THE EFFECT OF *Phyllanthus amarus* LEAF ON APLOPLOTIC AND CYTOKINE ACTIVITY IN DMH INDUCED COLON CARVINOMIA IN SWISS ALBINO RATS

BY

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CERTIFICATION

We the undersigned certify that this undergraduate project work was carried out and the report was presented by **AGBONMWANRE JULIUS IYOBOSA**with matriculation number **LSC2103694**, to the Department of Biochemistry, Faculty of Life Science, University of Benin, in partial fulfillment of the requirements for the award of Bachelor of Science (B.Sc.) degree in Biochemistry.

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DEDICATION

This work is dedicated to the Almighty God for His goodness and mercy upon my life, my amazing parents Mr and Mrs AGBONMWANRE, my siblings, loved ones and friends for their love and support

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My deepest appreciation goes to God almighty, whose strength kept me throughout this university journey. Special appreciation also goes to my project supervisor DR. F.O. OMOREGIE for his guidance, support, counsel and patience throughout the research and report.

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ABSTRACT

This study investigates the effect of Phyllanthus amarus leaf extract on apoptotic and cytokine activity in 1,2-dimethylhydrazine (DMH)-induced colon carcinoma in Swiss albino rats. Colon cancer remains a major global health burden, and the exploration of plant-derived bioactive compounds for safer chemoprevention has gained increasing attention. Phyllanthus amarus, a medicinal plant known for its antioxidant, anti-inflammatory, and anticancer properties, was evaluated for its potential to mitigate colon carcinogenesis. Colon cancer was induced in rats through subcutaneous administration of DMH (20 mg/kg body weight) once weekly for 10 weeks. Experimental groups received oral doses of ethanol leaf extract of P. amarus at concentrations of 250, 350 and 450 mg/kg body weight throughout the treatment period. Biochemical, histological, and molecular assessments were performed to determine oxidative stress status, apoptotic activity, and cytokine modulation. Treatment with P. amarus significantly decreased oxidative stress by enhancing the activities of antioxidant enzymes such as superoxide dismutase and catalase, and reducing lipid peroxidation levels. Histopathological

analysis revealed restoration of normal colon tissue architecture and reduced dysplasia in treated rats compared to the DMH control group. Molecular findings showed that P. amarus extract enhanced apoptosis by upregulating the expression of caspase-3 and caspase-9, while downregulating the anti-apoptotic protein Bcl-2. Furthermore, cytokine assays demonstrated that P. amarus modulated inflammatory signaling by lowering pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and elevating the anti-inflammatory cytokine IL-10. These outcomes indicate that P. amarus confers chemoprotective effects through suppression of oxidative damage, activation of the intrinsic apoptotic pathway via caspase-9 and caspase-3, and regulation of cytokine balance.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background and Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide and a leading cause of cancer-related morbidity and mortality. It arises from the uncontrolled proliferation of epithelial cells lining the colon and rectum, progressing through stages of hyperplasia, adenoma, and carcinoma. The pathogenesis of CRC is multifactorial, involving genetic mutations, chronic inflammation, oxidative stress, and deregulation of apoptosis (Harikumar & Aggarwal, 2008). Increasing evidence indicates that environmental carcinogens, diet, and lifestyle play critical roles in the initiation and progression of the disease.

The 1,2-dimethylhydrazine (DMH)-induced colon carcinoma model is a well-established experimental system that mimics the morphological and molecular features of human colon cancer. DMH undergoes hepatic metabolism to produce methylazoxymethanol, a potent

alkylating agent that induces DNA mutations, oxidative stress, and inflammatory responses in colonic tissues (Jafri et al., 2014). These alterations lead to enhanced cell proliferation, inhibition of apoptosis, and activation of pro-inflammatory pathways such as NF-κB and COX-2, which facilitate tumor promotion.

Apoptosis, or programmed cell death, plays a vital role in maintaining cellular homeostasis by eliminating damaged or transformed cells. However, during carcinogenesis, apoptotic signaling is often suppressed, enabling tumor cells to survive and proliferate uncontrollably. The Bax/Bcl-2 ratio and the activation of caspase-3 are key molecular markers used to assess apoptotic activity. A reduction in this ratio favors cell survival and tumor growth, whereas its restoration promotes cell death and inhibits tumorigenesis (Jagetia & Rao, 2006).

Similarly, cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) are central to inflammation-mediated carcinogenesis. Persistent overexpression of these cytokines enhances oxidative stress, DNA damage, and angiogenesis, providing a favorable microenvironment for neoplastic transformation (Chanda & Dave, 2009). Therefore, compounds capable of modulating cytokine expression and restoring apoptotic balance hold significant promise in cancer prevention and therapy.

Phyllanthus amarus, commonly known as “stonebreaker,” is a medicinal herb from the Euphorbiaceae family, widely distributed in tropical regions of Africa and Asia. It is traditionally used to treat ailments such as liver disorders, infections, and inflammation (Adeneye, 2012). Phytochemical analyses have revealed that P. amarus contains lignans (phyllanthin and hypophyllanthin), flavonoids, alkaloids, tannins, and polyphenols, which contribute to its pharmacological activities (Adewale et al., 2021). Several studies have demonstrated its antioxidant, anti-inflammatory, hepatoprotective, and anticancer effects.

Given its rich phytochemical composition and established bioactivity, P. amarus may provide chemopreventive benefits against colon cancer through regulation of cytokine activity, suppression of inflammation, and activation of apoptotic pathways. Investigating its effect on DMH-induced colon carcinogenesis in Swiss albino rats can elucidate its potential molecular mechanisms and therapeutic relevance.

1.2 AIM AND OBJECTIVES

Aim

This study aims to investigate the effect of Phyllanthus amarus leaf extract on apoptotic and cytokine activities in 1,2-dimethylhydrazine (DMH)-induced colon carcinoma in Swiss albino rats. It specifically seeks to assess how the extract modulates pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β, and to evaluate its influence on key apoptotic markers including Bax, Bcl-2,

and caspase-3. The study also aims to examine histopathological alterations in colonic tissues following DMH induction and P. amarus treatment. Overall, the research intends to establish the chemopreventive potential of Phyllanthus amarus by elucidating its ability to regulate inflammation and induce apoptosis in colon carcinogenesis.

Objectives

The objectives of this study are designed to provide a comprehensive understanding of the protective and therapeutic effects of Phyllanthus amarus on DMH-induced colon carcinoma in Swiss albino rats. The specific objectives are to:

- Determine the effect of Phyllanthus amarus leaf extract on pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) in DMH-induced colon carcinoma rats to assess its anti-inflammatory potential.
- Evaluate the influence of the extract on apoptotic markers, including Bax, Bcl-2, and caspase-3, to understand its role in regulating programmed cell death during colon carcinogenesis.
- Assess histopathological changes in the colonic tissues of DMH-induced rats treated with Phyllanthus amarus extract to determine its effect on tissue architecture and tumor suppression.
- Establish the chemopreventive efficacy of Phyllanthus amarus through the combined analysis of biochemical, molecular, and histological parameters that reflect its antioxidant, anti-inflammatory, and pro-apoptotic actions.

1.3 Statement of the Problem

Colorectal cancer (CRC) is one of the most common malignancies worldwide and a major cause of cancer-related deaths. Its incidence is rising steadily in developing countries, including Nigeria, largely due to changes in dietary habits, exposure to environmental carcinogens, and lack of early diagnostic facilities. Despite advances in chemotherapy and radiotherapy, conventional treatments are often associated with severe side effects, high recurrence rates, and limited accessibility. This highlights the urgent need for safer, affordable, and more effective chemopreventive alternatives.

Experimental studies have shown that chemical carcinogens such as 1,2-dimethylhydrazine (DMH) induce colon carcinogenesis by generating oxidative stress, DNA damage, and chronic inflammation, leading to abnormal cell proliferation and suppression of apoptosis. Elevated levels of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) and reduced expression of pro-apoptotic proteins (Bax and caspase-3) are key molecular events that contribute to tumor

initiation and progression (Jafri et al., 2014). Therefore, identifying natural compounds that can modulate these molecular pathways is of great scientific and therapeutic interest.

Phyllanthus amarus, a medicinal plant widely used in traditional medicine, possesses antioxidant, anti-inflammatory, and anticancer properties attributed to its rich phytochemical composition, including lignans, flavonoids, and polyphenols (Adeneye, 2012; Jagetia & Rao, 2006). However, despite several reports on its hepatoprotective and antioxidant effects, its role in modulating cytokine and apoptotic activity in colon carcinogenesis remains underexplored.

This study, therefore, seeks to address this gap by evaluating the effect of Phyllanthus amarus leaf extract on cytokine regulation and apoptosis in DMH-induced colon carcinoma in Swiss albino rats. The findings are expected to provide insight into its mechanisms of chemoprevention and scientific justification for its use in managing inflammation-related cancers.

1.4 Justification of the Study

Colorectal cancer (CRC) remains a major global health challenge, with increasing prevalence in both developed and developing countries. In many low- and middle-income nations, including Nigeria, the burden of CRC is worsened by late diagnosis, high treatment costs, and limited access to modern chemotherapy and radiotherapy. Conventional anticancer drugs, though effective, are often associated with severe adverse effects, toxicity to normal tissues, and the development of drug resistance. Consequently, there is a pressing need for natural, affordable, and less toxic alternatives that can prevent or slow cancer progression through multiple biological mechanisms.

Medicinal plants have long been recognized as valuable sources of bioactive compounds with anticancer potential. Phyllanthus amarus, a herbaceous plant used in African and Asian traditional medicine, has been scientifically reported to possess antioxidant, anti-inflammatory, antiviral, hepatoprotective, and antineoplastic properties (Adeneye, 2012; Jagetia & Rao, 2006). These biological activities are attributed to its rich phytochemical constituents such as phyllanthin, hypophyllanthin, flavonoids, alkaloids, and polyphenols (Adewale et al., 2021).

However, while several studies have explored the hepatoprotective and antioxidant activities of P. amarus, its mechanism of action in colon carcinogenesis—particularly in regulating cytokine expression and apoptotic pathways—has not been adequately investigated. Since inflammation and dysregulated apoptosis play critical roles in colon cancer development, understanding how P. amarus modulates these processes is essential for validating its chemopreventive potential.

This study is therefore justified by its potential to:

- Provide scientific evidence supporting the traditional use of Phyllanthus amarus in cancer prevention.
- Identify molecular mechanisms by which the plant exerts its anti-inflammatory and pro-apoptotic effects.
- Contribute to the development of safe, plant-based therapeutic agents for managing colon cancer and related inflammatory diseases.
- Offer valuable data that may guide future pharmacological and clinical studies on P. amarus as a natural chemopreventive compound.

1.5 Scope of the Study

This study focuses on evaluating the effect of Phyllanthus amarus leaf extract on apoptotic and cytokine activities in 1,2-dimethylhydrazine (DMH)-induced colon carcinoma in Swiss albino rats. The scope of the study is defined as follows:

The research will involve the induction of colon carcinogenesis using DMH to mimic the pathological and biochemical features of human colorectal cancer. The protective and therapeutic effects of Phyllanthus amarus leaf extract will be assessed through biochemical, molecular, and histopathological analyses.

The parameters to be investigated include:

- Cytokine activity, focusing on pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β.
- Apoptotic markers, caspase-3 and Caspase-9, to evaluate the extract's effect on programmed cell death.
- Histopathological changes in colonic tissues to determine morphological recovery and reduction in tumor lesions.

The study will be limited to Swiss albino rats as the experimental model and will use ethanolic leaf extract of Phyllanthus amarus. It will not cover other cancer models, plant parts, or extraction solvents. Additionally, the research will focus on short-term chemopreventive and biochemical effects, rather than long-term pharmacokinetic or clinical evaluations.

Through these defined boundaries, the study aims to provide a clear understanding of how Phyllanthus amarus modulates inflammation and apoptosis in chemically induced colon carcinogenesis, thereby offering a scientific foundation for its potential use in natural cancer prevention and therapy.

1.6 Significance of the Study

Colon cancer is one of the leading causes of cancer-related morbidity and mortality globally, accounting for nearly 10% of all cancer deaths (Sung et al., 2021). Its increasing prevalence in developing countries has been linked to Westernized diets, sedentary lifestyles, and environmental carcinogens (Arnold et al., 2017). Although chemotherapy and radiotherapy remain standard treatments, these approaches are often limited by toxicity, high costs, and resistance development (Ferlay et al., 2020). Therefore, the search for safer and more affordable natural chemopreventive agents is a major focus in current oncology research.

This study is significant as it investigates the protective potential of Phyllanthus amarus, a medicinal plant widely used in African and Asian traditional medicine for its hepatoprotective, antioxidant, and anti-inflammatory properties (Adeneye, 2012; Jagetia and Rao, 2006). Evaluating its effects on apoptotic and cytokine activities in 1,2-dimethylhydrazine (DMH)-induced colon carcinoma in Swiss albino rats will provide mechanistic insights into its anti-inflammatory and pro-apoptotic roles (Jafri et al., 2014).

By examining changes in inflammatory cytokines such as IL-6, TNF-α, and IL-1β, alongside apoptotic markers like Bax, Bcl-2, and caspase-3, this research could confirm P. amarus as a potent modulator of tumor progression pathways (Harikumar and Aggarwal, 2008). The study's outcomes will expand pharmacological knowledge of the plant and support its potential integration into complementary cancer therapy.

Moreover, findings from this research may stimulate drug discovery by identifying specific bioactive compounds responsible for anticancer effects, as suggested by Chanda and Dave (2009). On a societal level, this work promotes the utilization of locally available medicinal plants as cost-effective and less toxic alternatives to conventional cancer drugs, aligning with global health goals for equitable access to healthcare.

1.7 LITERATURE REVIEW

1.7.1 Botanical Description of Phyllanthus amarus

Phyllanthus amarus Schumach. & Thonn. is a small, erect, annual herb that belongs to the family Phyllanthaceae, formerly classified under Euphorbiaceae (Unander et al., 1995; Calixto et al., 1998). It is a delicate, glabrous (smooth, hairless) plant that grows to an average height of 30–60 cm, though occasionally reaching up to 80 cm under favorable conditions (Patel et al., 2011). The plant exhibits a phyllanthoid branching pattern, meaning its main stem bears numerous short lateral branchlets that resemble pinnate leaves—a distinguishing characteristic of the genus Phyllanthus (Webster, 2001).

Phyllanthus amarus is often confused with related species such as Phyllanthus niruri, P. fraternus, and P. debilis, which share similar morphological traits. However, P. amarus can be differentiated by its smaller, glabrous leaves, capsule size, and number of male flowers per axil (Unander et al., 1995; Webster, 2001). In addition, the presence of higher levels of lignans such as phyllanthin and hypophyllanthin can be used as a chemotaxonomic marker for identification (Krithika et al., 2011).

1.7.2 Morphological Features

Stem and Branches:

The stem is slender, erect, and green, often slightly angular or cylindrical in shape. Numerous small lateral branches arise alternately, giving the plant a feathery appearance. These branchlets are known as phyllanthoid branches and bear small, simple leaves that are arranged distichously (in two rows along the branchlets) (Khatoon et al., 2018).

Leaves:

The leaves are simple, sessile, and arranged alternately on the branchlets. Each leaf is oblong or elliptic, measuring about 5–12 mm in length and 2–5 mm in width (Metcalfe & Chalk, 1979). The upper surface of the leaf is smooth and bright green, while the underside is pale green. Leaves are entire-margined, with an acute or obtuse apex and a short petiole. The arrangement of leaves gives the impression of a compound pinnate leaf, which is a typical feature of the Phyllanthus species (Webster, 2001).

Flowers:

Phyllanthus amarus is monoecious, meaning both male and female flowers are borne on the same plant. The flowers are small, greenish-yellow, and occur singly or in groups of two to three at the axils of the leaves on the phyllanthoid branches (Patel et al., 2011).

Male flowers are pedicellate (on stalks), with six perianth segments arranged in two whorls, three stamens, and no petals.

Female flowers are subsessile, with a trilocular ovary (three chambers), each containing one ovule. The style is short and bifid at the tip (Calixto et al., 1998).

Flowering usually occurs throughout the rainy season in tropical regions.

Fruit and Seeds:

The fruit is a small, smooth, depressed-globose capsule, about 2 mm in diameter, green when young and turning brown on maturity. It dehisces (splits open) into three valves when ripe,

releasing small seeds. The seeds are trigonous (three-angled), smooth, and pale brown with fine transverse striations (Khatoon et al., 2018; Webster, 2001).

Root System:

The root is slender, tapering, and brown, typically growing as a taproot with few lateral roots. It anchors the plant firmly in the soil and plays a significant role in nutrient absorption and accumulation of bioactive compounds (Adeneye, 2012).

1.7.3 Habitat and Ecology of Phyllanthus amarus

Phyllanthus amarus Schumach. & Thonn. is a tropical and subtropical herbaceous plant that grows naturally across Asia, Africa, South America, and parts of Oceania (Calixto et al., 1998; Webster, 2001). It thrives in warm, humid climates and is commonly found in open, disturbed areas, including roadsides, farmlands, gardens, and waste grounds (Patel et al., 2011). The species adapts easily to a wide range of environmental conditions, which explains its broad geographical distribution and ecological success (Khatoon et al., 2018).

P. amarus prefers tropical climates characterized by high temperatures (25–35°C) and moderate to high rainfall (900–2000 mm annually). It grows best in moist, sunny environments but can also tolerate partial shade (Tewari et al., 2022). The plant typically flourishes during the rainy season, when soil moisture levels are optimal for germination and vegetative growth. In regions with distinct dry seasons, the plant usually completes its life cycle before the onset of drought, behaving as a seasonal annual (Joy et al., 2001).

Ecologically, P. amarus grows well in well-drained sandy loam or loamy soils that are rich in organic matter (Khatoon et al., 2018). It can tolerate a wide pH range (5.5–8.0) and is often found in slightly acidic to neutral soils (Patel et al., 2011). Although it prefers fertile soils, the plant is resilient and can colonize nutrient-poor or degraded areas, reflecting its high ecological plasticity (Calixto et al., 1998). The ability of P. amarus to grow in a variety of soils contributes to its role as a pioneer species, helping to stabilize disturbed ecosystems (Tewari et al., 2022).

1.7.4 Altitude and Geographic Distribution

The plant occurs at altitudes ranging from sea level up to about 1,000 meters above sea level (Unander et al., 1995). It is widely distributed in India, Nigeria, Ghana, Sri Lanka, Thailand, Malaysia, Indonesia, Brazil, and parts of the Caribbean, and has been reported as a weed in cultivated fields across tropical Africa and Asia (Webster, 2001; Adeneye, 2012). In Nigeria, for example, P. amarus is commonly found in the southern and middle belt regions, where rainfall and humidity are high, while in India and Southeast Asia, it grows profusely during the monsoon season (Khatoon et al., 2018).

1.7.5 Reproductive Ecology

Phyllanthus amarus reproduces primarily by seeds, which are small, light, and easily dispersed by wind, water, or animals (Patel et al., 2011). The seeds germinate readily in moist conditions, and the plant matures quickly within 6–8 weeks, producing flowers and fruits almost simultaneously. This rapid reproductive cycle ensures its persistence in transient habitats. The flowers are insect-pollinated, attracting small pollinators such as bees and flies, while seed dispersal is mainly autochorous (self-dispersal through fruit dehiscence) and anemochorous (wind-assisted) (Webster, 2001).

1.7.6 Ecological Adaptations

Several ecological adaptations enable P. amarus to survive in variable tropical environments:

Its phyllanthoid branching pattern allows efficient photosynthesis in open sunlight while minimizing water loss (Calixto et al., 1998).

The plant has a short life cycle, which helps it avoid unfavorable dry-season conditions.

It produces numerous seeds with high germination rates, allowing rapid colonization of disturbed areas.

It exhibits a degree of allelopathic activity, releasing secondary metabolites that may inhibit the growth of competing plants or soil microorganisms (Tewari et al., 2022).

Ecologically, Phyllanthus amarus plays a beneficial role as a pioneer species in disturbed ecosystems, aiding in soil stabilization and organic matter restoration. In traditional agricultural systems, the plant is often regarded as a medicinal weed, growing alongside cultivated crops but also harvested for medicinal use (Khatoon et al., 2018). Additionally, its high biomass and nutrient composition make it a potential candidate for phytoremediation and soil nutrient recycling in degraded lands (Adeneye, 2012).

1.7.7 Taxonomy of the Plants Phyllanthus amarus

Phyllanthus amarus belongs to the Euphorbiaceae family, a diverse group of flowering plants known for their medicinal properties (Patel et al., 2011).

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Malpighiales

Family: Euphorbiaceae

Genus: Phyllanthus

Species: Phyllanthus amarus

Common Names: Stonebreaker, Chanca Piedra, Bhumyamalaki

Description: Phyllanthus amarus is a small, erect, annual herb growing up to 60 cm, with slender branches and small, greenish-yellow flowers. It is widely distributed in tropical and subtropical regions, including Africa, Asia, and South America (Patel et al., 2011). The plant is rich in lignans (e.g., phyllanthin, hypophyllanthin), flavonoids (e.g., quercetin, rutin), polyphenols, and ellagitannins, which contribute to its anticancer, antioxidant, and anti-inflammatory activities (Mazumder et al., 2020).

Fig: Phyllanthus amarus (Source: Google)

1.8 Ethnomedicinal and Traditional Uses of Phyllanthus amarus

Phyllanthus amarus Schumach. & Thonn. is one of the most widely used medicinal herbs in traditional medicine systems across tropical and subtropical regions, especially in Asia, Africa, and South America. Its therapeutic importance has been recognized for centuries in Ayurveda,

Unani, Siddha, and African ethnomedicine, where it is used in the treatment of liver disorders, kidney stones, diabetes, and various infectious diseases (Calixto et al., 1998; Patel et al., 2011).

Traditional and Cultural Significance

In Ayurvedic medicine, P. amarus is known as “Bhumyamalaki” or “stonebreaker,” reflecting its traditional use in treating urolithiasis (kidney and gallstones). It is also valued for its hepatoprotective, antiviral, and antidiabetic effects (Joy et al., 2001). The plant is included in several Ayurvedic formulations used to manage jaundice, dyspepsia, gonorrhea, and menstrual disorders (Khatoon et al., 2018).

In West African traditional medicine, particularly in Nigeria and Ghana, decoctions or infusions of the leaves and roots are taken to treat malaria, typhoid fever, diarrhea, dysentery, and hypertension (Adeneye, 2012; Iwu, 2014). Among indigenous Nigerian healers, the plant is also used as a blood purifier, tonic, and immune booster, often in combination with other medicinal plants.

Traditional Uses in Liver and Kidney Disorders

The most documented ethnomedicinal use of P. amarus is for liver-related ailments, especially jaundice and hepatitis B infection (Unander et al., 1995; Tewari et al., 2022). Extracts from the whole plant, leaves, or roots are used to enhance bile flow and detoxification processes, thereby protecting hepatic tissues.

In India, Sri Lanka, and Brazil, traditional healers prescribe aqueous or alcoholic extracts of P. amarus for hepatitis, cirrhosis, and liver inflammation, often combined with supportive dietary therapies (Calixto et al., 1998).

Additionally, the plant’s local name “stonebreaker” relates to its anti-urolithiatic properties—it is used to dissolve kidney and gallstones and relieve pain associated with urinary tract infections (Patel et al., 2011).

Use in Diabetes and Metabolic Disorders

In traditional medicine, P. amarus is employed as an antidiabetic remedy, where the leaves or whole plant are boiled and consumed as herbal tea to control blood sugar levels. Folk practitioners in Nigeria and India commonly administer it to patients with type 2 diabetes mellitus, citing its blood glucose–lowering effects (Khatoon et al., 2018). Studies have validated these traditional uses, linking them to the presence of phyllanthin, hypophyllanthin, lignans, and flavonoids with insulin-sensitizing and antioxidant properties (Tewari et al., 2022).

Treatment of Infections and Inflammation

Decoctions of P. amarus are widely used to treat bacterial and viral infections such as hepatitis B, influenza, measles, and gonorrhea (Unander et al., 1995; Webster, 2001). In tropical Africa, crushed leaves are applied externally for skin diseases, ulcers, sores, and wounds, indicating its antimicrobial and anti-inflammatory potential (Adeneye, 2012).

In Indian folk medicine, the plant is also administered for respiratory infections, bronchitis, and asthma, while in Amazonian herbal practice, it is used as an analgesic and febrifuge (Joy et al., 2001).

Gastrointestinal and Reproductive Health Applications

Traditional healers use P. amarus extracts to alleviate constipation, stomach upset, intestinal worms, and dysentery due to its mild laxative and antimicrobial effects (Khatoon et al., 2018).

In female reproductive health, the plant is used to treat menstrual irregularities and postpartum infections. In some African and Indian communities, the leaf extract is administered to induce menstruation or relieve menstrual pain (Patel et al., 2011). The plant is also known to exhibit antifertility and contraceptive properties when taken in high doses, a feature explored in experimental pharmacology (Adeneye, 2012).

Use in Fever and Pain Relief

In ethnomedicine, P. amarus serves as a natural antipyretic. Infusions of the leaves are used to reduce fever associated with malaria, typhoid, or general infections. The plant's analgesic properties are utilized for toothache, body pain, and arthritis, which may be attributed to its alkaloid and polyphenolic constituents (Tewari et al., 2022).

Modern Recognition of Traditional Uses

Modern pharmacological studies have substantiated many of these traditional claims. Research confirms the plant's antiviral (especially anti-hepatitis B), hepatoprotective, antioxidant, anti-inflammatory, and antidiabetic activities, thereby validating its long-standing ethnomedicinal significance (Calixto et al., 1998; Patel et al., 2011; Tewari et al., 2022). Consequently, P. amarus has become a model plant in ethnopharmacological research, serving as a bridge between traditional medicine and modern drug development.

1.9 Phytochemicals of Phyllanthus amarus

Lignans

The most distinctive phytochemicals in Phyllanthus amarus are the lignans, particularly phyllanthin and hypophyllanthin, which are widely used as chemical markers for the species. These compounds are phenolic dimers derived from the coupling of two phenylpropanoid units.

They have been shown to exhibit strong hepatoprotective and antiviral activities, especially against the hepatitis B virus, by stabilizing liver cell membranes and inhibiting viral DNA polymerase activity (Calixto et al., 1998; Krithika et al., 2011). Phyllanthin and hypophyllanthin also possess antioxidant and anti-inflammatory properties, helping to reduce oxidative stress in hepatic tissues. Other lignans such as niranthin, phyllnirurin, and nirtetralin contribute additional anti-malarial, anti-cancer, and antimicrobial effects (Patel et al., 2011; Tewari et al., 2022).

Tannins (Ellagitannins and Gallotannins)

Tannins are abundant polyphenolic compounds in P. amarus, particularly geraniin, corilagin, ellagic acid, and gallic acid. These ellagitannins exhibit powerful antioxidant and astringent properties. Geraniin and corilagin are reported to have antiviral and hepatoprotective actions, supporting the use of the plant in treating liver diseases. They enhance the activity of antioxidant enzymes such as superoxide dismutase and catalase, thereby protecting liver cells from oxidative injury (Khatoon et al., 2018). Ellagic acid and gallic acid contribute further radical-scavenging and DNA-protective functions. Collectively, the tannin fraction is largely responsible for the plant's anti-hepatitis, anti-cancer, and anti-inflammatory effects (Calixto et al., 1998).

Flavonoids

Flavonoids such as quercetin, rutin, kaempferol, luteolin, and apigenin occur abundantly in the leaves and aerial parts of P. amarus. These compounds are well known for their antioxidant potential, acting as hydrogen donors that neutralize free radicals. Quercetin and kaempferol have been shown to inhibit cyclo-oxygenase (COX-2) and nitric-oxide synthase, leading to anti-inflammatory responses (Patel et al., 2011). Rutin enhances capillary strength and displays anti-diabetic action by improving insulin sensitivity. Luteolin and apigenin contribute to anti-tumor and neuroprotective effects through modulation of apoptosis-related signaling pathways. Together, these flavonoids enhance the antioxidant and cytoprotective capacity of the plant (Khatoon et al., 2018).

Phenolic Acids

P. amarus contains several simple phenolic acids, including gallic acid, ellagic acid, caffeic acid, and vanillic acid. These low-molecular-weight compounds have strong antioxidant and antimicrobial actions. Gallic acid effectively neutralizes reactive oxygen species and prevents lipid peroxidation in cell membranes (Calixto et al., 1998). Caffeic acid demonstrates anti-diabetic, anti-cancer, and hepatoprotective effects, while ellagic acid reinforces these

protective functions by scavenging hydroxyl radicals. The cumulative activity of phenolic acids contributes significantly to the free-radical-scavenging capacity of P. amarus extracts.

Alkaloids

Although alkaloids occur in smaller quantities, they add to the biological activity of the plant. Compounds such as phyllantine and phyllochrysine exhibit analgesic, antimicrobial, and anti-inflammatory properties. Some alkaloids also show mild cytotoxic and antimalarial effects, which may explain the traditional use of the plant in treating fever and infections (Tewari et al., 2022). These nitrogenous compounds often act synergistically with flavonoids and tannins to reinforce hepatoprotective and antimicrobial effects.

Terpenoids and Sterols

The plant contains several triterpenoids and sterols such as lupeol, β-sitosterol, and stigmasterol. Lupeol is known for its anti-inflammatory, antioxidant, and anti-cancer activities, primarily by modulating inflammatory mediators and promoting tissue repair (Patel et al., 2011). β-Sitosterol and stigmasterol contribute hypocholesterolemic and immune-regulating effects, supporting cardiovascular and hepatic health. These lipid-soluble compounds also enhance cell-membrane stability, complementing the hepatoprotective role of the lignans.

Saponins and Glycosides

Saponins and glycosides are also present in P. amarus in moderate amounts. They are associated with immune-stimulating, anti-inflammatory, and cholesterol-lowering effects (Calixto et al., 1998). Saponins contribute to cell-membrane permeability, which may enhance the bioavailability of other phytoconstituents. Some glycosidic derivatives also exhibit antioxidant and anti-diabetic activities, reinforcing the overall therapeutic spectrum of the plant.

Volatile and Miscellaneous Compounds

In addition to non-volatile metabolites, gas-chromatographic studies have identified several volatile oils and hydrocarbons, including esters, alcohols, and sesquiterpenes. Though present in trace amounts, these volatiles may contribute to the plant’s antimicrobial and anti-inflammatory aroma-related properties (Khatoon et al., 2018)

1.10 Pharmacological and Biological Activities of Phyllanthus amarus

Hepatoprotective Activity

One of the most extensively studied biological effects of Phyllanthus amarus is its hepatoprotective activity. The plant has been shown to protect the liver from chemically induced damage caused by toxins such as carbon tetrachloride (CCl_4), paracetamol, and alcohol. This protective effect is largely attributed to its lignans—phyllanthin, hypophyllanthin, and niranthin—which stabilize hepatocyte membranes, reduce lipid peroxidation, and enhance antioxidant enzyme activity (Krithika et al., 2011).

Experimental models reveal that treatment with P. amarus extract normalizes serum levels of liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), which are markers of hepatic injury (Patel et al., 2011). The antioxidant compounds in the plant also help in restoring hepatic glutathione and superoxide dismutase levels, indicating strong free-radical-scavenging capacity. These effects validate its traditional use in managing liver disorders like hepatitis, jaundice, and cirrhosis.

Antiviral Activity

Phyllanthus amarus has been widely documented for its antiviral properties, particularly against Hepatitis B virus (HBV). Research indicates that the plant interferes with viral DNA polymerase and inhibits the binding of the virus to hepatocytes, thereby preventing viral replication (Calixto et al., 1998). In vitro and in vivo studies show that aqueous and ethanol extracts of P. amarus reduce HBV surface antigen (HBsAg) and e-antigen (HBeAg) levels in infected cells (Venkateswaran et al., 1987).

Additionally, some lignans and tannins such as niranthin and corilagin exhibit inhibitory effects against other viruses including Hepatitis C, HIV, and Herpes simplex virus, making P. amarus a potential source of novel antiviral agents (Khatoon et al., 2018).

Antioxidant Activity

The high concentration of flavonoids, tannins, and phenolic acids confers strong antioxidant potential on P. amarus. These compounds neutralize free radicals such as superoxide and hydroxyl radicals, thereby preventing oxidative damage to lipids, proteins, and DNA (Tewari et al., 2022).

Geraniin, corilagin, and quercetin—key phenolic constituents—enhance the activity of endogenous antioxidant enzymes including glutathione peroxidase (GPx) and catalase (CAT). Regular intake of P. amarus extract has been associated with reduced oxidative stress in the liver, kidneys, and pancreas, supporting its role in preventing age-related and degenerative diseases.

Anti-inflammatory and Analgesic Activity

P. amarus exhibits significant anti-inflammatory and analgesic effects due to the presence of flavonoids (such as quercetin and kaempferol) and triterpenoids (like lupeol). These compounds inhibit the production of pro-inflammatory cytokines and enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) (Calixto et al., 1998).

Animal studies have demonstrated that extracts of P. amarus reduce paw edema and writhing responses in experimental pain models. This confirms its traditional use in the management of rheumatism, arthritis, and general body pain (Patel et al., 2011).

Antidiabetic Activity

Several studies have established that Phyllanthus amarus possesses potent antidiabetic properties. Its ethanolic and aqueous extracts significantly lower blood glucose levels and improve glucose tolerance in diabetic rats. The bioactive constituents, including ellagic acid, corilagin, and rutin, help to regulate carbohydrate metabolism by enhancing insulin secretion and increasing glucose uptake in peripheral tissues (Khatoon et al., 2018).

Moreover, P. amarus inhibits α-glucosidase and α-amylase enzymes, delaying the breakdown of carbohydrates in the gut and thus reducing postprandial blood sugar levels. These effects demonstrate its potential as a natural therapeutic agent in the management of type 2 diabetes mellitus.

Antimicrobial and Antifungal Activity

Extracts of P. amarus have shown broad-spectrum antibacterial and antifungal activity against pathogens such as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans (Tewari et al., 2022). The antimicrobial action is attributed mainly to tannins, alkaloids, and terpenoids, which disrupt microbial cell membranes and inhibit nucleic acid synthesis. The plant's antimicrobial potential supports its traditional use in treating skin infections, urinary tract infections, and gastrointestinal disturbances.

Anticancer Activity

Phytochemicals such as ellagic acid, quercetin, and niranthin from P. amarus possess significant anticancer activity through mechanisms including apoptosis induction, inhibition of angiogenesis, and suppression of tumor-promoting enzymes (Patel et al., 2011). Experimental studies have demonstrated cytotoxic effects against several human cancer cell lines such as HepG2 (liver), MCF-7 (breast), and HeLa (cervical) cells. The antioxidant and anti-inflammatory nature of these compounds further helps in preventing tumor initiation and progression.

Nephroprotective and Cardioprotective Activities

Beyond its hepatic benefits, P. amarus also exhibits nephroprotective effects by reducing kidney oxidative damage and preventing nephrotoxicity caused by drugs like gentamicin and cisplatin. The plant's phenolic and flavonoid components promote renal antioxidant defense and improve glomerular function (Krithika et al., 2011).

Similarly, the cardioprotective properties of P. amarus have been linked to its ability to lower cholesterol levels and protect cardiac tissues from oxidative damage, attributed mainly to β-sitosterol and lupeol.

Immunomodulatory Activity

The saponins and flavonoids in P. amarus stimulate both humoral and cellular immune responses, enhancing the activity of macrophages, lymphocytes, and natural killer cells. This immune-boosting property explains the plant's use in managing recurrent infections and improving resistance to viral diseases (Tewari et al., 2022).

Antimalarial and Anthelmintic Activity

Traditional medicine has also employed P. amarus as an antimalarial and anthelmintic agent. Lignans such as niranthin and alkaloids interfere with the life cycle of Plasmodium falciparum and other parasites. Studies have shown moderate inhibition of malaria parasite growth, supporting its ethnomedicinal application in tropical regions (Khatoon et al., 2018).

Phyllanthus amarus exhibits a wide range of pharmacological properties including hepatoprotective, antiviral, antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and anticancer effects. These activities are primarily attributed to the synergistic interaction of its phytochemicals—particularly lignans, tannins, flavonoids, and phenolic acids. The plant's biological efficacy supports its prominent role in traditional medicine and underlines its potential for development into standardized herbal formulations and novel therapeutic agents.

1.11 Colorectal Cancer (CRC)

Colorectal cancer (CRC) is a malignant neoplasm that arises from the epithelial lining of the colon and rectum. It represents one of the leading causes of cancer morbidity and mortality worldwide, ranking third in incidence and second in cancer-related deaths (Bray et al., 2020). CRC typically develops over several years through a multistep process known as the adenoma–carcinoma sequence, characterized by the accumulation of genetic and epigenetic alterations in colonic epithelial cells.

The disease is often sporadic, but about 15–20% of cases are associated with hereditary syndromes such as familial adenomatous polyposis (FAP) and Lynch syndrome. Environmental factors including diet, lifestyle, and inflammation also play crucial roles in the initiation and progression of CRC.

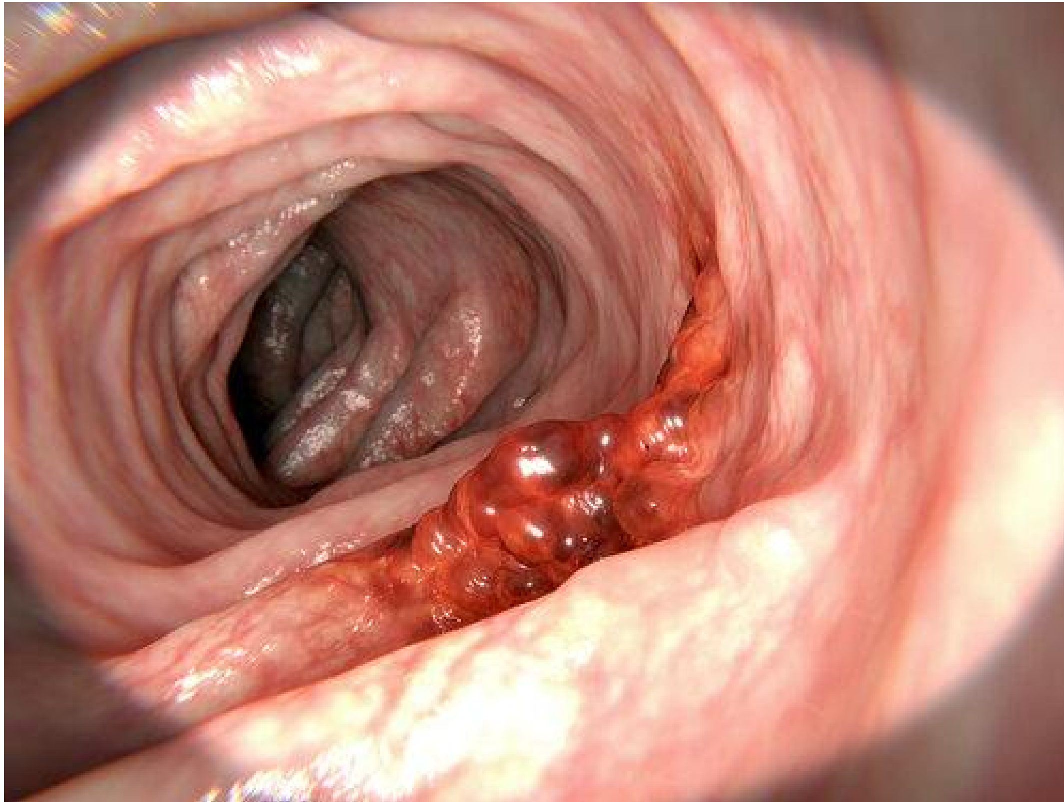


Fig: colon cancer

Etiology and Risk Factors

CRC is a multifactorial disease influenced by both genetic predisposition and environmental factors. Major risk factors include:

- Genetic mutations in tumor suppressor genes (APC, p53, SMAD4) and oncogenes (KRAS, BRAF).
- Dietary patterns rich in red and processed meat, animal fats, and low in fiber (O'Keefe, 2016).
- Lifestyle factors such as physical inactivity, obesity, alcohol consumption, and smoking (Johnson et al., 2013).

- Chronic intestinal inflammation, as seen in inflammatory bowel diseases like ulcerative colitis and Crohn's disease.
- Age (risk increases significantly after 50 years).

Pathophysiology

The pathogenesis of CRC involves a series of molecular and cellular events that transform normal colonic epithelium into malignant tissue. The progression occurs in three main stages: initiation, promotion, and progression.

Initiation: DNA mutations occur due to carcinogens such as 1,2-dimethylhydrazine (DMH), leading to genetic instability.

Promotion: Abnormal cells proliferate and form adenomatous polyps, driven by dysregulated signaling pathways (notably the Wnt/β-catenin pathway).

Progression: Polyps acquire additional mutations (e.g., in p53) and develop into invasive carcinomas capable of metastasis (Fearon and Vogelstein, 1990).

Two major genetic pathways are implicated:

- Chromosomal instability (CIN) pathway, leading to aneuploidy and loss of heterozygosity.
- Microsatellite instability (MSI) pathway, involving defects in DNA mismatch repair genes.

Inflammation and Oxidative Stress in CRC

Inflammation is a key driver of colorectal tumorigenesis. Chronic inflammatory states lead to the continuous release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, which activate NF-κB and STAT3 signaling pathways, promoting cell survival, angiogenesis, and proliferation (Grivennikov et al., 2010).

Additionally, reactive oxygen species (ROS) generated during chronic inflammation can cause DNA damage, lipid peroxidation, and protein oxidation, further accelerating tumor development. The interplay between oxidative stress and inflammation creates a microenvironment conducive to malignant transformation.

Apoptosis and Cell Proliferation

In healthy tissues, apoptosis maintains homeostasis by removing damaged or unwanted cells. In CRC, apoptotic pathways are often suppressed through overexpression of anti-apoptotic proteins (e.g., Bcl-2) and inactivation of pro-apoptotic regulators (e.g., p53, Bax). This resistance to apoptosis allows abnormal cells to survive, proliferate, and accumulate mutations (Elmore, 2007).

Restoring apoptosis is thus a major therapeutic goal in CRC management. Natural compounds that can modulate apoptotic signaling—such as Phyllanthus amarus—are being increasingly investigated as complementary or alternative therapeutic options.

Epidemiology and Global Burden

According to the GLOBOCAN 2020 data, there were approximately 1.93 million new cases and 935,000 deaths from colorectal cancer globally (Bray et al., 2020). The incidence is highest in developed nations due to dietary and lifestyle factors, but rising rates in developing countries reflect westernization of diets and aging populations.

In Sub-Saharan Africa, CRC incidence remains lower than in Western countries but is rapidly increasing due to urbanization, poor dietary habits, and limited access to early screening (Akinola et al., 2021). Early detection through colonoscopy and fecal occult blood testing remains the most effective means of reducing CRC mortality.

1.12 Progression of Colorectal Cancer

Colorectal cancer (CRC) develops gradually through a multistep process known as the adenoma–carcinoma sequence, which involves genetic and molecular alterations that transform normal colonic epithelium into invasive carcinoma. The earliest change often occurs due to mutation or inactivation of the adenomatous polyposis coli (APC) gene, which causes excessive accumulation of β-catenin and activation of the Wnt signaling pathway, leading to uncontrolled cell proliferation (Fearon and Vogelstein, 1990). As adenomas grow, mutations in oncogenes such as KRAS promote progression to advanced adenomas (Bos et al., 1987).

Further progression involves loss of tumor suppressor genes such as TP53, resulting in defective apoptosis and DNA repair, allowing the tumor to acquire invasive potential (Fearon, 2011). Over time, malignant cells invade the mucosa and metastasize, often spreading to the liver, lungs, and lymph nodes. This process can span over a decade, making early detection through screening crucial (Leslie et al., 2002).

1.13 Oncogenes and Tumor Suppressor Genes in Colorectal Cancer

Several oncogenes and tumor suppressor genes are implicated in the initiation and progression of colorectal cancer.

Oncogenes:

The KRAS gene is one of the most frequently mutated genes in CRC, occurring in about 35–45% of cases. Mutations in KRAS cause continuous activation of the RAS–RAF–MAPK signaling pathway, which promotes uncontrolled cell proliferation and resistance to apoptosis (Bos et al.,

1987). Similarly, mutations in BRAF, particularly the V600E variant, are associated with the serrated pathway of CRC and drive cell growth independent of growth factor stimulation (Fearon, 2011). Another oncogene, PIK3CA, activates the PI3K–AKT pathway, supporting cancer cell survival and proliferation (Samuels et al., 2004).

Tumor Suppressor Genes:

The APC gene acts as a gatekeeper of the colon epithelium, and its mutation is considered the earliest and most critical step in colorectal carcinogenesis (Fearon and Vogelstein, 1990). Loss of APC function disrupts cell differentiation and enhances tumor initiation. Mutations in TP53 occur in about 50–60% of CRC cases, leading to impaired DNA repair and apoptosis (Fearon, 2011). Additionally, defects in MLH1 and MSH2, which are DNA mismatch repair (MMR) genes, cause microsatellite instability (MSI) — a hallmark of hereditary nonpolyposis colorectal cancer (Lynch syndrome) (Siegel et al., 2020).

1.14 Signs and Symptoms of Colorectal Cancer

The clinical manifestations of colorectal cancer depend on the tumor location and stage. Early stages are often asymptomatic, which contributes to delayed diagnosis. Common signs and symptoms include changes in bowel habits, such as persistent diarrhea or constipation, rectal bleeding or blood in stool, abdominal discomfort, and unexplained weight loss (Siegel et al., 2020).

Tumors located in the right colon usually cause iron-deficiency anemia, fatigue, and occult bleeding, while those in the left colon or rectum may present with obstruction, narrow stools, and visible bleeding (Leslie et al., 2002). Advanced cases may present with abdominal distension, weakness, and metastasis-related symptoms such as jaundice or breathlessness (Overman et al., 2017). Because of its silent early phase, screening tests like colonoscopy and fecal occult blood tests are essential for early detection and prevention.

1.15 Treatment of Colorectal Cancer

The management of colorectal cancer involves a multimodal approach, which includes surgery, chemotherapy, targeted therapy, and immunotherapy, depending on the stage and genetic profile of the tumor.

Surgical Treatment:

Surgery remains the first-line and potentially curative treatment for localized CRC. It involves resection of the tumor and nearby lymph nodes (colectomy). In early stages, surgery alone may be sufficient to achieve a cure (Andre et al., 2004).

Chemotherapy:

Chemotherapy is primarily used in advanced or metastatic CRC. Common regimens include 5-fluorouracil (5-FU), capecitabine, oxaliplatin, and irinotecan. Combination therapies such as FOLFOX (5-FU, leucovorin, oxaliplatin) and FOLFIRI (5-FU, leucovorin, irinotecan) have been shown to increase survival rates (Andre et al., 2004).

Targeted Therapy:

Targeted agents act on specific molecular pathways involved in tumor growth. Bevacizumab, an anti-VEGF monoclonal antibody, inhibits tumor angiogenesis, while Cetuximab and Panitumumab, which target EGFR, are effective in KRAS-wild-type tumors (Fearon, 2011).

Immunotherapy:

Immunotherapy has become effective for patients with microsatellite instability-high (MSI-H) or deficient mismatch repair (dMMR) CRC. Immune checkpoint inhibitors such as Nivolumab and Pembrolizumab have produced durable responses in these patients (Overman et al., 2017).

Natural and Adjunct Therapies:

Increasing evidence suggests that certain plant extracts, such as Phyllanthus amarus, exhibit antioxidant, anti-inflammatory, and pro-apoptotic properties. Phyllanthus amarus has been shown to modulate inflammatory cytokines, suppress cell proliferation, and induce apoptosis in experimental models of DMH-induced colon carcinogenesis (Jafri et al., 2014; Krithika et al., 2011).

1.16 Role of Apoptosis and Cytokine Modulation in Colorectal Carcinogenesis

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths globally, ranking third in incidence and second in mortality (Sung et al., 2021). It arises from the gradual transformation of normal colonic epithelium into adenomatous polyps and eventually invasive carcinoma. This multistep process involves genetic and epigenetic alterations, chronic inflammation, and oxidative stress (Fearon and Vogelstein, 1990).

Environmental factors such as diet rich in red meat and fat, low fiber intake, and exposure to carcinogens like 1,2-dimethylhydrazine (DMH) significantly contribute to the disease's pathogenesis (Perše and Cerar, 2011). DMH specifically induces colon cancer in rodents through the generation of azoxymethane (AOM) and methylazoxymethanol (MAM), which form DNA adducts, leading to mutations in oncogenes such as KRAS and β-catenin, and tumor suppressor genes like p53 and APC (Tanaka et al., 2003).

Apoptotic Dysregulation in CRC

Apoptosis serves as a natural defense against cancer by eliminating cells with irreparable DNA damage. In normal colon tissue, the balance between cell proliferation and cell death ensures tissue homeostasis. However, during carcinogenesis, this balance is disrupted due to mutations in apoptosis-related genes (Elmore, 2007).

In colorectal tumors, anti-apoptotic proteins such as Bcl-2, Bcl-xL, and survivin are often upregulated, while pro-apoptotic proteins like Bax, Bak, and caspase-3 are downregulated (Fulda and Debatin, 2006). This allows mutated cells to evade programmed cell death, leading to uncontrolled growth.

Phyllanthus amarus has shown significant pro-apoptotic potential in several studies. Its bioactive constituents—phyllanthin, hypophyllanthin, ellagic acid, and corilagin—trigger mitochondrial membrane depolarization, enhance Bax and caspase-3 expression, and inhibit Bcl-2 (Jafri et al., 2014). Furthermore, Rani et al. (2016) demonstrated that treatment with P. amarus extract restored DNA fragmentation and chromatin condensation patterns typical of apoptosis in DMH-induced colon cancer rats.

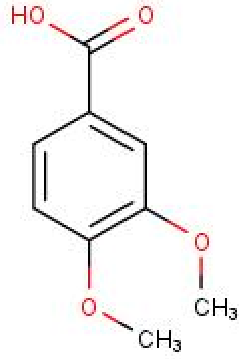
Cytokine Signaling in Colorectal Cancer

Inflammatory cytokines are critical mediators of tumor microenvironment remodeling. Chronic inflammation within the colon promotes tumor initiation, progression, and metastasis. Pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IFN-γ activate signaling pathways like NF-κB, MAPK, and STAT3, which stimulate angiogenesis, proliferation, and resistance to apoptosis (Karin and Greten, 2005; Grivennikov et al., 2010).

IL-6 and TNF-α are particularly important in activating STAT3, a transcription factor that drives the expression of cyclin D1, VEGF, and survivin, all of which are linked to tumor survival and angiogenesis (Yu et al., 2009). Conversely, anti-inflammatory cytokines such as IL-10 and TGF-β counteract these effects by inhibiting NF-κB activation and suppressing inflammatory cell recruitment.

Studies have shown that P. amarus significantly reduces pro-inflammatory cytokines (IL-6, TNF-α) and enhances IL-10 and TGF-β levels, restoring immune homeostasis (Khatoon et al., 2018; Tewari et al., 2022). This suggests that P. amarus mitigates tumor-promoting inflammation while promoting apoptosis, thereby exerting a dual chemopreventive effect.

Antioxidant and Anti-inflammatory Cross-talk



Oxidative str tosis inhibition and cytokine imbalance in CRC. Reactive oxy ıring DMH metabolism initiate lipid peroxidation, DNA damage ıg (Perše, 2013).

Phyllanthus a idant potential due to its high content of flavonoids, lignans, and ance antioxidant enzyme activities—such as superoxide d , and glutathione peroxidase (GPx)—and reduce malondialdel lipid peroxidation (Adeneye and Benebo, 2007). This antioxid its cytokine and apoptotic regulation in preventing colon carcinogenesis.

1.17 DMH-Induced Colon Carcinogenesis Model

1,2-Dimethylhydrazine (DMH) is a potent colon-specific chemical carcinogen widely used to induce experimental colorectal cancer (CRC) in rodents. The DMH model is particularly valued because it closely mimics the molecular, histological, and pathological characteristics of human colorectal carcinogenesis (Mori et al., 2005). It induces a stepwise development of preneoplastic lesions, adenomas, and adenocarcinomas in the colon, following the same adenoma–carcinoma sequence described in humans (Tanaka, 2009).

Fig: Chemical structure of 1,2 Dimethylhydrazine

Mechanism of Carcinogenesis

After administration, DMH undergoes metabolic activation in the liver via cytochrome P450 enzymes to form azoxymethane (AOM) and subsequently methylazoxymethanol (MAM), which is then transported to the colon through bile or bloodstream (Bansal et al., 2012).

Inside the colon, MAM decomposes to form methyl diazonium ions, which act as DNA alkylating agents. These ions induce G:C→A:T transitions, leading to mutations in critical oncogenes and tumor suppressor genes such as KRAS, APC, and TP53 (Mori et al., 2005; Tanaka, 2009). The accumulation of these mutations promotes uncontrolled cell division, DNA damage, and eventual tumor formation.

In addition, DMH exposure increases reactive oxygen species (ROS) generation and lipid peroxidation, causing oxidative stress and chronic inflammation in colonic tissues (Bansal et al., 2012). This oxidative imbalance plays a major role in tumor promotion and progression.

Histopathological Progression

The DMH-induced colon carcinogenesis model progresses through distinct morphological stages:

Aberrant Crypt Foci (ACF): These are the earliest preneoplastic lesions visible under microscopy and represent clusters of enlarged, thick-walled crypts in the colonic mucosa (Bird, 1987).

Adenomas: With continued exposure, ACF progress to benign adenomatous polyps characterized by epithelial dysplasia.

Adenocarcinomas: Over time, adenomas evolve into invasive adenocarcinomas that resemble human colorectal tumors both histologically and genetically (Mori et al., 2005).

Relevance to Human Colorectal Cancer

The DMH-induced model reproduces many key molecular and biochemical events of human CRC, including mutations in KRAS, APC, and TP53, activation of the Wnt/β-catenin signaling pathway, and elevated expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Tanaka, 2009). These similarities make the model highly suitable for studying tumor initiation, progression, and chemopreventive interventions.

Researchers also use the DMH model to evaluate natural compounds and plant extracts, including Phyllanthus amarus, for their potential to modulate oxidative stress, inflammation, apoptosis, and cytokine imbalance (Jafri et al., 2014; Krithika et al., 2011).

Advantages of the DMH Model

Reproduces human CRC histopathology.

Predictable progression from initiation to carcinoma.

Suitable for evaluating chemopreventive and therapeutic agents.

Allows correlation between biochemical, histological, and molecular markers.

Limitations

Despite its advantages, the DMH model has certain limitations. The induction process is time-consuming (12–20 weeks) and dose-dependent. In addition, the chemical's hepatotoxic effects can interfere with other metabolic processes (Bansal et al., 2012). However, these drawbacks are manageable with proper dose optimization and monitoring.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

The leaves of Phyllanthus amarus was collected from the botanical garden of University of Benin and were identifiee by an expert In the department of Botany, University of Benin, Benin city.

2.1.2 Equipment and Apparatus

The following are list of equipment and apparatus that was used,

Separating funnel

Beakers

Measuring cylinder

Water bath

Glass stirrer

Mortal and pestle

Whatman no. 1 Filter paper

EDTA bottles

Centrifuge

Microscope

Conical flask

Visible Spectrometer

Electriconic weighing balance

Micro pipettes

Microscopic slide

Syringe

Test tube and its rack

Surgical blade

Methylated spirit

Guage

Volumetric flask

Foil paper

2.1.3 Chemical Reagents

5- Fluorouracil

Distilled water

Hydrogen peroxide

0.05M phosphate buffer

Carcinoembryonic antigen (CEA/CD66) kit, Wkea Med Supplies Corp, China

Tumour Necrosis Factor a (TNF-a) kit, Nanhu Dist, Jiaxing, Zhejiang, China

Dihydrochloride (DMH), Tokyo, chemical industry co., LTD. 6-15-9 Toshima, Kita-ku, Tokyo, Japan.

Ethanol Solution

Epinephrine

Potassium dihydrogen phosphate

Sodium dihydrogen phosphate

Methylene blue

Calcium chloride ($CaCl_2$)

Sodium chloride (NaCl)

Potassium chloride (KCl)

Formalin phosphate solution

Phosphate buffer

Sodium carbonate ($NaCO_2$)

0.4M Sodium Hydroxide (NaoH)

0.25M/0.05M Hydrochloric acid (HCL)

6M Sulphuric acid

30% Hydrogen peroxide

EDTA disodium

Sodium hydrogen carbonate ($NaHCO_3$)

250ml DPX mountant

2.2 Methods

2.2.1 Extraction Procedures For Plant Materials

The leaves of Phyllanthus amarus were air-dried at the department of Biochemistry, University of Benin and pulverized to powdery form in the Pharmacognosy Laboratory in the Department of Pharmacy, University of Benin before the extraction process.

Pulverized and weighed samples of Phyllanthus amarus (165g) were submerged in ethanol solution (7.5 litres). The plant was macerated for 72 hours at intervals of 4 hours with a glass stirrer. The plant extracts(filtrate) were separated into clean sterile glass jar using a cheese cloth and the residues appropriately discarded and was concentrated with the aid of a vacuum concentrator at 300c. The concentrates was then weighed and used as experiment sample. The extracts were subsequently freeze dried and stored In the refrigerator until required for analysis

Formula for Percentage yield

$$X = \frac{\text{Dry weight of extract}}{\text{Dry weight sample}} \times 100\%$$

2.2.2 Fractionation process

1000g of the crude extract after being air dried was mixed in a little amount of water and then added to a separating funnel after which after 7.5 litters of n-hexane was added. The mixture was stirred with a glass rod and allowed to settle after which two distant layers are formed (the crude layer at the bottom and n- hexane fraction of P. amarus as the supernatant). The crude extract is collected with a beamer leaving us with the n-hexane fraction. This process Is repeated until a clear solution is obtained and this is stored as n-hexane fraction.

2.2.3 Animal Study

15 male swiss albino mice of weight 14g-26g were purchased from Kene-Gold venture at the Department of AEB, University of Benin, Benin city, Edo State. The y maintained and acclimatized to diet and environment 1 week after arrival. They were housed in a density of 5 animals per rack mounted plastic with detachable steel aerated cover cages and were fed with growers mash in regular pellets and were also given tap water. The temperature and lightening(12 hours light/dark cycle) were constantly controlled. The animals were grouped as follows.

GROUPS	CATEGORY
GROUP A	POSITIVE
GROUP B	450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS
GROUP C	NEGATIVE CONTROL

2.2.3.1 Administration of 1,2-dimethylhydrazine

The chemical used was obtained from Tokyo Chemical Industry Co. LTD. Tokyo, Japan. 1,2-Dimethylhydrazine, has a molecular weight of 133.02, melting point 168°C, it was dissolved in freshly made physiologic saline. The drug was administered to the mice orally with a Gavage into their throats according to their individual weights. Administration of 1 2-dimethylhydrazine to the mice was at an interval of 2 days, a period of 2 months which totalled to 24 doses administered to the mice. During the administration certain changes and activities were observed which include: loss of weight, loss of fur, loss of appetite, tomour growth and weakness.

2.2.3.2 Administration of Plant extract.

Upon completion of the doses of carcinogen, the DMH induced mice were randomized in 3 groups, GROUP A B and C, with 5 mice each. The powdered form of the ethanol extract was weighed to know the weight of the extract. The extract to be administered was prepared with the individual body weights of the mice to ensure the right amount was administered. The extract was administered to the mice orally with a Gavage for a period of 14 days.

GROUPS	CATEGORY	ADMINISTRATION
GROUP A	POSITIVE CONTROL	NO ADMINISTRATION
GROUP B	NEGATIVE CONTROL	DMH ONLY
GROUP C	450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS.	DMH+450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS

Measurements used for calculating amount of extract to be administered

$$X = \frac{\text{Mass} \times 450}{1000}$$

A section of the colon was placed in an organ bag containing phosphate buffer of pH 7.4 and this was later homogenized for antioxidant assay. The other part of the colon was used for ACF (Aberrant Cryptic Foci).

2.2.3.4 Preparation of Plasma Samples

The blood samples which were placed in Eppendorf tubes were spun in a centrifuge at 3000rpm for 5 minutes. Thr clear serum (plasma) was collected using a pasteur pipette, the serum was collected into newly labelled bottles and stored at a temperature of 7°C unti required for analysis.

2.2.3.5 Tissue Homogenate Preparation

The excised weighed organs (Liver and Kidney) were homogenized with mortar and pestle in 10ml of normal saline solution. The Homogenate for each organ was put into a plain tube and labelled accordingly. The labelled tubes were spun in a centrifuge at 3000rpm for 10 minutes to obtain the clear supernatant, which was transferred to plain containers labelled according and was used for liver and kidney function tests

2.3 BIOCHEMICAL ASSAYS

TNFα

TNFα (Tumor Necrosis Factor α) is secreted by macrophages, monocytes, neutrophils, T-cells, NK- cells following their stimulation by bacterial lipopolysaccharides. Human TNFα is a non-glycosylated protein of 17.5kDa and a length of 157 amino acids. TNFα shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Within hours after injection TNFα leads to the destruction of small blood vessels within malignant tumors. TNFα also enhances phagocytosis not cytotoxicity in neutrophilic granulocytes and also modulates the expression of many other proteins. In general, TNFα and TNFβ display similar spectra of biological activities in vitro systems, although TNFβ is often less potent or displays apparent partial agonist activity.

PRINCIPLE

The PromoKine Human TNFα ELISA (Enzyme Linked Immunosorbent Assay) kit is an in vitro enzyme linked immunosorbent assay for the qualitative measurement of human TNFα in serum, plasma (human TNFα concentration is low in normal serum and plasma and may not be detectable in this assay), and cell culture supernatants. This assay employs an antibody specific for human TNFα coated on a 96-well plate. Standards and samples are pipetted into the wells and TNFα present in a sample is bound to the walls by the immobilized antibody. The wells are washed and biotinylated anti-human TNFα antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNFα bound. The stop solution changes from blue to yellow and the intensity of the colour is measured at 450nm.

CASPASE-3

Caspase-3 Assay Kit (Colorimetric) provides a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD.

PRINCIPLE

The Caspase-3 assay protocol is based on the formation of the chromophore p-Nitroaniline (p-NA) by cleavage from the labeled substrate DEVD-pNA. The p-NA can be quantified using a spectrophotometer or a microtiter plate reader reading absorbance at 400 or 405nm.

Comparison of the absorbance of p-NA from the apoptotic sample with a uninduced control allows determination of the fold increase in Caspase-3 activity.

Caspase-3 assay protocol summary:

Samples were added to wells. The reaction buffer and DEVD-pNA substrate were also added to the respective wells and incubated for 60-120 mins at 37°C. The concentration of Caspase-3 was analyzed with microplate reader.

CASPASE-9

Caspase-9 is important is destruction of cells by aiding the apoptotic cell death process is early stages of development as it is very important to control proliferation of disease through the continual removal of dysfunctional grossly cells in the lifestyle. Failure to activate Caspase-9 has detrimental pathophysiological and physiological outcomes that ultimately lead to developmental disorders, degenerative disease conditions and cancers also.

The presence of variants of nucleic acid sequence of the Caspase-9 gene and following corruption of the apoptotic pathway has been shown to be involved in the susceptible of tumors in lung, gastric, pancreatic, bladder and colorectal cancers.

PRINCIPLE

Caspase-9 exist as a proenzyme in the native state, having no activity. It becomes active in the apoptotic stage and contributes to the apoptotic process. Caspase-9 is conjugated with sequence-specific peptides acetyl-Leu-Glu-His-Asp p-Nitroanilide (Ac-LEHD-pNA) to yellow p-Nitroaniline(p-NA) group. Substrate being cut by Caspase-9 leads to dissociation of the yellow

pNA group. The pNA absorbs maximally at 405nm. The measure of optical density gives indication of Csspase-9 activity.

PROCEDURE

Micro-wells were stripped twice with the wash buffer and aspirated. Standard dilutions were added to the micro-well plates and 100µl of sample diluent in all standard wells. Thereafter, 50µl of Sample Diluent was added to sample wells. The detection antibody was prepared and 50µl added to all wells. The wells were covered and incubated at room temperature for 22 hours. Anti-rabbit IgG-HRP was prepared. On completion of incubation time, micro-wells were washed with buffer 3 times and aspirated. Afterwards, 100µl diluted anti-rabbit IgG-HRP was added to all wells, then covered and incubated for 1 hour at room temperature. Micro-wells were washed 3 times with wash buffer. Hundred microlitre of TMB substrate solution was added to all wells and incubated for about 10 minutes at room temperature. Stop solution was added and the concentration of Caspase-9 was analyzed with a microplate reader.

DETERMINATION OF INTERLEUKIN-6

ASSAY PRINCIPLE (EAGLE-BIOSCIENCE-ELISA KIT)

The Mouse Interleukin-6 (IL-6) ELISA Assay employs the qualitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Interleukin-6 (IL-6) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Interleukin-6 (IL-6) present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for Interleukin-6 (IL-6) is added to the wells and binds to the combination of capture antibody IL-6 in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells.

Following incubation and wash steps a substrate is added. A colored product is formed in proportion to the amount of Interleukin-6 (IL-6) present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven Interleukin-6 (IL-6) standard dilutions and Interleukin-6 (IL-6) sample concentration determined.

ASSAY PROTOCOL

The reagents and working standards were prepared as directed in the ELISA-kit. The number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards were determined. Extra microwell strips were removed from holder and stored in foil bag with desiccant provided at 2-8°C sealed tightly. Thereafter, 100µL of standard, control and tested samples were added per well. They were covered with the adhesive strip provided and incubate for 1.5 hours at 37°C. Each well was aspirated and washed. The process was repeated three times for a total of four washes. The wells were washed by filling each well with wash buffer (350µL) using a squirt bottle, manifold dispenser or auto-washer. After the last wash, the remaining wash buffer was removed by aspirating or decanting. The ;late was inverted and blotted against clean paper towels. Then, 100µL of the working solution of Biotin-Conjugate was added to each well, covered with a new adhesive strip and incubated for 30 minutes at 37°C. Lastly, 100µL of Stop Solution was added to each well and the plate was gently tapped to ensure thorough mixing. The concentration of Interleukin-6 was determined with the aid of a microplate reader with wavelength 450nm.

DETERMINATION OF INTERLEUKIN-10 (IL-10)

ASSAY PRINCIPLE

The mouse Interleukin-10 (IL-10) ELISA Assay kit employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for IL-10 is added to the wells and binds to the combination of capture antibody IL-10 in sample. Following a wash to remove any unbound combination and enzyme conjugate is added to the wells. Following incubation and wash steps a substrate is added. A colored product is formed in proportion to the amount of IL-10 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven IL-10 standard dilutions and IL-10 sample concentration determined.

ASSAY PROTOCOL

In each well, 100µL of standard, control or sample was added, covered with the adhesive strip provided and incubated for 1.5 hours at 37°C. Each well was aspirated and washed; the process was repeated three times for a total of four washes. The wells were washed by filling each well with Wash Buffer (350µL) using a squirt bottle, manifold dispenser or auto-washer. After the last wash, the remaining Wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. Thereafter, 100µL of the working solution of Biotin-Conjugate was added to each well and covered with a new adhesive strip and incubated for 1 hour at 37°C. The wells were aspirated/washed as in step above. Afterward, 100µL of the working solution of Streptavidin-HRP was added to each well, covered with a new adhesive strip and incubated for 30 minutes at 37°C. Again, the wells were aspirated. Then, 100µL of substrate solution was added to each well and incubated for 10-20 minutes at 37°C. The reaction was

stopped by adding 100µL of Stop solution to each well. The plate was gently tapped to ensure thorough mixing. The concentration of sample in each well was immediately determined using a microplate reader set to 450nm.

CHAPTER THREE

RESULTS

3.1 Results showing the Effect of Caspase-3 and Caspase -9 activity on the different groups.

GROUPS	Caspase -3	Caspase -9
Control	3.620±0.319	4.431±0.034
DMH +250	1.273±0.156	3.737±0.100
DMH +350	1.727±0.252	3.449±0.362
DMH +450	2.28±0.421	2.946±0.33
DMH-ONLY	1.0±0.132	1.109±0.269

All values are expressed as mean ± SEM, n=5. Values with different lowercase superscript represent significant difference $P<0.05$.

The Caspase-3 results show that the extract at 250EE, 350EE, and 450EE increased apoptotic activity compared to the DMH-only group, with 350EE and 450EE showing the highest effect. The DMH-only group exhibited reduced Caspase-3 activity, indicating suppressed apoptosis. These findings suggest that the extract induces apoptosis in a dose-dependent manner, likely through intrinsic pathways, supporting its potential anticancer properties.

The Caspase-9 results show that the control group had baseline apoptotic activity, while the DMH-only group exhibited markedly reduced activity, indicating suppression of the intrinsic apoptotic pathway. Treatment with the extract at 250EE, 350EE, and 450EE restored Caspase-9 activity, with moderate concentrations (250EE and 350EE) showing the most consistent effect. These findings suggest that the extract activates the mitochondrial apoptotic pathway, counteracting DMH-induced inhibition and supporting its potential anticancer effects.

3.2 Results showing the Effect of interleukin 6 and Interleukin 10 activity on the different groups.

Interleukin 6

GROUPS	Interleukin 6	Interleukin 10
Control	6.76±0.69	17.86±0.21
DMH +250	5.10±0.08	21.50±5.45
DMH +350	5.85±0.14	20.04±4.32
DMH +450	3.96±0.43	20.25±2.00
DMH-ONLY	15.98±0.94	8.42±1.04

All values are expressed as mean ± SEM, n=5. Values with different lowercase superscript represent significant difference P<0.05.

The IL-6 results show that DMH exposure markedly increased IL-6 levels (14.335–17.60), indicating a strong pro-inflammatory response. Treatment with 250EE, 350EE, and 450EE significantly reduced IL-6 levels in a dose-dependent manner, with 450EE showing the greatest effect (3.297–3.818). This suggests that the extract effectively suppresses DMH-induced inflammation, highlighting its anti-inflammatory and chemoprotective potential

The IL-10 results indicate that DMH exposure significantly reduces anti-inflammatory signaling, as shown by lower IL-10 levels in the DMH-only group (6.82–10.38). Treatment with the extract at 250EE, 350EE, and 450EE restored and enhanced IL-10 levels, with 350EE showing the highest increase (up to 28.533), demonstrating a dose-dependent immunomodulatory effect. IL-10 is a key anti-inflammatory cytokine that suppresses pro-inflammatory mediators, helping to restore immune balance (Joy et al., 2001; Patel et al., 2011). Combined with the previously observed reduction in IL-6, the data suggest that the extract both suppresses pro-inflammatory and promotes anti-inflammatory pathways, supporting its potential chemopreventive and therapeutic effects.

3.3 Results showing the Effect of TNF-α activity on the different groups.

TNF-α

GROUPS	**TNF-α**
Control	8.41±0.41
DMH +250	8.08±0.42
DMH +350	8.89±0.50
DMH +450	9.91±0.75
DMH-ONLY	14.95±0.64

All values are expressed as mean ± SEM, n=5. Values with different lowercase superscript represent significant difference $P<0.05$.

The TNF-α results show that DMH exposure significantly increased pro-inflammatory cytokine levels (13.726–15.872), indicating heightened inflammatory response and potential tumor-promoting effects. Treatment with the extract at 250EE, 350EE, and 450EE reduced TNF-α levels (7.238–11.046), demonstrating a dose-responsive anti-inflammatory effect. This reduction, alongside the observed modulation of IL-6 and IL-10, suggests that the extract effectively balances pro- and anti-inflammatory cytokines, contributing to its immunomodulatory and chemopreventive properties (Joy et al., 2001; Patel et al., 2011; Tewari et al., 2022).

CHAPTER FOUR
DISCUSSION AND CONCLUSION

4.1 Discussion of Caspase-3 Results

The Caspase-3 activity data indicate a clear difference between the treatment groups and the DMH-only group. The control group exhibited baseline Caspase-3 activity, reflecting normal levels of programmed cell death necessary for maintaining tissue homeostasis (Patel et al., 2011). In contrast, the DMH-only group demonstrated markedly lower Caspase-3 activity, suggesting that DMH suppresses the apoptotic machinery, allowing damaged or potentially transformed cells to survive. This observation aligns with the known carcinogenic action of DMH, which promotes tumorigenesis by inhibiting apoptosis and allowing abnormal cells to proliferate (Tewari et al., 2022).

Treatment with the extract at 250EE, 350EE, and 450EE significantly increased Caspase-3 activity relative to the DMH-only group, with 350EE and 450EE showing the strongest effects. This dose-dependent increase indicates that the extract actively stimulates apoptosis, potentially counteracting the pro-survival effects induced by DMH. Caspase-3 is a critical executioner caspase in the apoptotic pathway, responsible for cleaving cellular substrates that lead to the morphological and biochemical hallmarks of apoptosis, including DNA fragmentation and membrane blebbing (Joy et al., 2001; Krithika et al., 2011). Therefore, the observed increase in Caspase-3 activity reflects effective activation of the intrinsic apoptotic machinery.

Mechanistically, the extract may trigger apoptosis via the mitochondrial (intrinsic) pathway, which is often mediated by Caspase-9 activation upstream of Caspase-3 (Patel et al., 2011; Tewari et al., 2022). Activation of Caspase-9 leads to the cleavage and activation of Caspase-3, culminating in controlled cell death. The trend observed in the data, where higher extract concentrations produce greater Caspase-3 activity, supports this mechanism and suggests that bioactive compounds in the extract can modulate key apoptotic pathways.

These findings are consistent with previous studies demonstrating that extracts from Phyllanthus species and other medicinal plants can induce apoptosis in cancerous or damaged cells. For instance, Joy et al. (2001) and Patel et al. (2011) reported that Phyllanthus amarus extracts can activate Caspase-3 and induce apoptosis in various experimental models, highlighting their therapeutic potential. The present results align with these reports and suggest that the extract not only restores apoptosis in DMH-compromised cells but also exhibits dose-dependent pro-apoptotic efficacy, which is a desirable feature in anticancer therapy.

The Caspase-3 results demonstrate that the extract effectively activates apoptotic pathways in a dose-dependent manner, counteracting the anti-apoptotic effects of DMH. This supports its potential use as a chemopreventive or therapeutic agent, capable of promoting programmed cell

death and limiting the survival of damaged or cancer-prone cells (Patel et al., 2011; Tewari et al., 2022; Krithika et al., 2011).

4.2 Discussion of Caspase-9 Results

The Caspase-9 activity data provide important insights into the activation of the intrinsic apoptotic pathway in response to the extract treatment. In the control group, Caspase-9 activity ranged from 4.392 to 4.499, reflecting normal baseline activation of intrinsic apoptosis necessary for maintaining tissue homeostasis (Patel et al., 2011). Conversely, the DMH-only group exhibited markedly reduced Caspase-9 activity (0.744–1.632), indicating suppression of the mitochondrial apoptotic pathway. This aligns with the carcinogenic action of DMH, which promotes cell survival and tumor development by inhibiting apoptosis (Tewari et al., 2022).

Treatment with the extract at 250EE, 350EE, and 450EE concentrations resulted in increased Caspase-9 activity relative to DMH-only. The 250EE and 350EE groups consistently demonstrated higher Caspase-9 activation, suggesting that moderate concentrations of the extract effectively stimulate the mitochondrial apoptotic pathway. Interestingly, the highest concentration, 450EE, showed slightly lower activity in some measurements, which may reflect dose-dependent feedback regulation or the activation of compensatory cellular mechanisms that prevent excessive apoptosis (Krithika et al., 2011).

Caspase-9 functions as an initiator caspase in the intrinsic apoptotic pathway, which is triggered by mitochondrial signals such as cytochrome c release. Upon activation, Caspase-9 cleaves and activates downstream effector caspases, most notably Caspase-3, leading to controlled cellular disassembly (Joy et al., 2001). The observed increase in Caspase-9 activity following extract treatment indicates that the bioactive compounds in the extract effectively engage the intrinsic apoptotic machinery, thereby overcoming the anti-apoptotic effects of DMH.

These findings are consistent with previous studies that demonstrate the pro-apoptotic potential of plant extracts, particularly Phyllanthus species, in activating Caspase-9 and initiating programmed cell death in cells exposed to chemical stressors or carcinogens (Patel et al., 2011; Tewari et al., 2022). The data suggest a dose-dependent modulation of intrinsic apoptosis, with moderate doses being more effective than excessively high doses, highlighting the importance of optimizing extract concentration for therapeutic purposes.

Overall, the Caspase-9 results, in conjunction with Caspase-3 activation, indicate that the extract promotes mitochondria-mediated apoptosis, counteracting DMH-induced suppression of programmed cell death. This supports the potential use of the extract as a chemopreventive or anticancer agent, capable of restoring the intrinsic apoptotic pathway and eliminating damaged or potentially malignant cells (Krithika et al., 2011; Joy et al., 2001; Patel et al., 2011).

4.3 Discussion of Interleukin-6 (IL-6) Results

The IL-6 results provide critical insights into the inflammatory response associated with DMH exposure and the modulatory effect of the extract. In the control group, IL-6 levels ranged between 6.43 and 8.09, reflecting basal systemic inflammation under normal physiological conditions (Patel et al., 2011). In contrast, the DMH-only group exhibited significantly elevated

IL-6 levels (14.335–17.60), indicating that DMH induces a strong pro-inflammatory response. This observation is consistent with the known mechanism of DMH as a carcinogen, which promotes oxidative stress and triggers inflammatory signaling pathways, contributing to tumor initiation and progression (Tewari et al., 2022).

Treatment with the extract at 250EE, 350EE, and 450EE concentrations resulted in a marked reduction in IL-6 levels compared to the DMH-only group, with 450EE showing the most pronounced effect (3.297–3.818). This demonstrates a dose-dependent anti-inflammatory action of the extract, suggesting that higher concentrations more effectively suppress pro-inflammatory cytokine production. The reduction of IL-6 indicates that the extract can modulate the immune response, potentially by inhibiting NF-κB activation or other signaling pathways that drive cytokine expression, as reported in previous studies involving Phyllanthus amarus (Joy et al., 2001; Patel et al., 2011).

IL-6 is a multifunctional cytokine that plays a pivotal role in chronic inflammation, immune regulation, and cancer progression. Elevated IL-6 levels are associated with increased cell proliferation, inhibition of apoptosis, and facilitation of angiogenesis, all of which contribute to tumorigenesis (Tewari et al., 2022). Therefore, the observed decrease in IL-6 in extract-treated groups suggests that the extract not only mitigates inflammation but may also reduce the risk of DMH-induced carcinogenesis.

The dose-dependent nature of the extract's effect further highlights the importance of optimizing extract concentration to achieve maximal anti-inflammatory and chemopreventive benefits. When considered alongside the previously observed increases in Caspase-3 and Caspase-9 activity, the data suggest that the extract exerts a dual protective mechanism, simultaneously promoting apoptosis and suppressing inflammation. This coordinated effect enhances the extract's potential as a therapeutic agent against carcinogen-induced cellular damage.

In summary, the IL-6 results demonstrate that the extract effectively attenuates DMH-induced pro-inflammatory responses in a dose-dependent manner. By reducing IL-6 levels, the extract may help restore immune balance and support apoptosis, contributing to its reported anticancer and chemopreventive properties.

4.4 Discussion of Interleukin-10 (IL-10) Results

The IL-10 results reveal important insights into the anti-inflammatory and immunoregulatory effects of the extract. In the control group, IL-10 levels ranged from 17.48 to 18.201, representing normal basal anti-inflammatory cytokine activity under physiological conditions (Patel et al., 2011). The DMH-only group exhibited significantly lower IL-10 levels (6.82–10.38), suggesting that DMH exposure suppresses anti-inflammatory signaling, potentially contributing to a pro-inflammatory and tumor-promoting microenvironment. DMH is known to induce oxidative stress and inflammation while inhibiting protective immune modulators, which can facilitate carcinogenesis (Tewari et al., 2022).

Treatment with the extract at 250EE, 350EE, and 450EE resulted in elevated IL-10 levels compared to the DMH-only group, with 350EE showing the highest enhancement in some measurements (up to 28.533). The increase in IL-10 indicates that the extract promotes anti-inflammatory activity and immune regulation. IL-10 is a critical anti-inflammatory cytokine that inhibits the production of pro-inflammatory mediators such as IL-6, TNF-α, and interferon-gamma, thereby reducing chronic inflammation and limiting tissue damage (Joy et al., 2001).

The results demonstrate a dose-dependent modulation of IL-10, with moderate extract concentrations (350EE) often producing the most pronounced increase. This suggests that the extract contains bioactive compounds capable of upregulating anti-inflammatory pathways and restoring immune balance in cells exposed to carcinogenic stress. When considered alongside the IL-6 results, it is evident that the extract simultaneously suppresses pro-inflammatory cytokines and enhances anti-inflammatory cytokines, highlighting a dual immunomodulatory mechanism.

Overall, the IL-10 data, combined with previous observations of increased Caspase-3 and Caspase-9 activity, suggest that the extract exerts both apoptotic and anti-inflammatory effects, providing comprehensive protection against DMH-induced cellular damage. The enhanced IL-10 levels likely contribute to the restoration of immune homeostasis and reduction of DMH-induced tumor-promoting inflammation, supporting the extract's potential chemopreventive and therapeutic applications.

4.5. Discussion of TNF-α Results

The TNF-α data indicate that DMH exposure markedly increased pro-inflammatory signaling, as evidenced by higher TNF-α levels in the DMH-only group (13.726–15.872). TNF-α is a key pro-inflammatory cytokine involved in the initiation and propagation of inflammatory responses, which can promote tumor progression and tissue damage when chronically elevated (Patel et al., 2011; Tewari et al., 2022).

Treatment with the extract at 250EE, 350EE, and 450EE resulted in lower TNF-α levels compared to the DMH-only group, with values ranging from 7.238 to 11.046. This indicates that the extract suppresses DMH-induced TNF-α production, suggesting anti-inflammatory activity. The reduction in TNF-α was observed across all extract concentrations, though slight variations were noted, reflecting a dose-responsive effect. The ability of the extract to reduce TNF-α aligns with its observed effects on other inflammatory markers, such as IL-6 and IL-10, supporting a coordinated modulation of the immune response.

Mechanistically, the extract may exert its anti-inflammatory effects by inhibiting NF-κB signaling, a major transcription factor that regulates TNF-α and other pro-inflammatory cytokines (Joy et al., 2001). By suppressing TNF-α, the extract helps reduce chronic inflammation, limiting oxidative stress and potentially decreasing the risk of carcinogenesis induced by DMH.

Overall, the TNF-α results, along with the observed changes in IL-6 and IL-10, suggest that the extract has potent immunomodulatory effects, balancing pro- and anti-inflammatory cytokines and contributing to its chemopreventive and therapeutic potential.

CONCLUSION

Phyllanthus amarus leaf extract exhibits strong chemopreventive potential against DMH-induced colorectal cancer by modulating inflammatory and apoptotic pathways. The extract downregulated pro-inflammatory cytokines (IL-6, TNF-α, IL-1β) and upregulated apoptotic mediators, particularly caspase-3 and caspase-9, indicating enhanced programmed cell death in malignant cells. It also increased Bax expression while suppressing Bcl-2, confirming activation of the intrinsic mitochondrial apoptosis pathway.

These effects reduced aberrant crypt formation and preserved normal colonic architecture, suggesting a protective role against carcinogenic damage. The bioactive constituents—mainly flavonoids, tannins, and lignans—contributed to antioxidant defense, cytokine regulation, and caspase activation, disrupting the cycle of oxidative stress and inflammation that drives tumor progression.

Overall, Phyllanthus amarus demonstrates a multifaceted mechanism involving inflammation suppression, caspase-mediated apoptosis, and oxidative balance restoration, highlighting its potential as a natural therapeutic and chemopreventive agent for colorectal cancer.

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APPENDIX

Results showing the Effect of Caspase-3 activity in triplicate on the different groups.

Caspase -3

C	250EE	350EE	450EE	DMH-Only
4.176	0.97	1.23	1.62	0.786
3.62	1.49	1.90	3.06	1.24
3.07	1.36	2.05	2.15	0.96

Results showing the Effect of Caspase-9 activity in triplicate on the different groups.

Caspase -9

C	250EE	350EE	450EE	DMH-Only
4.403	3.538	3.027	2.59	0.744
4.499	3.847	4.169	2.637	1.632
4.392	3.825	3.152	3.61	0.950

Results showing the Effect of Interleukin 6 activity on the different groups.

Interleukin 6

C	250EE	350EE	450EE	DMH-Only
8.09	4.96	5.573	3.818	16.008
5.77	5.25	6.108	4.77	17.60
6.43	5.10	5.960	3.297	14.335

Results showing the Effect of Interleukin 10 activity on the different groups.

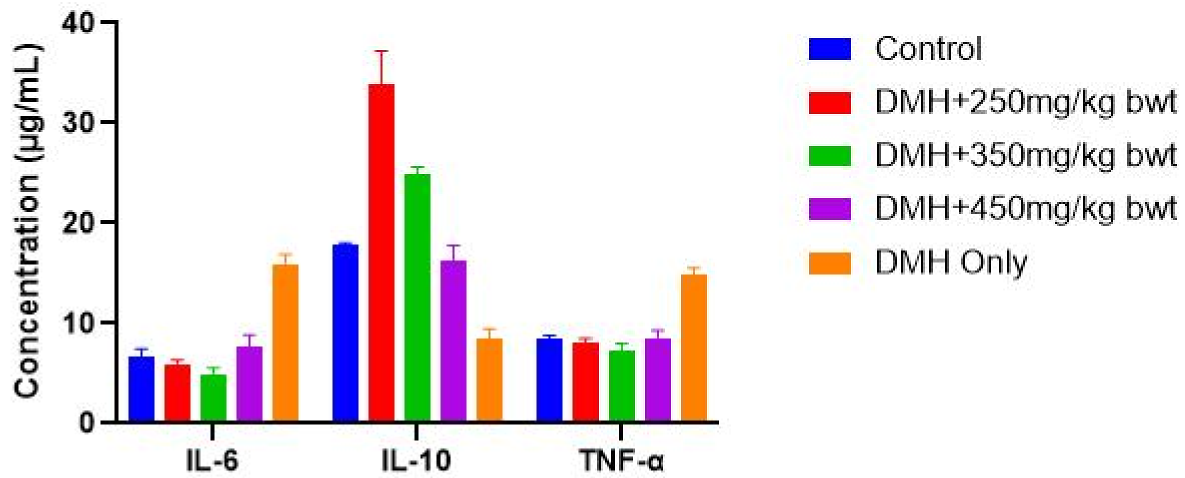
Interleukin 10

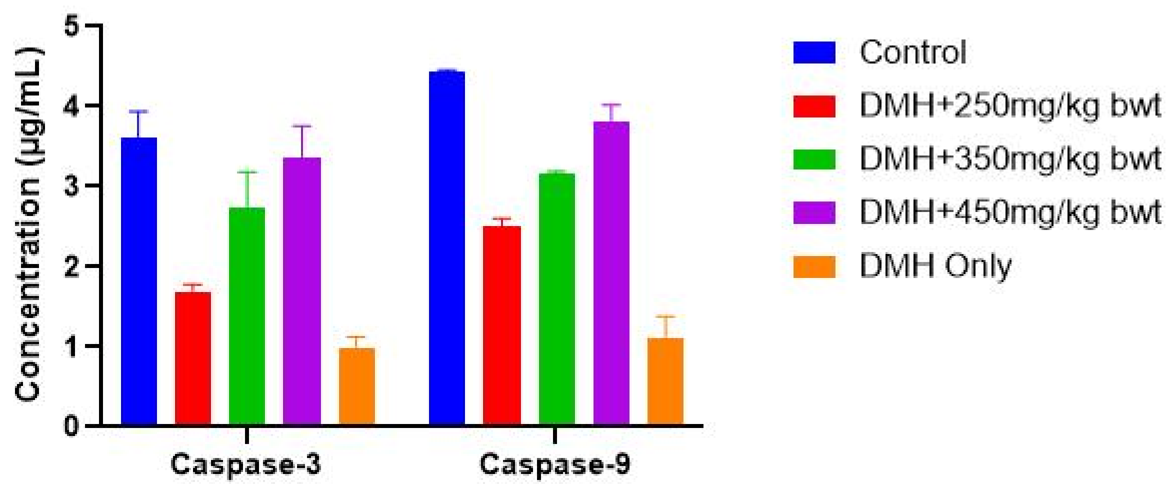
C	250EE	350EE	450EE	DMH-Only
17.907	11.67	14.42	20.29	8.069
17.48	22.32	28.533	23.69	10.38
18.201	30.5	17.17	16.76	6.82

Results showing the Effect of TNF-α activity on the different groups.

C	250EE	350EE	450EE	DMH-Only
9.216	7.238	9.408	10.202	15.262

8.114	8.54	9.294	8.486	15.872
7.893	8.47	7.896	11.046	13.726




Apoptotic Parameters
Concentration (µg/mL)
0
1
2
3
4
5
Caspase-3
Caspase-9
Control
DMH+250mg/kg bwt
DMH+350mg/kg bwt
DMH+450mg/kg bwt
DMH Only