rats in the normal control group. In addition, the average number and size of tumor produced in DMH treated rats were significantly higher ($p < 0.001$) and the tumor incidence was found to be 100% (Table 1). Representative macroscopic view of tumors induced is presented in Figure 2.

Besides, in DMH20 rats, there was a significant reduction in serum concentration of total protein ($p < 0.01$) as compared to normal control. On the other hand, an increase in serum concentration of triglyceride ($p < 0.05$) and total cholesterol ($p < 0.05$) was recognized in DMH20 rats (Table 1).



A.

B.

Figure 2: Representative macroscopic view of the colorectal tumors; A. DMH treated group; B. normal control.

5.3. Effect of the extract on number and size of tumors

At the end of the experimental period, a total of 30 and 41 tumors were examined from the pre-initiation and post-initiation treatment groups, respectively (Table 2). But, only 2 tumors were identified in aspirin treated group. Treatment initiated with middle and high doses of the extract produced significant ($p < 0.05$) reduction of average tumor size as compared to DMH20 in both pre and post-initiation groups. Likewise, the mean tumor number of PIV400 and POV400 showed a significant reduction ($p < 0.05$) than DMH20. On the other hand, no significant change

in number and size of tumors was observed between the extract and standard in both phases. Similarly, statisticaly significant difference were not detected between pre and post-initiation regimens. Furthermore, aspirin treated rats exhibited a substantial decrement ($p < 0.05$) of the average number and size of tumor in comparison with DMH20 (Table 2).

Table 2: Effect of *Vernonia auriculifera* on the number and size of tumors in colon and rectum of dimethylhydrazine-treated rats

Groups	No. rats examined	No. rats with tumor	Total no. of Tumor	Average no-of tumor	Tumor size (mm^3)
DMH20	6	6	26	4.33 ± 0.42	138.33 ± 26.25
ASP60	6	2	2	$0.33 \pm 0.21^{d*}$	$29.50 \pm 18.66^{d*}$
Pre-initiation phase					
PIV100	6	4	14	2.33 ± 0.84	42.83 ± 20.56
PIV200	6	4	12	2.00 ± 0.63	$34.17 \pm 10.98^{b*}$
PIV400	6	3	4	$0.67 \pm 0.33^{b*}$	$35.00 \pm 21.60^{b*}$
Post-initiation phase					
POV100	6	4	24	3.33 ±1.67	46.67 ± 15.84
POV200	6	4	13	2.17 ± 0.91	$35.00 \pm 11.97^{b*}$
POV400	6	4	4	$0.67 \pm 0.33^{b*}$	$36.66 \pm 16.5^{b*}$

Data are expressed as Mean ± SEM (n=6); Analysis was performed by one-way ANOVA; b: Extract versus DMH control; c: Extract versus Standard; d: Standard versus DMH control; NC, normal control without dimethyl hydrazine; DMH20, carcinogenic group taking 20mg/kg dimethyl hydrazine; ASP60, aspirin 60mg/kg; PIV and POV, pre-initiation and post initiation *V. auriculifera* at 100, 200, and 400mg/kg respectively. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

5.4. Effect of the extract on tumor incidence

Treatment with the extract was associated with a decrease in tumor incidence and maximum the lowest tumor incidence was obtained in group of rats treated with aspirin (33%) (Table 3). Treatment initiated with a high dose of the extract before DMH administration produced a higher reduction (50%) compared to other doses as well as the post-initiation regimen.

Table 3: Effect of *Vernonia auriculifera* on tumor incidence in dimethylhydrazine-treated rats

Groups	No. rats examined	No. rats with tumor	Tumor incidence (%) No. rats with tumor/rats examined
DMH20	6	6	100
ASP60	6	2	33.33
Pre-initiation phase			
PIV100	6	4	66.67
PIV200	6	4	66.67
PIV400	6	3	50
Post-initiation phase			
POV100	6	4	66.67
POV200	6	4	66.67
POV400	6	4	66.67

NC, normal control without dimethyl hydrazine; DMH20, carcinogenic group taking 20mg/kg dimethyl hydrazine; ASP60, aspirin 60mg/kg; PIV and POV, pre-initiation and post-initiation *V. auriculifera* at 100,200, and 400mg/kg respectively.

5.5. Effect of the extract on change in body weight and growth rate

Body weight of the rats in all groups increased gradually during the 25 weeks experimental period (Table 4). Accordingly, from week 0 to 25 variable changes were observed in weight increment and growth rate of rats assigned in all groups. Weight increment in the DMH20 group was significantly lower ($p < 0.01$) than the normal control group. Treatment with aspirin increased the weight of rats by 120 % ($p < 0.05$). Percent weight increment of rats` in the pre-initiation groups was not significantly different from DMH20 rats, except with the highest dose, where the difference reached statistical significance ($p < 0.05$). Low and middle dose of the extract produced significantly lower body weight increment compared to the normal control. However, no significant difference in weight increment was observed between all pre-initiation groups and the standard. On the other hand, weight increment in post-initiation groups tended to be lower than the pre-initiation groups. Statistically significant weight change difference was not recorded in post-initiation groups as compared to DMH20. Regardless of the dose, treatment with the extract produced lower percent weight increment as compared to the normal control ($p <$

0.01). Moreover, no significant change was obtained between the post-initiation regimens and the standard.

In terms of change in growth rate, both pre- and post-initiation treatment didn't bring a significant change as compared to DMH20. However, in comparison with normal control, treatment with low and medium doses of the extract showed a significant reduction of growth rate in both phases ($p < 0.01$ for pre-initiation regimen and $p < 0.05$ for the post-initiation regimen). Unlike the pre-initiation phase, treatment with high dose in the post-initiation phase didn't sustain growth rate increment ($p < 0.05$) as compared to the normal control. On the other hand, treatment with the extract in both phases brought no significant change in growth rate ($p < 0.05$) as compared to the standard (Table 4). Moreover, the difference in growth rate between pre and post-initiation regimens was not statistically significant.

Table 4: Effect of *Vernonia auriculifera* on body weight increment and growth rate of rats with dimethylhydrazine induced colorectal cancer

Groups	% Weight increment	Growth rate
NC	180.62 ± 18.44	1.27 ± 0.13
DMH20	91.47 ± 10.84	0.67 ± 0.08
ASP60	120.00 ± 13.26^{d*}	1.01 ± 0.08
Pre-initiation phase		
PIV100	109.33 ± 11.28 ^{a*}	0.84 ± 0.07^{a*}
PIV200	107.52 ± 4.06 ^{a*}	0.83 ± 0.03^{a*}
PIV400	123.80 ± 7.26 ^{b*}	0.99 ± .03
Post-initiation phase		
POV100	96.56 ± 11.23 ^{a**}	0.78 ± 0.12^{a**}
POV200	92.32 ± 18.08 ^{a**}	0.75 ± 0.14 ^{a**}
POV400	102.26 ± 10.92^{a**}	0 .79 ± 0.064^{a*}

Data are expressed as Mean ± SEM (n=6); Analysis was performed by one-way ANOVA; a: Extract versus normal control; b: Extract versus DMH control; c: Extract versus Standard; d: Standard versus DMH control; NC, normal control without dimethyl hydrazine; DMH20, carcinogenic group taking 20mg/kg dimethyl hydrazine; ASP60, aspirin 60mg/kg; PIV and POV, pre-initiation and post initiation *V. auriculifera* at 100, 200, and 400mg/kg respectively. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

5.6. Effect on morphologic pathology

Histopathological analysis showed that, out of the total tumors, 41 were assigned to tubular adenoma, 5 to villous adenoma, 17 to tubulovillous adenoma, and 36 tumors to invasive adenocarcinoma. Table 5 summarizes tumor classification of each treatment group. DMH20 produced 5 tubular adenomas and 21 invasive adenocarcinomas. The number of invasive adenocarcinomas decreased in rats treated with the extract. However, more reduction was obtained by treatment in the pre-initiation than the post-initiation phase (4 vs. 11). In addition, no villous adenomas were presented in the pre-initiation groups, while 3 found in post-initiation groups. Furthermore, no tubulovillous adenoma was presented in treatment with high dose of the extract in both phases. However, treatment with a low and medium doses of the extract in post-initiation phase demonstrated more reduction than in pre-initiation phase (5 vs 12).

Table 5: Effect of *Vernonia auriculifera* on total tumor number in dimethylhydrazine-treated rats based on the tumor classifications

Groups	Tubular adenoma	Villous adenoma	Tubulovillous adenoma	Invasive adenocarcinoma
DMH20	5	-	-	21
ASP60	2	-	-	-
Pre-initiation phase				
PIV100	5	-	5	4
PIV200	5	-	7	-
PIV400	4	-	-	-
Post-initiation phase				
POV100	8	2	2	10
POV200	9	-	3	1
POV400	3	3	-	-
Total	41	5	17	36

NC, normal control without dimethyl hydrazine; DMH20, carcinogenic group taking 20mg/kg dimethyl hydrazine; ASP60, aspirin 60mg/kg; PIV and POV, pre-initiation and post-initiation *V. auriculifera* at 100,200, and 400mg/kg respectively.

Histopathological variations among the colon and rectum of different treatment groups were examined under a light microscope in high power (40X) magnification. The photomicrographs of the rat's colorectal tumor section histology displayed diverse histological architectures among different experimental groups (Figure. 3).

The colon and rectum of control rats showed normal glands with normal mucosal and sub-mucosal layers and typical colonic architecture with no signs of apparent abnormality (Figure. 3a). There was also normal proportion of glands lined by regular cells with scattered goblet cells. By contrast in DMH20 rats, histopathological analysis revealed not only the presence of colorectal tumors but also histological features of invasive adenocarcinoma with invasive single cells and glands made of highly malignant epithelial cells dissecting muscle proper (Figure. 3b). On the other hand, representative colon tumors taken from PIV100 (Figure. 3c) rats were found to be tubulovillous with villous and tubular components are clearly observed and lined by regular epithelial cells while in and PIV200 (Figure. 3d), villous adenoma having a villous projections from mucosa which is lined by regular epithelial cells was detected.

A tubular adenoma with no evidence of invasion in the lamina propria and sub-mucosa of PIV400 (Figure. 3e) was also identified. Colorectal tumor taken from rat of POV100 (Figure. 3f) group was confirmed to be an invasive carcinoma with proliferation of highly pleomorphic epithelial cells having dysplastic nuclei forming glandular structures and invading the sub-mucosa. Similarly, tumor sectioned from group POV200 rat was confirmed to be carcinoma with invasive glandular structures in muscle proper lined by pleomorphic epithelial cells with frequent mitotic activity (Figure. 3g). Moreover, a pedunculated polyp, gray-brown was taken from group POV400 rat and after microscopic examination; this was confirmed to be a tubular adenoma with branching tubules embedded in the lamina propria and rounded structures in cut cross section (Figure. 3h). Finally, tumor taken from group ASP60 rat was confirmed to be tubular adenoma with increased number of glands with few villous components (Figure. 3i).

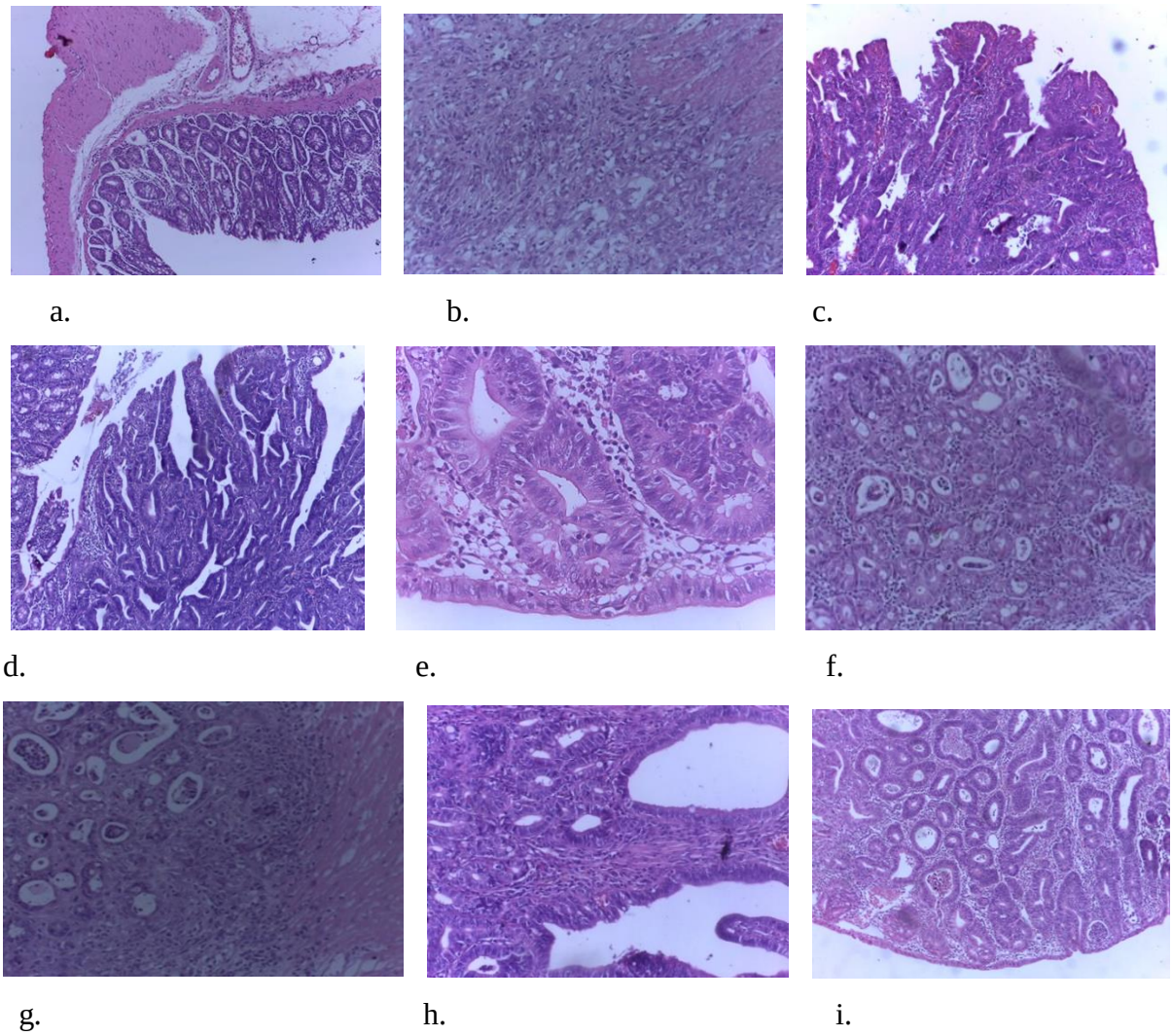


Figure 3: Photomicrographs showing hematoxylin and eosin stained colorectal tumor pathological sections in dimethylhydrazine induced colorectal tumorigenesis in rats treated with *Vernonia auriculifera* ; **a:** NC; b: DMH20; c: PIV100; d: PIV200; e: PIV400; f: POV100; g: POV200; h: POV400; i: ASP60

5.7. Effect of the extract on biochemical parameters

The level of biochemical parameters among the different treatment groups was measured at the end of the study period and presented in Table 6. An increase in total cholesterol ($p < 0.05$) and triglycerides ($p < 0.01$) as well as a reduction in total protein ($p < 0.05$) were observed in DMH20 compared to normal rats. Aspirin produced a significant reduction of total cholesterol ($p < 0.05$) and triglycerides ($p < 0.01$), but couldn`t produce significant change in total protein. Treatment with the extract exhibited variable response depending on the parameter measured. Triglycerides were significantly decreased by all doses ($p < 0.05$) in the pre-initiation phase, but only the medium and larger doses were capable of reducing triglycerides in the post-initiation

phase ($p < 0.05$). Total cholesterol, on the other hand, was significantly reduced only by PIV400 ($p < 0.05$). The extract, regardless of the dose, had effect on total protein neither in pre-initiation nor in the post-initiation phase. Total cholesterol ($p < 0.01$) and triglycerides ($p < 0.05$) were significantly reduced by aspirin as compared to DMH20. Total protein, on the other hand, was not significantly changed by aspirin. Total cholesterol and triglycerides in extract treated rats were not significantly different from rats in the normal control. Whereas, treatment with low ($p < 0.01$) and middle ($p < 0.05$) dose regimens in post- but not in pre-initiation phase didn`t protect reduction of total protein. The levels of all the three parameters in extract treated rats were not significantly different from aspirin treated rats. Furthermore, there were no significant changes in total cholesterol, triglycerides and total protein level between the pre- and post-initiation regimens.

Table 6: Effect of *Vernonia auriculifera* on serum biochemical parameters in dimethylhydrazine-induced colorectal cancer

Groups	TG (mg/dl)	TCHOL (mg/dl)	TP (mg/dl)
NC	59.82 ± 4.25	55.57 ± 1.24	6.54 ± 0.08
DMH20	89.25 ± 8.0	71.43 ± 5.84	5.60 ± 0.14
ASP60	61.33 ± 5.05 ^{d**}	55.82 ± 0.84^{d*}	6.27 ± 0.22
Pre-initiation phase			
PIV100	62.73 ± 1.62 ^{b*}	58.35 ± 1.27	5.75 ± 0.17
PIV200	63.20 ± 4.83 ^{b*}	59.95 ± 1.77	5.85 ± 0.14
PIV400	60.00 ± 2.68 ^{b**}	57.33 ± 4.07 ^{b*}	6.10 ± 0.16
Post-initiation phase			
POV100	67.97 ± 2.82	63.52 ± 3.22	5.56 ± 0.22^{a**}
POV200	65.55 ± 6.36 ^{b*}	61.20 ± 2.05	5.63 ± 0.20^{a*}
POV400	63.77± 6.43 ^{b*}	58.72 ± 2.69	5.87 ±0.20

Data are expressed as Mean ± SEM (n=6); Analysis was performed by one-way ANOVA; a: Extract versus normal control; b: Extract versus DMH control; c: Extract versus Standard; d: Standard versus DMH control; NC, normal control without dimethyl hydrazine; DMH20, carcinogenic group taking 20mg/kg dimethyl hydrazine; ASP60, aspirin 60mg/kg; PIV and POV, pre-initiation and post initiation *V. auriculifera* at 100, 200, and 400mg/kg respectively. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

6. Discussion

The development of CRC involves three distinct stages: initiation, promotion and progression phases. In the initiation phase, there is an alteration of the molecular message of a normal cell while in promotion and progression phases a phenotypically altered transformed malignant cell is usually generated (Suwannalert *et al.,* 2016). Animal model of DMH-induced colorectal carcinogenesis is widely applicable to study the pathogenesis of CRC and chemopreventive effects of different agents. Accordingly, in chemoprevention studies using this model has several advantages. First, the evolution of colorectal tumors in the DMH model is similar to that in humans, including the progression of ACF to adenomas (often polyps), and ultimately carcinomas. Second, the histopathological features of DMH-induced colorectal tumors are similar to those of human tumors. Finally, 30–60% of DMH-induced colorectal tumors possess K-RAS mutations as seen in human colorectal tumors (Chang, 1997).

Chemoprevention involves prolonged administration of a synthetic, natural or biological agent to reduce or delay the occurrence of malignancy (Steward, 2013). For instance, factors in the Asian diet such as tea, spices, and medicinal herbs are being screened for potential use as cancer chemopreventive in man and showed promising effects. There is also a continuous interest in systematically testing ancient medicinal herbs for this activity (Wargovich, 2001). In the present study, the induction of CRC by DMH was performed and the chemopreventive effect of *V. auriculifera* was conducted in two phases i.e. pre-initiation and post-initiation phases.

Administration of DMH caused a significant increase in number, size, and incidence of tumors as compared to the normal control. DMH is known to produce an active carcinogenic metabolite AOM and methylazoxymethanol in the liver, which further is transported to the colon and rectum to generate free radical producing metabolite, diazonium ion, that induces oxidative DNA damage in the colon (Fiala, 1977). The diazonium ion elicits oxidative stress by methylating biomolecules of colonic epithelial cells and leads to promutagenic events as a result of inflammation and tumor promotion (Bird, 1995).

In the pre-initiation phase, the extract was administered for two weeks before the initiation of DMH in order to assess its repeated dose safety profile in rats. Administration of the extract continued until the end of the experiment to investigate its inhibitory effect upon the

entire period of administration on DMH-induced colorectal carcinogenesis. On the other hand, in the post-initiation phase, the extract was initiated followed by two doses of DMH administration to assess its ability to slow down progression of the disease after development of the preneoplastic lesions, as it is known that administration of DMH during this period can induce the formation of preneoplastic lesions (Bird, 2018). In this phase, it was aimed to explore the effect of the extract on the growth of preneoplastic lesions to neoplastic lesions by determining the number, size and incidence of tumors, as well as the degree of progression to aggressive invasive adenocarcinoma.

Both in pre and post-initiation groups, high dose treatment with the extract produced comparable reduction ($p < 0.05$) of the average number of tumor. The average size of tumor also significantly decreased in rats treated with medium and high doses of the extract ($p < 0.05$) (Table 3). However, the mean tumor size was lower in pre-initiation groups than post initiation groups. This suggests that the extract could inhibit the growth of colorectal tumor in the early stage.

Reduction of tumor number and size is commonly associated with anti-oxidative and anti-inflammatory properties, induction of phase II enzymes, cell cycle arrest, apoptosis, autophagy, and changes in gut microbiota by the chemopreventive agents (Zhao *et al.*, 2018). Plants classified under the genus *Vernona* exhibit chemopreventive properties (Thomas *et al*., 2016). This is attributed to their abilities to scavenge free radicals, induce detoxification, inhibit stress response proteins and interfere with DNA binding activities of some transcription factors (Farombi and Owoeye, 2011). Study revealed that *V.auriculifera* contains tannins, flavonoids, phlobatannins, terpenoids, and saponins (Albejo *et al.*, 2015).

Flavonoids act as antioxidants, scavengers of a wide range of reactive oxygen species, and inhibitors of lipid peroxidation (Ammar *et al*., 2009). Flavonoids and other phenolic compounds might also exert direct protective effects on the gastrointestinal tract by scavenging reactive species and/or preventing their formation. Polyphenols can inhibit hemeprotein-induced peroxidation in the stomach and decrease DNA base deamination or nitrosamine formation by nitrous acid-derived reactive nitrogen species (Halliwell B, 2007). Furthermore, the methanol extracts of *V.auriculifera* are shown to contain triterpenoids (Kiplimo *et al.*, 2011) and a large number of triterpenoids are known to exhibit cytotoxicity against a variety of tumor cells as well as anticancer efficacy in preclinical animal models commonly due to their potent anti-inflammatory effects (Leake, 2014).

One of the species from the genus *Vernonia*, *V. amygdalina* could mitigate cycasin-induced oxidative damage in colonic tissues (Lolodi and Eriyamremu, 2013). Moreover, supplementation with *V. amygdalina* leaves to rats exposed to N-Methyl-N-Nitrosurea, showed a decrease in carcinoembryonic antigen level (a biomarker for CRC), moderate necrosis of colon epithelium and increased superoxide dismutase level (Farombi, 2014). The anti-inflammatory and anticancer effects of flavonoids from this plant are indispensable in the human body (Kumar *et al*, 2013). As a result, this reinforces the results of this study and may suggest that there might be similar constituents found in these two species.

The standard agent aspirin also produced a significant ($p < 0.05$) reduction of the average number and size of the tumor compared to untreated group. This result sustained the study of randomized controlled trial on CRC prevention which revealed treatment with aspirin significantly reduced polyp number and size (Burn *et al.*, 2011). Mechanistically, aspirin effects may be by inhibiting an elevated COX-1&2 enzymes activity and Wnt/β-catenin signaling pathway (Sheng *et al.*, 1998).

Early changes within the colonic epithelium that persist throughout tumor genesis represent ideal targets for chemopreventive intervention (Tanaka, 2009). As a result, pre-initiation high dose showed a lower incidence of tumors (50%) as compared to other doses and post-initiation regimens. This might be attributed to the collective effects of early treatment initiation and the presence of a higher concentration of phytochemicals within this dose of the extract. While rats from pre-initiation phase which received low and medium doses of the extract exhibited similar tumor incidence with post-initiation regimens, even though the average number and size of tumor between the rats in each group were different. This might be associated with a low concentration of phytochemicals and late initiation of the treatment. Rats treated with aspirin produced the lowest incidence of colorectal tumor. This finding was supported by the previous study. Aspirin therapy has reduced CRC incidence and perhaps mortality (Chubak *et al.*, 2016).

From histopathological observation, oral administration of *V. auriculifera* leaf extract further suppressed the progression of adenoma to invasive carcinoma in the pre-initiation phase than in post-initiation (4 vs 11). Likewise, no invasive adenocarcinoma was presented in medium and high doses of pre-initiation regimens. On the other hand, in the post-initiation phase treatment with a medium dose of the extract exhibited a single invasive adenocarcinoma. A low dose of the extract was presented a greater number of invasive adenocarcinoma in the

post-initiation phase, but with a lower number of villous and tubulovillous adenomas which have a higher risk of malignant transformation than tubular adenomas. This is attributed to the suppressive effect of phytochemicals on tumor progression when the extract was given throughout the experimental period. This assertion is further reinforced by a study; supplementation of *Amorphophallus campanulatus* (Roxb.) Blume tuber during the post-initiation and entire period of the study significantly suppressed colonic neoplastic progression. But, the entire period treatment regimen was found to be the most effective treatment approach (Ansil *et al.*, 2014).

On the other hand, colorectal tumor section of rats treated with aspirin showed, only a few benign tubular adenomas after histopathological microscopic examinations. Low-dose aspirin therapy is known to reduce risk of Dukes stages B–D CRC, suggesting a role for low-dose aspirin in the progression of established CRC; a substantial reduction in the risk of Dukes A CRC (Garcea *et al*., 2017). It may be through blockage of the COX enzyme. Because, blockage of COX enzyme suppresses the eicosanoid production of prostaglandins that affect the cell proliferation, tumor growth and immune responsiveness (Sheng *et al.*, 1998). In addition, the anti-inflammatory efficacy of NSAID had the potential to reduce the progression of preneoplastic cells to adenocarcinomas (Higuchi *et al.*, 2007).

Moreover, the body weight increment in DMH20 was found to be significantly lower as compared to the normal control group ($p < 0.01$) at the end of the experiment, which might be due to induction of cancer by DMH. DMH induced decreased weight increment or inability to sustain normal body weight in rats showed a prognosis in CRCs growth (Roshni, 2013). Since weight loss is an important prognostic factor in cancer; the higher the extent of weight loss, the shorter the survival time (Al-Henhena, 2014).

Body weight increment was more prominent with pre-initiation than post-initiation groups. Maximum body weight increment obtained in PIV400. Thus, the presence of greater percent body weight increment upon administration of *V. auriculifera* observed in this group, emphasizes early treatment with a higher dose of the extract had chemopreventive potential against DMH induced CRC. This might be due to the higher concentration of the active components in the high dose of the extract given and prior initiation of the extract before the tumurogenesis was progressed. On the other hand, low and medium doses of the pre-initiation phase ($p < 0.05$) and all doses of post-initiation ($p < 0.01$) groups showed lower

percent weight increment as compared to the normal control. This reduced percent weight increment may be due to the tumor burden in the colon and rectum, which in turn leads to decreased food intake. From this, one can conclude that late administration of the extract couldn't significantly suppress the proliferation of cancer cells and their development to a tumor. Since body weight loss is an indicative parameter of CRC progression (Al-Henhena, 2014).

In addition, significantly ($p < 0.01$) reduced growth rate observed in DMH exposed rats as compared to the normal control might be another indicative parameter of the occurrence of tumors in the colonic tract. In the pre-initiation phase, PIV400 showed no comparable change in growth rate as compared to the normal control. This has not occurred in post-initiation high dose and rather exhibited significant reduction ($p < 0.05$). This implies early treatment with the extract exhibited a better effect on maintaining normal growth rate and inhibition of tumor development than late initiation. On the other hand, despite the difference in significant levels low and medium doses of the extract in pre ($p < 0.01$) and post-initiation ($p < 0.05$) phases showed significant growth rate reduction as compared to normal control. This might be due to the presence of low level of phytochemicals in these doses of the extract. However, rats in both phases which received the extract at the three dose levels didn`t show any significant ($p < 0.05$) change in growth rate as compared to both DMH20 and standard control.

Furthermore, biochemical measurements were carried out in sera of different treatment groups. The result indicated that serum levels of cholesterol and triglyceride in DMH20 were significantly elevated in comparison with the normal control ($p < 0.05$) and aspirin-treated ($p < 0.01$) rats. This is because DMH treatment results in doubling of the biological membrane and gross distortion of cell shape with changes in lipid composition, membrane fluidity, and morphology (Cooper *et al*., 1980). In addition, the increased cholesterol content in the serum of DMH treated rats might be associated with one of the properties of malignant cells. Colorectal polyps were significantly associated with an increased total cholesterol and triglycerides levels and an elevated serum triglyceride concentration is positively associated with bile acid synthesis (Haggar and Boushey, 2009; Byun *et al.*, 2010; Yang *et al*., 2013). Besides, serum triglyceride and fecal bile acids may be biologically related to each other (Åkerlund *et al.*, 1994). Both may promote carcinogenesis in the large intestine.

Moreover, the other likely mechanisms of the relationship between colorectal polyps and serum lipids might explain these findings. Primarily, hypercholesterolemia has a strong association with hyperinsulinemia and insulin resistance (Leake, 2014). Hyperinsulinemia and insulin resistance also can induce CRC risks (Laws and Reaven, 1992). Secondly, serum triglyceride concentration may be positively associated with bile acid synthesis and fecal bile acids. An increase in synthesized and secreted bile acids may provide abundant substrates for the formation of secondary bile acids and promote carcinogenesis in the large bowel (Borena *et al.*, 2011).

Serum levels of cholesterol in PIV400 and triglyceride in all pre initiation groups were lower than those measured in DMH20. However, no significant reduction in total cholesterol was obtained from post-initiation groups. POV200 and POV400 showed a significant effect on triglyceride. This suggests that *V. auriculifera* had the potential to inhibit serum cholesterol and triglyceride production and thereby can exert a negative impact on cell proliferation when it was given early. Accordingly, it could be the suppressive effect of *V.auriculifera* on serum cholesterol and triglyceride production, which may contribute to the chemopreventive and tumor growth inhibitory effects in this animal model of colon carcinogenesis.

Protein loss is usually associated with most cancer and may justify DMH induced decrease in serum total protein in this finding. This could be attributed to increased protein degradation and /or decreased protein synthesis by the carcinogenic agent. In support of this finding, a variety of metabolic disorders were related to the occurrence and development of different kinds of malignant tumor and CRC (Reeves *et al.*, 2007). In comparison with rats given DMH alone, those received the extract in pre- and post-initiation phases as well as aspirinhave shown an increased level of serum total protein. This suggests that DMH may have more apparent metabolic changes and treatments with *V. auriculifera* decreases these metabolic alterations.

7. Conclusion

In summary, the salient finding emerging from this study is that the 80 % methanol extract of *V. auriculifera* leaves in the pre-initiation phase presented better chemopreventive efficacy against DMH-induced colon carcinogenesis than the post-initiation phase. Particularly, the maximum effect was obtained from high dose of the extract in this phase. Results from this study also showed that early initiation of *V. auriculifera* treatment demonstrated a significant delaying of progression, restriction of invasion and attenuation of aggressiveness of colon tumors. Moreover, *V. auriculifera* had the potential to inhibit serum cholesterol and triglyceride production and prevent serum protein level reduction and thereby can exert a suppressive effect of malignant cell proliferation. Thus, this might have contributed to the chemopreventive and tumor growth inhibitory effects of the plant.

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8. Suggestions for future work

Based on the present study the following suggestions are proposed:

- *In vitro* and *in vivo* anti-CRC effects of the extract on other models should be performed to estimate its tumor cell proliferation suppressive effects.
- Further experiments like, mechanistic studies are necessary to fully evaluate its cancer preventive properties and to understand its mode of action.
- Isolation and chemical characterization of the major compounds that contribute to the suppression of inflammation, scavenging free radicals, altering transcriptional factors, and inhibition of carcinogenesis are needed.

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