INVESTIGATION OF ANTI-CARCINOGENIC ACTIVITIES OF *MUSSAENDA MACROPHYLLA* WALL. (FAMILY: RUBIACEAE)

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INVESTIGATION OF ANTI-CARCINOGENIC ACTIVITIES OF *MUSSAENDA MACROPHYLLA* WALL. (FAMILY: RUBIACEAE)

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In partial fulfillment of the requirements of the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

CERTIFICATE

I certify that the thesis entitled "**Investigation of anti-carcinogenic activities of *Mussaenda macrophylla* Wall. (Family: Rubiaceae)**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Marina Lalremruati** is a record of research work carried out during the period of 2017-2022 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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I, **Marina Lalremruati**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

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CHAPTER I

GENERAL INTRODUCTION

Cancer is a heterogeneous disease with multiple genotoxic and oncogenic aberrations characterized by uncontrolled proliferation, invasion and metastasis of cells (Hanahan and Weinberg, 2000). It is a multifactorial disease where a group of abnormal cells uncontrollably grow defying all the natural rules and regulatory mechanisms of cell division and growth (Hejmadi, 2010). There are more than 36 major types of cancer with a striking fatality rate of 9.6 million people per year. It is a major health burden worldwide and is expected to rank as the leading cause of death and the most important barrier to increasing life expectancy throughout the world in the 21st century. According to the International Agency for Research on Cancer (IARC), one-in-five men and one-in-six women will develop cancer throughout their lifetime, with one-in-eight men and one-in-eleven women dying from it. Some of the factors that appear to be driving the rise in diseases; most notably, population expansion and ageing, are also one of the global incidences associated to increased cancer risk factors associated with social and economic development countries (Bray et al., 2018; Rawla and Barsouk, 2019). The most commonly diagnosed cancer is female breast cancer accounting for 11.7% of total cases and is closely followed by lung (11.4%) and colon (10%) cancers. Lung cancer is the leading cause of cancer related deaths (18% of total cancer deaths), followed by colon (9.4%) and liver (8.3%) cancers. In 2020, the global incidence rate of all cancers was 19% higher among men (222 per 100,000) than in women (186 per 100,000), though rates varied greatly by region. Overall, cancer mortality is twice as high in men as it is in women, with death rates 43% higher in men than in women, owing in part to differences in cancer type distribution. Asia accounts for half of all cancer diagnoses and 58.3% of cancer deaths for both sexes, with Asia accounting for 59.5% of the global population. Despite accounting for 9.7% of the global population, Europe contributes for 22.8% of all cancer cases and 19.6% of cancer deaths, followed by the Americas with 20.9% of incidence and 14.2% of mortality.

Because of the distinct distribution of cancer types and greater case fatality rates in these regions, the share of cancer fatalities in Asia (58.3%) and Africa (7.2%) is larger than the share of cancer incidence (49.3% and 5.7%, respectively). Worldwide, a 47% rise in cancer cases is expected by the year 2040, with an estimated 28.4 million new cancer cases (Sung et al., 2021).

History

According to paleopathologic findings, tumors have already occurred in animals in prehistoric times, long before men appeared on Earth. The earliest written description of cancer, written in 1600 BC, was breast cancer found in the Edwin Smith Papyrus. The Ebers Papyrus, which dates from around 1500 BC, has the first mention of a fatty tumor, a soft-tissue tumor, as well as references to skin, stomach, uterus and rectum tumors (Ebbell, 1937). The word cancer is derived from Latin, which literally means crab and the Greek word is karkinos which also means crab. The term originated from the observation of Hippocrates (the father of modern medicine) that the blood vessels around malignant tumors appear like the claws of a crab, and hence the name cancer. The word metastasis was introduced in 1829 by Joseph Recamier. He was able to identify the invasion of blood vessel by cancer with the naked eye by watching the growth and spread of cancers. In 1838, Johannes Muller defined cancers as "special clusters of aberrant cells and stroma." He described cancer to be caused by the development of new cells in damaged organs that have the potential to be destructive and spread to other parts of the body through vascular invasion. He linked cancer to ageing and identified tumor necrosis (apoptosis) as a regressive symptom. He was able to tell the difference between epithelial and connective tissue cancers using microscopy. For the treatment of cancer, tumor removal surgeries have been documented in ancient Egypt, hormone therapy and radiotherapy were developed in the late 19th Century. Immunotherapy, chemotherapy and newer targeted therapies were introduced in the 20th century (Diamandopoulus, 1996; Kardinal, 1979).

Characteristics of Cancer

Under normal condition, human body cells are formed, divided and differentiated each day to restore the dead and worn-out cells, these cells will receive the mitogenic growth signals in order to enter the active proliferative phase and will not multiply in the absence of these signals. However, cancer cells have acquired the ability to proliferate whether these stimulatory signals are present or not and can produce their own growth factors imitating the normal growth factors thus making them independent of the normal growth factors (Fedi et al., 1997; Hanahan and Weinberg, 2000).

There are more than 100 distinct kinds of cancers and tumors till date with each specific organ having subtypes and these human cancers share numerous fundamental properties in spite of this diversity. A set of six characteristic properties of cancers termed the 'hallmarks of cancer' has been proposed by Hanahan and Weinberg (2000) which are as follows:

1. Growth signal autonomy

Tumor cells do not depend strictly on exogenous growth signals for their proliferation as they have the ability to generate their own growth signals by altering the extracellular growth signal, the transducers and intracellular circuits that translate these signals into action thereby hampering the normal homeostatic mechanism. For example, glioblastomas PDGF (platelet-derived growth factor) and sarcomas produce TGFα (tumor growth factor α).

2. Insensitivity to antigrowth signals

Tumor cells evade antiproliferative signals by altering the retinoblastoma protein (pRb) pathway, which prevents cells from progressing through the G1 phase of the cell cycle, allowing them to proliferate and becoming resistant to antigrowth factors (Weinberg, 1995).

3. Resistance to apoptosis

Resistance to apoptosis in cancer cells is evident in more than half of human cancers. This resistance can be achieved by mutation of the tumor suppressor gene p53 (Harris, 1996).

4. Unlimited replicative potential

The three main acquired characteristics of cancer cells, such as growth signal autonomy, antigrowth signal insensitivity and apoptosis resistance, can lead to a decoupling of a cell's growth programme from external signals. This can be achieved through upregulation of the expression of telomerase enzyme.

5. Induction and sustenance of angiogenesis

Because the rapid clonal proliferation of tumor cells requires the development of new blood vessels, tumor cells tend to activate the angiogenic switch by altering the balance of angiogenesis inducers like VEGF and countervailing inhibitors like thrombospondin-1 (Hanahan and Folkman, 1996).

6. Metastasis and tissue invasion

During the progression of most human cancers, tumor cells spread out and infect surrounding organs, where they may succeed in forming new colonies through a process known as metastasis. Metastases are responsible for approximately 90% of all cancer deaths in humans (Sporn, 1996). The loss of function of E-cadherin owing to mutational inactivation, transcriptional repression, or proteolysis of the extracellular cadherin domain in the majority of epithelial malignancies is one of the reasons for this capability (Christofori and Semb, 1999).

Causes and Risk factors

Exogenous factors (biological, chemical or physical carcinogens) as well as endogenous factors (inherited mutations, immune conditions, hormones and mutations that occur from metabolism) play a role in the initiation and progression of cancer. These factors can work in unison or in sequence at different stages of tumor development, through different mechanisms, causing altered cell activity and excessive proliferation which leads to metastasis. Lifestyle, environment, and age all play a profound role in the development of cancer, with tobacco consumption accounting for 22% of deaths and poor diet, obesity, lack of physical activity, excessive alcohol consumption accounting for 10% of deaths. Other factors include exposure to ionizing radiation, environmental pollutants and infection. Infections such as hepatitis B, hepatitis C, *Helicobacter pylori*, human papillomavirus infection and the immunodeficiency virus (HIV), Epstein-Barr virus contribute to

approximately 15% of cancer worldwide. These factors are at least partially responsible for the gene alterations. 5-10% of cancer cases are caused by inherited genetic mutations from the patient's parents.

Cancer is caused by a combination of genetic factors and three types of exogenous chemicals that humans absorb, including:

i. *Physical Carcinogens*: Ionizing radiations such as ultraviolet rays from sunlight, uranium, radon, radiation from alpha, beta, gamma and X-ray-emitting sources.

ii. *Chemical Carcinogens*: Compounds including n-nitrosamines, cadmium, asbestos, benzene, nickel, vinyl chloride and benzidine as well as about 60 known powerful cancer-causing toxins or compounds, are found in cigarette smoking or tobacco use, a food contaminant (aflatoxin) and a drinking water contaminant (arsenic).

iii. *Biological Carcinogens*: Infections from certain viruses, bacteria or parasites and pathogens such as human papillomavirus (HPV), hepatitis B and C, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus (KSHV), Markel cell polyomavirus, *Helicobacter pylori* and *Schistosoma* spp. (Blackadar, 2016; Saini et al., 2020).

Treatments

There are a variety of cancer therapies available, depending on the type of cancer and how far it has progressed. Some cancer patients have only one treatment, but the majority receive a combination of treatments, such as surgery and radiation therapy. The following are the different types of treatments:

***Surgery*:** Surgery is the oldest cancer treatment, and it continues to be the mainstay of solid tumor treatment to this day. It works best for treating isolated primary tumors with regional lymphatic invasion. En bloc surgical methods aim to encompass gross and microscopic tumors in all neighbouring and contiguous anatomic regions (Kufe et al., 2003). In some circumstances, it may be combined with chemotherapy and radiation therapy. Prostatectomy for prostate cancer and lung cancer surgery for non-small cell lung cancer are two examples of cancer surgical methods. The primary purpose of surgery can be to remove either the entire organ or only the tumor. Other surgeries available for cancer treatment that do not require any incisions include:

i. Cryosurgery / Cryotherapy: This therapy uses argon and nitrogen gas to remove abnormal cells in the cervix and skin, and it is used to treat precancerous development on the cervix and skin.

ii. Lasers: Lasers are utilized for more precise procedures since they have a precise focus on small areas and can shrink or eliminate tumors. This intense beam of light is used to treat skin conditions as well as the lining of internal organs such as vaginal, cervical and basal cell carcinomas.

iii. Hyperthermia: This therapy involves exposing a small body part to a high temperature, which destroys cancer cells and allows radiation or chemotherapy treatments to work. Heat is provided by high-energy radio waves.

iv. Photodynamic Therapy: Photodynamic drugs are employed to treat cancer cells in this therapy. These drugs react to a specific wavelength of light, and when tumors are exposed to that wavelength of light, the drug becomes active and kills cancer cells nearby. This therapy is used to treat non-small cell lung cancer and skin cancer (Subotic et al., 2012).

***Radiation therapy/Radiotherapy*:** This therapy uses high doses of radiation to reduce tumors, kill cancer cells, and inhibit the growth of cancer cells by damaging their DNA. Damaged DNA does not repair, and the cell dies, and the body removes it. The external beam is used to treat pain, loss of bowel and bladder control and difficulty in breathing caused by the shrinking tumor, whereas radiopharmaceuticals are drugs used in systemic radiation treatment to treat pain that has spread to the bones. Systemic refers to treatment that travels through the bloodstream to tissues all over the body, as well as treatment given via IV and swallowing (Formenti and Demaria, 2013). For example, brachytherapy is used to treat cancers of the breast, neck, cervix, eye, and head using an external beam. Radioactive iodine, or i-131, is used to treat thyroid cancer. The gastro-entero-pancreatic neuroendocrine tumor and advanced prostate cancer are both treated with radio nucleotide therapy.

***Chemotherapy*:** This therapy involves the use of chemicals to treat cancer by targeting cells that divide rapidly and interfering with cell division, which is the cancer cell's principal feature. For the treatment of various forms of cancers, there are over 100 distinct types of chemotherapeutic drugs that can be used, either alone or in combination (Colvin, 2003). Chemotherapy's effectiveness is dependent on the type

of cancer and its stage. Breast cancer, pancreatic cancer, colorectal cancer, ovarian cancer, testicular cancer and some lung cancers have all benefitted from the combination of surgery and chemotherapy (NCI, 2014). Since certain treatments work better together than they do separately, combination therapy is a frequently used chemotherapy approach to treat cancer patients. Therefore, combination therapy is used in which two or more treatments are given at the same time. Cancer cells retain many of the regulatory activities that regular cells do not. As a result, they continue to divide even when normal cells do not. Chemotherapeutic drugs are more sensitive to cancer cells with this characteristic. A substantial collection of useful chemotherapeutic drugs has been established after almost five decades of systemic medication research and development. However, chemotherapeutic therapies are not devoid of drawbacks and can result in a wide range of toxic side effects. For example, 5-fluorouracil is a common chemotherapeutic agent that has been demonstrated to produce cardiotoxicity (Rexroth and Scotland, 1994), myelotoxicity (Macdonald, 1999) and even act as a vasospastic agent in rare but recorded cases (Rastogi et al., 1993). Doxorubicin, another commonly used chemotherapeutic agent, causes cardiac toxicity (Kilickap et al., 2005), myelotoxicity (Manil et al., 1995) and renal toxicity (Gibaud et al., 1994). Similarly, the chemotherapy drug bleomycin is known for its pulmonary toxicity (Karam et al., 1995). Furthermore, bleomycin has been reported to show cutaneous toxicity (Cohen et al., 1973). Cyclophosphamide, a drug used to treat various malignant conditions, has been shown to be associated with bladder toxicity including hemorrhagic cystitis, alopecia, immunosuppression and cardiotoxicity at high dosages (Fraiser et al., 1991).

***Immunotherapy*:** Immunotherapy is the use of medicinal drugs to stimulate the immune system of a patient so that the body can establish a strong system to combat cancer cells. Intravesical BCG immunotherapy for bladder cancer is one of several techniques for generating immune responses in the body. Interferons and cytokines are used to stimulate immune responses in individuals with renal cell carcinoma and melanoma (Waldmann, 2003). Allogeneic hematopoietic stem cell transplantation, or bone marrow transplantation from a genetically non-identical donor, is another type of immunotherapy. In this procedure, the tumor is frequently attacked by the donor's immune cells, a process known as the graft-versus-tumor effect. This treatment is

often combined with other cancer treatment strategies such as radiation, surgery or chemotherapy and is referred to as Autologous Immune Enhancement Therapy (AIET) (Damodar et al., 2006; Sivaraman et al., 2008).

***Targeted Therapy*:** Targeted therapy entails the use of specific agents to disrupt the protein regulation of cancer cells. Within the cancer cell, small molecule targeted therapy agents inhibit the enzymatic domains of mutant, overexpressed, or essential proteins. Examples include tyrosine kinase inhibitors gefitinib and imatinib. The therapeutic agent in monoclonal antibody therapy is an antibody that attaches to a protein on the surface of cancer cells. Photodynamic therapy (PDT) is a cancer treatment that uses a photosensitizer, tissue oxygen, and light (usually a laser). It can be used to treat basal cell carcinoma (BCC) or lung cancer, as well as to remove vestiges of malignant tissue after massive tumors have been surgically removed (Dolmans et al., 2003).

***Precision/Personalized Medicine*:** It is a newer strategy in which genetic testing is used to select the optimal treatment for a patient. Nowadays, cancer treatments are the same for patients with the same cancer and stage of cancer, although some patients' reactions differ. Precision medicine, according to scientists, has a bright future because it aids in receiving the best cancer therapy. Many medications are employed as treatments known as target therapies in order to test treating patients with treatments that target the cancer-causing genetic alterations in the tumor (Walko and McLeod, 2014).

Medicinal plants for cancer treatment

Despite significant improvements in the currently available treatments for cancer patients and the positive impact of these treatments on survival, these treatments can cause a variety of traumatic side effects, including hair loss, fatigue, sleep disturbances, changes in taste, appetite loss, sore mouth, fever, anxiety, depression, vomiting and nausea. These side effects can be difficult to alleviate or manage, and they can have a major impact on a cancer patient's quality of life (QOL). Second tumors following chemotherapy, hormonal and reproductive difficulties, impacts on the immune system, cardiac disease, effects on 6fv the urinary bladder, kidney, gastrointestinal organs, psychological and neurological disorders, and so on are all

possible side effects of these treatments (Boivin, 1990; Yates et al., 2005). Complementary and alternative therapies that do not employ recognized cancer medications or approaches that are not widely used in the medical world are now considered mainstream ways to control symptoms. Researching and understanding the risks and advantages of these therapies is important. Medicinal plants continue to play an important role in the healthcare systems of many people across the world. The medical and economic benefits of plants are being more widely recognized and promoted in both developing and developed countries. Herbal supplements, botanicals, and phytomedicines are all terms for products manufactured from plants that are used to improve or maintain one's health. Plant-based pharmaceuticals constitute around a quarter of the modern pharmacopoeia, according to estimates. Traditional herbal medications are naturally occurring plant-derived drugs that have been utilized to treat illness in local or regional healing practices with little or no industrial processing. Health promotion, negative prognosis and limited treatment options for a serious disease, disease prevention, exhaustion of conventional treatments, significant side effects or risks associated with conventional medicine, lack of efficacy of conventional treatments and belief that natural and herbal products are safer and better are all common reasons for using herbal and natural products (Jain et al., 2016).

Plants have been well recognized for their anticancer effects for centuries. The isolation of podophyllotoxin and other lignans from *Podophyllum peltatum* led to the development of drugs for treating testicular and small cell lung cancer (Pettit et al., 1995). The National Cancer Institute (NCI) has evaluated more than 35,000 plant species for anticancer properties and around 3,000 plant species have been found to exhibit anticancer activities. Many researches have investigated the anticarcinogenic characteristics of plants, such as anticarcinogenic activities of *Abrus precatorius* on fibrosarcoma in mice, Yoshida sarcoma in rats and ascites tumor cells (Reddy and Sirsi, 1965). Similarly, Dhar et al. (1968) investigated the anticancer effects of *Albizzia lebbeck* in mice with sarcoma and *Alstonia scholaries* in humans with benzo[a]pyrene-induced gastrointestinal cancer. Other plants that have been reported to show anticarcinogenic activities include *Anacardium occidentale* (against hepatoma), *Ageratum conyzoides* (against human lung carcinoma and mice p388

leukemia), *Asparagus racemosa* (against human epidermoid carcinoma), *Boerhaavia diffusa* (against breast cancer), *Boswellia serrata* (against nasopharynx carcinoma), *Derris scandens* (against human colon cancer), *Euphorbia hirta* (against Freund virus leukemia), *Khaya senegalensis* (against human cervical and colon cancers), *Nigella sativa* (against Lewis lung carcinoma), *Picrorrhiza kurroa* (against hepatic cancers), *Peaderia foetida* (against human epidermoid carcinoma of the nasopharynx) and *Withania somnifera* and *Zingiber officinale* against various cancer cell lines (Adebayo et al., 2010; Muthulingam and Chaithanya, 2018; Androulakis et al., 2006; Li et al., 2018; Ohiagu et al., 2021). Plant-based phytochemicals and derivatives have the potential to improve therapy efficacy and reduce side effects in cancer patients. Several of these phytochemicals with high anticancer potential are physiologically active substances found in nature. The basis for developing effective phytochemical based anticancer treatment free from side effects begins with the screening of natural (dry/wet plant material) extracts for potential anticancer bioactivity and evaluating for *in vitro* and *in vivo* effects. The beneficial effects of plants in cancer treatment have been extensively researched and have yielded promising results. Furthermore, various studies and research have demonstrated the beneficial effects of plants in the treatment of diabetes, fertility and sterility, thyroid diseases, anaemia, and psychological disorders (Choudhari et al., 2020).

***Mussaenda macrophylla* Wall.**

The genus *Mussaenda* has been reported to be a significant source of natural products in the field of pharmacology (Vidyalakshmi et al., 2008). *Mussaenda macrophylla* Wall., belonging to the Rubiaceae family, is a flowering shrub with an orange-colored inflorescence and is reported to occur in south-east Asia, India, China and Myanmar in association with herbs and other shrubs (Arunachalam et al., 2015; Manandhar, 2002). Traditionally, different parts of *M. macrophylla* has been used to treat various health problems such as cancer, cough, sore mouth, fever, chronic ulcer, diarrhea, dysentery, indigestion and snake bites (Manandhar, 1994; Sharma et al., 2001; Rosangkima and Jagetia, 2015). Previous studies with *M. macrophylla* also revealed its anti-bacterial, anti-coagulant, anti-inflammatory and hepatoprotective activities (Dinda et al., 2008).

Antioxidant activity

The antioxidant potential of *M. macrophylla* extract was evaluated by the phosphomolybdenum assay method in an experiment performed by Islam et al. (2012). The dichloromethane soluble fraction of *M. macrophylla* leaves revealed DPPH radical scavenging activity which may be correlated to its high phenolic content or due to synergistic activity of different chemical substance present in the extracts (Islam et al., 2012). An antioxidant activity assay performed by Bhandari et al. (2020) revealed that *M. macrophylla* root extracts inhibited DPPH free radical significantly which is almost similar to the standard ascorbic acid.

Anti-microbial activity

The root bark of *M. macrophylla* was found to inhibit the growth of *Porphyromonas gingivalis*, a gram-negative anaerobic oral bacteria typically associated with human gum disease, in an antimicrobial screening against two oral pathogens, *Porphyromonas gingivalis* and *Streptococcus mutans*. However, they were found to be inactive against *S. mutans*, a gram-positive facultative anaerobic coccus that is the etiologic agent of human dental caries (Kim et al., 1999). An experiment conducted by Chowdhury et al. (2013) in the leaf extracts of *M. macrophylla* exhibited significant inhibition against *Salmonella paratyphi*, *Aspergillus niger* and *Staphylococcus aureus*, respectively.

Anti-diabetic activity

Ethanol and methanol extract of *M. macrophylla* root revealed potent anti-diabetic activity in *in vitro* (Bhandari et al., 2020). The anti-diabetic activity was measured according to established method by determining the extent of glucose diffusion inhibition by ethanol and methanol extract through a semipermeable membrane that can correlate their ability to slow down the diffusion and movement of glucose in the intestinal tract.

Membrane stabilizing activity

Islam et al. (2013) demonstrated that the extracts of *M. macrophylla* leaves significantly protected the lysis of erythrocyte membrane mediated by hypotonic solution and heat when compared with the standard acetyl salicylic acid. In hypotonic solution and heat-induced conditions, the petroleum ether and carbon tetrachloride soluble partitionates of methanolic extract of *M. macrophylla* exhibited

significant inhibition of RBC hemolysis induced by hypotonic solution, respectively. In addition, the petroleum ether soluble partitionate of methanol extract of *M. macrophylla* also protected heat induced lysis of human erythrocyte membrane.

Given the present state of the scientific evidence on various pharmaceutical applications of *M. macrophylla*, high priority research is required to objectively assess the potential anticancer activity of *M. macrophylla*.

Aim of the study

The main objective of this study is to evaluate the anticancer activity *of M. macrophylla* extracts by conducting the following investigations:

- To prepare different extracts of *M. macrophylla* leaves and estimate the phytochemical contents.
- To determine free radical scavenging activities and *ex vivo* antioxidant activities of *M. macrophylla.*
- To investigate anticancer properties of *M. macrophylla* extracts in Dalton's Lymphoma Ascites bearing Swiss Albino mice.
- To study the anticancer activities and effects of *M. macrophylla* extract on differential gene expression involved in apoptosis in A549 cell line.
- To determine the anticancer activities and effects of *M. macrophylla* extract on differential expression of apoptotic genes in HCT 116 cell line.

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CHAPTER II

Preparation of extracts, estimation of phytochemical contents, and identification of bioactive compounds of different extracts of *Mussaenda macrophylla* Wall.

ABSTRACT

The leaves of *Mussaenda macrophylla* were collected, dried in shade and powdered. The powdered leaves were subjected to sequential Soxhlet extraction with increasing polarity of different solvents such as petroleum ether, chloroform, methanol and distilled water. The liquid extracts were filtered and concentrated using a rotary evaporator and finally freeze dried. We also investigate the phytochemical composition using qualitative and quantitative analysis and LC-MS profiling. Preliminary phytochemical screening revealed the presence of alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids from various extracts of *M. macrophylla*. Among the various extracts of *M. macrophylla*, aqueous extract has the highest total phenolic (387.61 ± 14.10 mg gallic acid equivalent/g) and flavonoid (5,761.65 ± 38.5 mg quercetin equivalent/g) contents. LC-MS analysis also revealed the presence of 74 major bioactive compounds in the aqueous extract of *M. macrophylla*. Some of the compounds such as Chaetocin, Daphnoretin and Brefeldin identified from the LC-MS analysis have been previously reported to exhibit anticancer activities.

1. INTRODUCTION

A wide range of plants have been used as primary source of traditional herbal medicine all over the world since prehistoric times (Doughari, 2012). Various natural products, particularly those derived from plants, have been studied for their qualities, medicinal properties and health impacts. Plants and natural products have largely influenced the evolution of successful and dependable traditional therapeutic techniques that have been used in China, India, and other nations for thousands of years (Sneader, 2005). Phytochemicals are natural bioactive chemical substances found in plants that defend them from environmental threats such as UV exposure, pollution, drought, pathogenic attack and stress (Ali et al., 2006). These bioactive chemical substances are also known as secondary plant metabolites and include organic substances like alkaloids, terpenoids, carotenoids, glycosides, tannins, steroids, mucilages, flavonoids, vitamins, minerals, organic acids etc. (Hahn, 1998). These chemicals are produced in practically every part of the plant, including the leaves, bark, stem, flower, root, seeds, fruits, suggesting that any part of the plant could be a source of active compounds (Charles et al., 2013). More than 100,000 phytochemicals are produced as secondary metabolites by plants, which can be categorized based on physical properties, chemical compositions and mechanisms involved in their formation. Despite this, only 150 phytochemicals had been thoroughly investigated (Qin et al., 2011). The relationship between phytochemicals and plant bioactivity is important to understand for the synthesis and production of compounds with specific activities that can be used to treat a variety of health problems and chronic diseases (Pandey et al., 2013). Phytochemicals appear to have strong anticancer potential, according to scientific findings. From 1940 to 2014, around half of all licensed anticancer drugs came from natural products or were developed directly from them (Newman and Cragg, 2016).

M. macrophylla, locally known as Vakep, is a flowering shrub belonging to Rubiaceae family. *M. macrophylla* is endemic to south-east Asia and is known to occur in India, China and Myanmar (Manandhar, 2002). Traditionally, different health problems such as sour mouth, sour throat, oral infections, fever, cough, chronic ulcer, diarrhea, dysentery, indigestion, cancers and snake bites have been treated using various parts of this plant (Kim et al., 1999; Rosangkima and Jagetia,

2015). *M. macrophylla* have also been reported to show multi-pharmaceutical activities including anti-microbial (Chowdhury et al., 2013), antioxidant (Islam et al., 2012), thrombolytic (Islam et al., 2013) and anti-diabetic (Bhandari et al., 2020). Despite the extensive use of *M. macrophylla* as traditional medicine, scientific validation of their pharmaceutical property is still scarce. The characterization of the constituents of plants are necessary to unravel their specific biological activity. Therefore, we aimed to investigate the phytochemical composition of various solvent extracts of *M. macrophylla*.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Gallic acid, quercetin, sodium nitrite, aluminium chloride, bismuth nitrate, folin-ciocalteau sodium hydroxide, sodium carbonate, ferric chloride, glacial acetic acid, hydrochloric acid, sulphuric acid and potassium iodide were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Petroleum ether, chloroform, methanol, olive oil and Whatman filter paper were procured from the local pharmacy.

2.2. Collection of plant and preparation of extracts

M. macrophylla was collected from Kolasib, District, Mizoram, India. It was identified and authenticated by the Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl (voucher sample: MZU/HAMP/2018/026). The leaves were dried in shade at room temperature and powdered. The pulverized leaves were then first defatted using petroleum ether in a Soxhlet apparatus at 40^{o}C for 30 cycles and dried at 40^{o}C overnight to remove all the traces of petroleum ether. The powdered leaves were further extracted with chloroform, methanol and distilled water according to their increasing polarity at their respective boiling points using Soxhlet apparatus for a minimum of 40 cycles each. The liquid extracts were filtered and concentrated using a rotary evaporator (Buchi, Germany) under reduced pressure at 40°C for about 5 h and finally freeze dried.

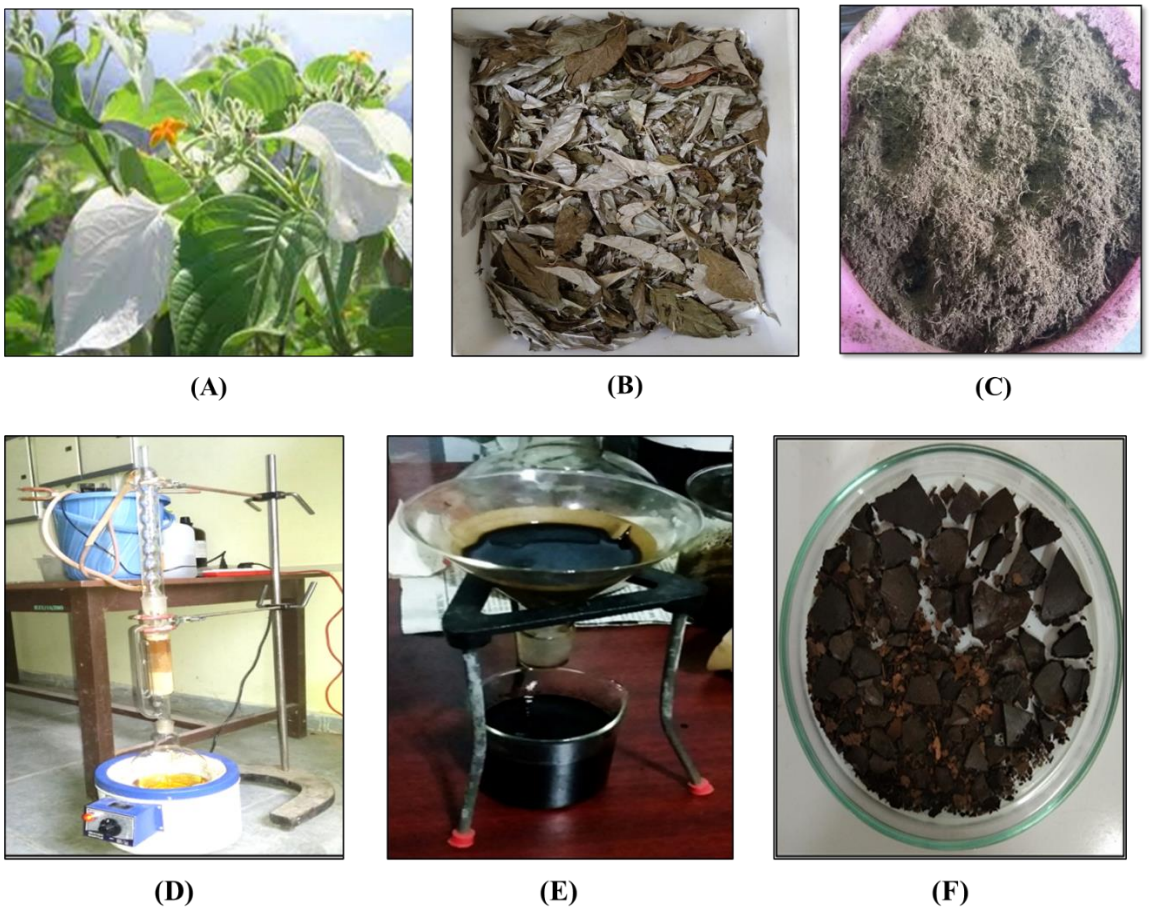


Figure 1.1. (A) *Mussaenda macrophylla* Wall.; (B) Dried leaves of *M. macrophylla*; (C) Powdered leaves; (D) Extraction using soxhlet apparatus; (E) Filtered liquid extracts; (F) Dried extracts.

2.3. Qualitative Phytochemical screening

Preliminary phytochemical screening was performed using standard methods (Bargah, 2015; Gangwar et al., 2014; Muthukrishnan and Manogaran, 2018). Samples of different fractions of *M. macrophylla* were tested for the presence of alkaloids, cardiac glycosides, saponins, steroids, tannins, terpenoids, and phlobatannins. Results are expressed as '+' for the presence and '−' for the absence of phytochemical. A description of methods used to conduct the tests are summarized below:

2.3.1. *Alkaloids*

0.1g of the extract was mixed with 0.5 mL of Draggendorff's reagent to determine the presence of alkaloids. The presence of alkaloids was indicated by the production of a reddish-brown precipitate.

2.3.2. *Cardiac glycosides*

5 mL of each extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution and underlain with 1 mL of concentrated sulphuric acid to assess the presence of cardiac glycosides. The presence of cardiac glycosides was indicated by the formation of a brown ring at the interface.

2.3.3. *Saponins*

The presence of saponins was determined by adding 3 drops of olive oil to the extracts and agitated vigorously for a few minutes. The presence of saponins was indicated by the production of a fairly stable emulsion.

2.3.4. *Steroids*

Salkowski's test was used to determine the presence of steroids in the extracts. A few drops of concentrated sulphuric acid were mixed with 0.1 g of different extracts dissolved in different solvents. The presence of steroids was indicated by the development of a red color in the lower layer.

2.3.5. *Tannins*

A ferric chloride test was used to determine the presence of tannins. A few drops of 1% ferric chloride were added to 0.1 g of dried samples dissolved in different solvents. The presence of tannins was indicated by the production of a brownish green or blue-black color.

2.3.6. *Terpenoids*

The presence of terpenoids was determined by mixing 5 mL of each extract with 2 mL chloroform and 3 mL concentrated sulphuric acid, then allowing the mixture to form a layer. The presence of terpenoids was indicated by the appearance of a reddish brown color at the interface.

2.3.7. *Phlobatannins*

The presence of phlobatannins was determined by boiling various extracts of *M. macrophylla* in 1% aqueous hydrochloric acid. The formation of a red precipitate indicated the presence of phlobatannins.

2.4. Quantitative Phytochemical analysis

2.4.1. *Estimation of total phenolic content*

The total phenolic content of *M. macrophylla* was estimated using standard method (Khan et al., 2012). Briefly, 5 mL of Folin-Ciocalteau's reagent (diluted ten-fold) was mixed with 1 mL of *M. macrophylla* extracts (0.25–8.0 mg/mL). After 5 min of incubation, 4 mL of sodium carbonate (0.115 mg/mL) was added to the mixture. The mixture was then incubated in the dark at room temperature for 2 h and the absorbance was recorded at 765 nm. Calibration curve was also prepared by mixing methanol solution of gallic acid (1 mL, 0.25–4.0 mg/mL) with the reagents above and absorbance was recorded at 765 nm using an ultraviolet-Visible spectrophotometer. The experiment was repeated three times and the total phenolic content was expressed as gallic acid equivalents (GAEs) mg/g of the dry extract.

2.4.2. *Estimation of total flavonoid content*

Total flavonoid content of various extracts of *M. macrophylla* was estimated according to the method previously described (Khan et al., 2012) with minor modifications. Briefly, 0.25 mL of *M. macrophylla* extracts (0.25–8.0 mg/mL) and standard quercetin solution was mixed with 75 µL of 5% (w/v) sodium nitrite solution and 1.25 mL of distilled water. After the addition of 150 µL of 10% (w/v) aluminum chloride, the solution was allowed to stand for 5 min followed by the addition of 0.5 mL of 1 M NaOH. The volume of the solution was made up to 2.5 mL using distilled water and mixed well. The absorbance was recorded immediately at 510 nm. The total flavonoid content of *M. macrophylla* was expressed in terms of quercetin equivalent (mg/g extract).

2.5. Liquid chromatography-mass spectrometry (LC-MS) Analysis

Liquid chromatography-mass spectrometry (LC-MS) was used to determine the chemical constituents of the aqueous extract of *M. macrophylla*. ACCUCORE HPLC (C18,150 X 2.1, 1.7 um) system coupled to a triple quadrupole tandem mass spectrometer (ACQ-TQD-QBB1152, Waters acuity PDA detector, Waters Corporation, Milford, MA, USA) equipped with an orthogonal ESI source was used to carry out the analysis. The mass acquisition spectra was recorded between 150-

2000 m/z and the source temperature and desolvation was set to 120 ℃ and 350 °C respectively. Running conditions were as follows: solvent composition was consisted of the mixture of two mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in 100% acetonitrile). Solvents were delivered at a total flow rate of 0.3 mL/min and a volume of 5 µL of the extracts were injected onto the analytical column for analysis. The detected chemicals were identified from their mass spectra by comparing the retention times of peaks with interpretation of MS fragmentation patterns from library data. All the data acquisitions were recorded and analyzed using OpenLynx and MassLynx MS Software.

2.6. Statistical analysis

Results are expressed as mean ± standard error of mean. One-way analysis of variance was performed to test the significant variations on phytochemical contents of various extracts followed by Tukey multiple comparisons of means. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Qualitative Phytochemical analysis

The present study revealed the presence of various naturally occurring compounds such as alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids in *M. macrophylla* extracts (Table 1.1). However, different solvent extracts of *M. macrophylla* contain different phytochemicals.

Table 1.1. Preliminary phytochemical screening of different extracts of *M. macrophylla.* ('+' indicates presence of phytochemical and '–' indicates absence of phytochemical).

Phytochemicals	Reagent	Colour Indication	MMCE	MMME	MMAE
Alkaloids	Dragendorff's Reagent	Reddish brown precipitate	-	+	+
Cardiac glycosides	Glacial acetic acid, ferric chloride, sulphuric acid	Brown ring	-	+	+
Saponins	Olive oil	Whitish Emulsion	-	+	+
Steroids	Sulphuric acid	Red Colour	-	+	-
Tannins	Ferric chloride	Brownish Green or blue-black	+	-	+
Terpenoids	Sulphuric acid	Reddish Brown	-	+	-
Phlobatannins	Hydrochloric acid	Red precipitate	-	-	-

4.2. Total phenolic and flavonoid contents

The total phenolic content of *M. macrophylla* extracts increased in a concentration-dependent manner (Figure 1.3). The total phenolic content showed significant variation between different extracts of *M. macrophylla* ($F_{2,6}$ = 252.14; $p < 0.001$). MMAE has the highest ($p < 0.001$) total phenolic content (387.61 ± 14.10 mg GAE/g) followed by MMME (301.29 ± 21.73 mg GAE/g) and MMCE (226.9 ± 21.04 mg GAE/g). Similarly, dose dependent increase in the total flavonoid content was observed among the various extracts of *M. macrophylla* (Figure 1.5). At 8 mg/ mL, there was a significant variation ($F_{2,6}$ = 1,189.4; $p < 0.001$) in the flavonoid contents among *M. macrophylla* extracts. MMAE has the highest flavonoid content (5,761.65 ± 38.5 mg quercetin equivalent/g) followed by MMME (4,864.68 ± 36.75 mg quercetin equivalent/g) and MMCE (4,830.09 ± 32.80 mg quercetin equivalent/g).

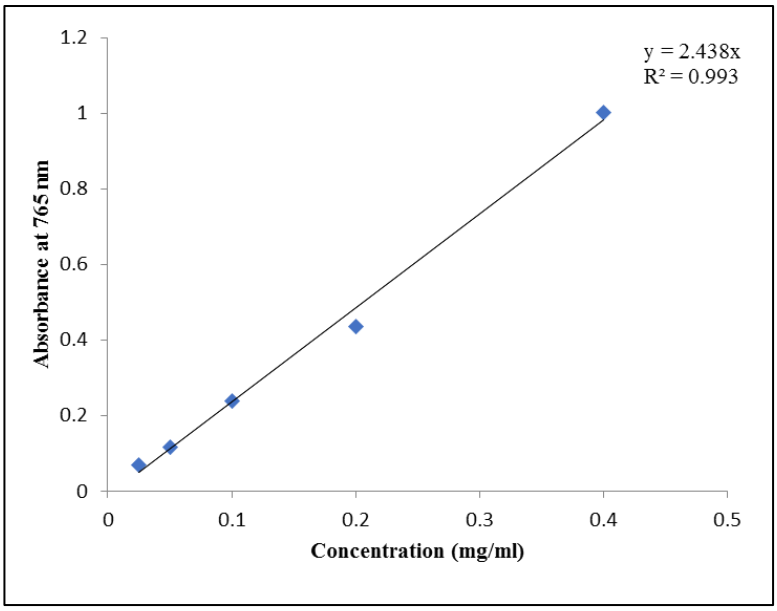


Figure 1.2. Standard graph of gallic acid.

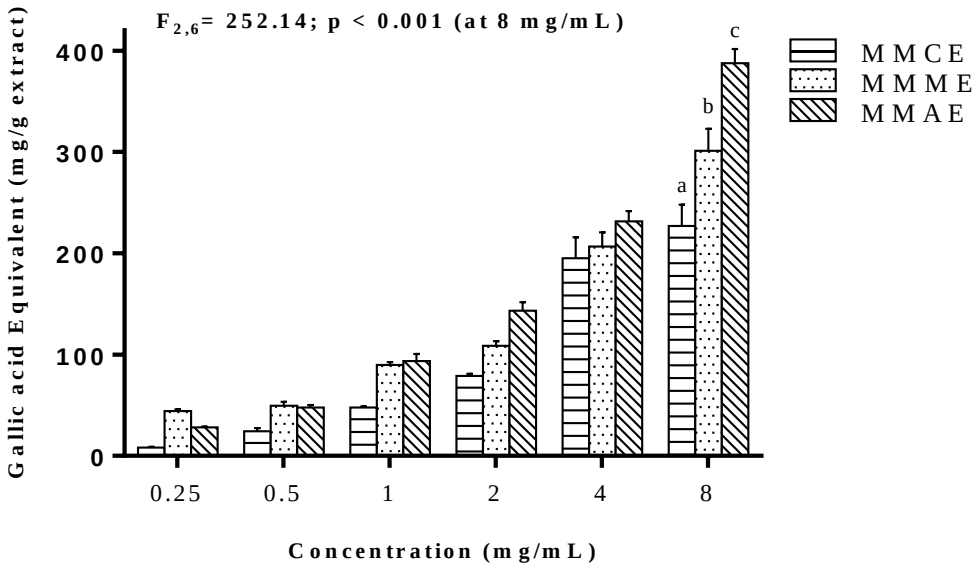


Figure 1.3. Total phenolic content of various extracts of *M. macrophylla* determined as GAE. Values are expressed as mean ± standard error mean (SEM), n= 3. Different letters indicate significant variation.

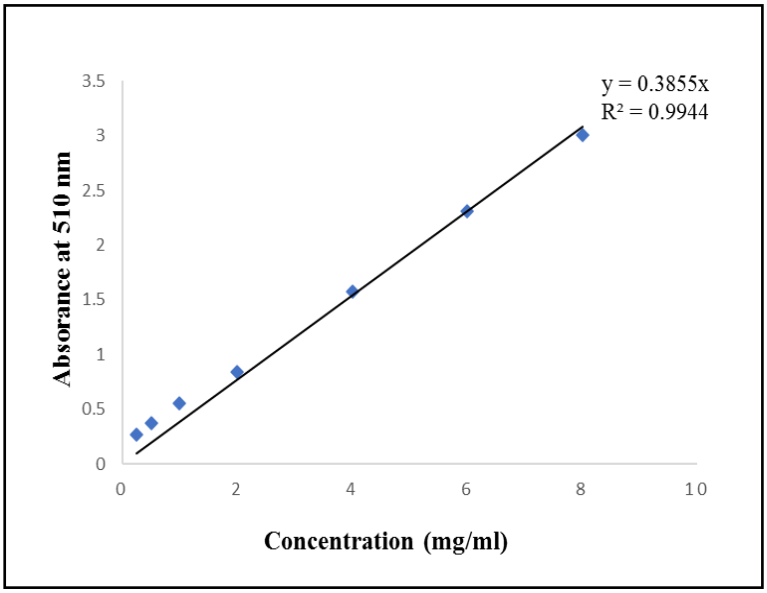


Figure 1.4. Standard graph of quercetin.

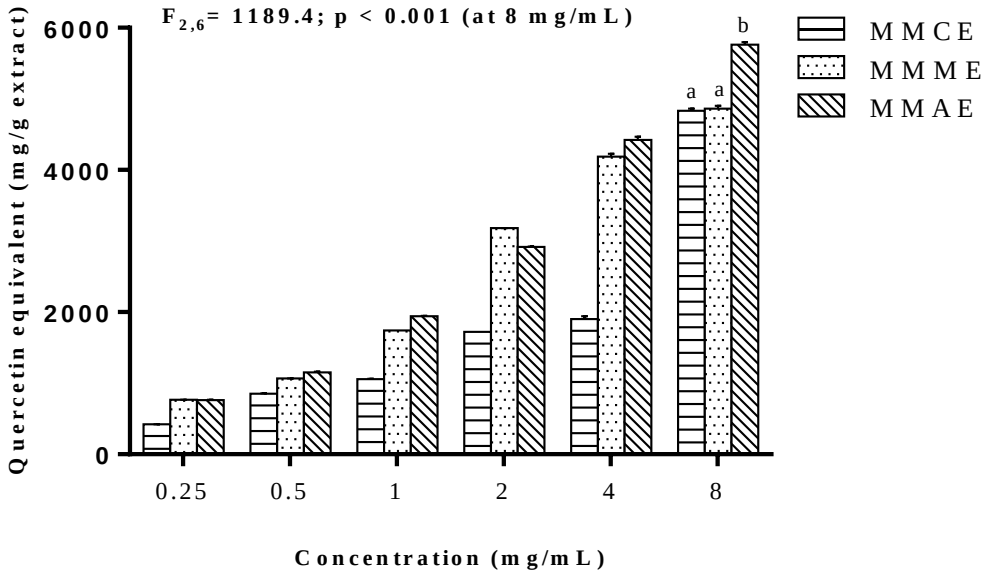


Figure 1.5. Total flavonoid content of various extracts of *M. macrophylla* determined as quercetin equivalent. Values are expressed as mean ± standard error mean (SEM), n = 3. Different letters indicate significant variation.

4.3. LC-MS analysis

The aqueous crude extract from the leaves of *M. macrophylla* (MMAE) was analyzed by LC-MS method. Untargeted analysis was performed to determine the chemical profile of the main compounds present in the extract. The tentative identification of the phytochemicals was performed based on the MS data (accurate mass, isotopic distribution and fragmentation pattern). The phytochemical analysis of MMAE by the LC-MS showed 73 major phytochemicals. The molecular formula, accurate and exact masses, retention time (RT) and characteristic fragment ions corresponding to each compound are shown in Table 1.2. The chemical structures of selected compounds with pharmacological applications are given in Figure 1.6.

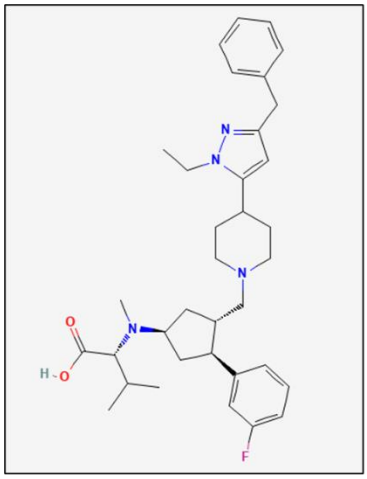
Table 1.2. LC-MS identification of major chemical constituents of aqueous extract of *M. macrophylla*.

ID	Compounds	Molecular weight	RT	Ions	Molecular formula
1	Pelargonic acid	158.23	1.14	[M-H]-	$C_9H_{18}O_2$
2	Bisphenol S	250.275	1.14	[M+H]+	$C_{12}H_{10}O_4S$
3	12-HETE	320.473	1.14	[M-H]-	$C_{20}H_{32}O_3$
4	Benzenesulfonic acid	158.17	1.14	[M-H]-	$C_6H_6O_3S$
5	MK-578	966.21	1.14	[M-H]-	$C_{35}H_{47}FN_4O2$
6	2,2-Dipyridyl	156.19	1.14	[M+H]+	$C_{10}H_8N_2$
7	Phenylphosphonic acid	141.08	1.14	[M-H]-	$C_6H_7O_3P$
8	Fumonisin Py2	705.82	1.14	[M-H]-	$C_{34}H_{56}O_{15}$
9	Norcantharidin	303.35	1.14	positive	$C_8H_8O_4$
10	Perfluorobutanoic acid	214.04	1.14	[M-H]-	$C_4HF_7O_2$
11	Perfluorooctanoic acid	414.07	1.14	[M-H]-	$C_8HF_{15}O_2$
12	DMST	214.28	1.14	[M-H]-	$C_9H_{14}N_2O_2S$
13	Perfluorobutyric acid	213.98	1.14	[M-H]-	$C_4HF_7O_2$
14	N-EtFOSAA	585.24	1.14	[M-H]-	$C_{12}H_8F_{17}NO_4S$
15	Perfluorotridecanoic acid	664.1	1.14	[M-H]-	$C_{15}H_{10}O_4$
16	Perfluoroheptanoic acid	364.06	1.14	[M-H]-	$C_7HF_{13}O_2$
17	Et-PFOSA-AcOH	557.18	1.14	[M-H]-	$C_{10}H_4F_{17}NO_4S$
18	Perfluorononanoic acid	464.08	1.14	[M-H]-	$C_9HF_{17}O_2$

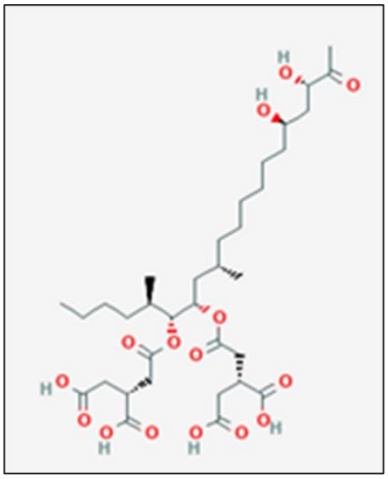
ID	Compounds	Molecular weight	RT	Ions	Molecular formula
19	Mefruside	382.9	1.14	[M+H]+	$C_{13}H_{19}ClN_2O_5S_2$
20	4-Sulfobenzoic acid	240.27	1.14	[M-H]-	$C_7H_6O_5S$
21	HT2 toxin	424.5	1.14	[M+H]+	$C_{22}H_{32}O_8$
22	Chaetocin	696.84	1.14	[M-H]-	$C_{30}H_{28}N_6O_6S_4$
23	Metamitron	202.24	1.14	[M-H]-	$C_{10}H_{10}N_4O$
24	Chlorcyclizine	300.82	1.14	[M+H]+	$C_{18}H_{21}ClN_2$
25	CPP_287.1310_14.5	286.8	1.14	[M+H]+	$C_{17}H_{19}ClN_2$
26	Harmalol	200.24	1.14	positive	$C_{12}H_{12}N_2O$
27	Cetirizine	388.89	1.14	[M+H]+	$C_{21}H_{25}ClN_2O_3$
28	Hydroxyzine	374.9	1.14	[M+H]+	$C_{21}H_{27}ClN_2O_2$
29	CPP_387.1471_17.0	386.9	1.14	[M+H]+	$C_{21}H_{23}ClN_2O_3$
30	Pterosin B	218.29	1.14	[M+H]+	$C_{14}H_{18}O_2$
31	Diclofenac	296.1	1.14	[M+H]+	$C_{14}H_{11}Cl_2NO_2$
32	Cilastatin	358.45	1.14	[M+Na]+	$C_{16}H_{26}N_2O_5S$
33	Cinchophen	249.26	1.14	[M+H]+	$C_{16}H_{11}NO_2$
34	Sulfapyridine	249.29	1.14	[M+H]+	$C_{11}H_{11}N_3O_2S$
35	7-Benzyloxytryptamine	266.34	1.14	[M+H]+	$C_{17}H_{18}N_2O$
36	Pethoxamide	295.81	1.14	[M+H]+	$C_{16}H_{22}ClNO_2$

ID	Compounds	Molecular weight	RT	Ions	Molecular formula
37	N(4)-AcSDM	352.37	1.14	[M-H]-	$C_{14}H_{16}N_4O_5S$
38	Aflatoxin G1	328.27	1.14	[M+Na]+	$C_{17}H_{12}O_7$
39	MUG trihydrate	352.29	1.14	Negative	$C_{16}H_{16}O_9$
40	Fipronil-desulfinyl	437.2	1.14	[M-H]-	$C_{12}H_4Cl_2F_6N_4$
41	Daphnoretin	352.29	1.14	Negative	$C_{19}H_{12}O_7$
42	Strychnine_N_Oxide	350.4	1.14	[M+H]+	$C_{21}H_{22}N_2O_3$
43	LTB4_20-Hydroxy	352.46	1.14	[M-H]-	$C_{20}H_{32}O_5$
44	Tebufenozide	352.5	1.14	[M-H]-	$C_{22}H_{28}N_2O_2$
45	Tetrahydroalstonine	352.4	1.14		$C_{21}H_{24}N_2O_3$
46	Vinpocetine	350.45	1.14	[M+H]+	$C_{22}H_{26}N_2O_2$
47	Jacobine N-oxide	367.4	8.183	[M+H]+	$C_{18}H_{25}NO_7$
48	Fipronil	437.15	8.183	[M+H]+	$C_{12}H_4Cl_2F_6N_4OS$
49	Ochratoxin B	369.37	8.183	[M-H]-	$C_{20}H_{19}NO_6$
50	Amisulpride	369.48	8.183	[M-H]-	$C_{17}H_{27}N_3O_4S$
51	Retrorsine N-oxide	367.39	8.183	[M+H]+	$C_{18}H_{25}NO_7$
52	Methylprednisolone	374.47	8.183	[M+Na]+	$C_{22}H_{30}O_5$
53	Mitragynine	398.49	8.183		$C_{23}H_{30}N_2O_4$
54	Thiazopyr	396.4	8.183	[M+H]+	$C_{16}H_{17}F_5N_2O_2S$
55	Sulfasalazine	398.39	8.183	[M-H]-	$C_{18}H_{14}N_4O_5S$

ID	Compounds	Molecular weight	RT	Ions	Molecular formula
56	BNPP	340.18	14.86	[M-H]-	$C_{14}H_{16}N_4O_5S$
57	Yohimbinic acid	340.4	14.86	negative	$C_{17}H_{12}O_7$
58	Behenic acid	340.58	14.86	[M-H]-	$C_{16}H_{16}O_9$
59	Brefeldin A	280.36	14.86	negative	$C_{12}H_4Cl_2F_6N_4$
60	NT 2 Toxin	466.52	14.86	[M-H]-	$C_{19}H_{12}O_7$
61	Pseudocopsinine	165.23	14.86		$C_{21}H_{22}N_2O_3$
62	Levamisole	204.29	22.38	[M+H]+	$C_{20}H_{32}O_5$
63	2,4-Di-tert-butylphenol	206.32	22.38	[M-H]-	$C_{22}H_{28}N_2O_2$
64	4-n-Octylphenol	206.32	22.38	[M-H]-	$C_{21}H_{24}N_2O_3$
65	DL-Thioctic acid	206.04	22.38	negative	$C_{22}H_{26}N_2O_2$
66	Diuron-desdimethyl	205.04	22.38	[M+H]+	$C_7H_6Cl_2N_2O$
67	Pyridoxal phosphate	247.14	22.38		$C_8H_{10}NO_6P$
68	Bufotenine	204.27	22.38	positive	$C_{12}H_{16}N_2O$
69	Ethambutol	294.31	22.38	[M+H]+	$C_{10}H_{24}N_2O_2$
70	Bromacil	261.11	22.38	[M+H]+	$C_9H_{13}BrN_2O_2$
71	4-Octylphenol	206.32	22.38	[M-H]-	$C_{14}H_{22}O$
72	4-tert-Octylphenol	296.33	22.38	[M-H]-	$C_{14}H_{22}O$
73	Phosphatidylcholine 16:0-22:6	790.1	23.647		$C_{46}H_{80}NO_7P$



(5) MK-578



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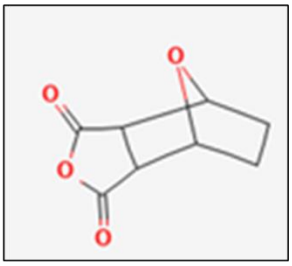

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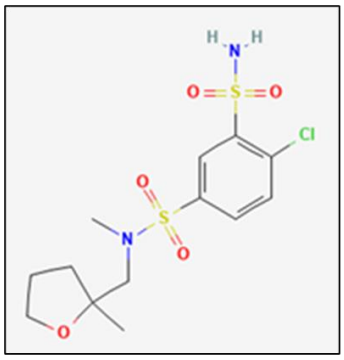

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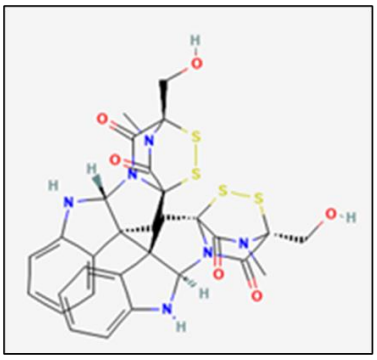

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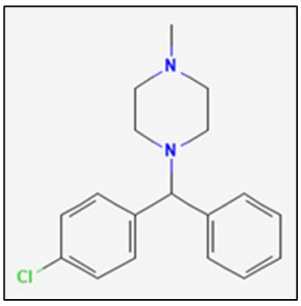

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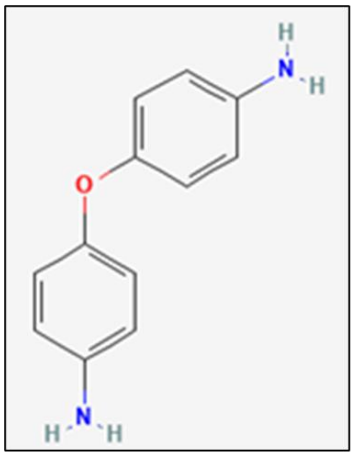

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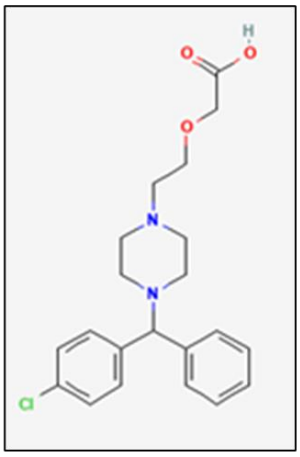

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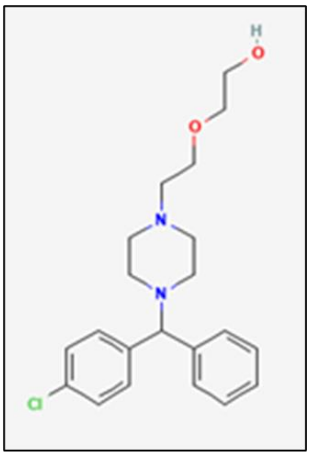
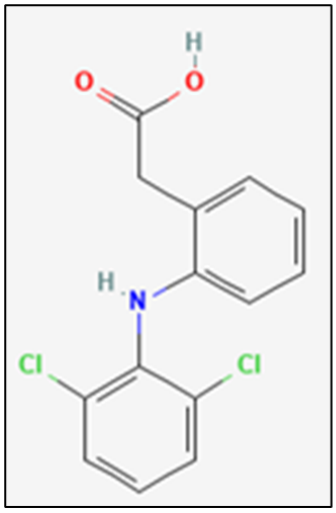
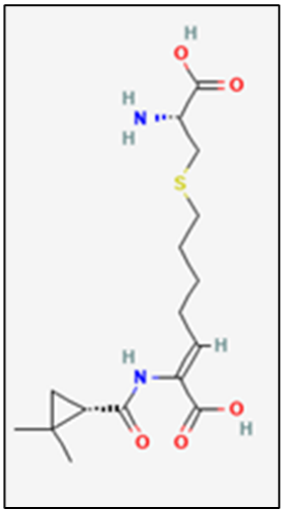


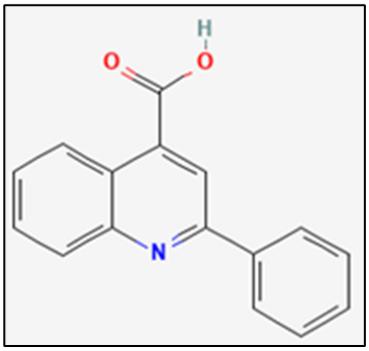
(28) Hydroxyzine



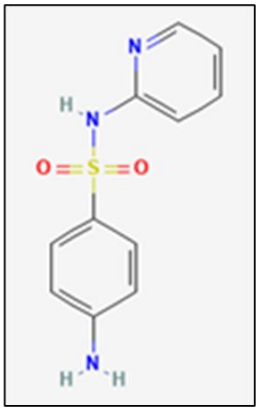
(31) Diclofenac



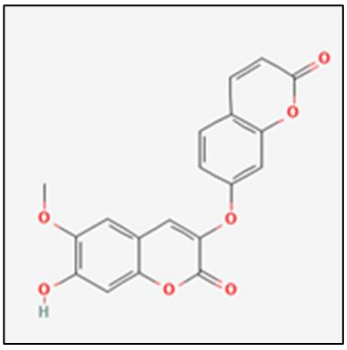
(32) Cilastatin



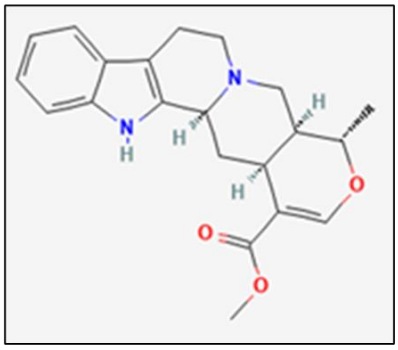
(33) Cinchophen



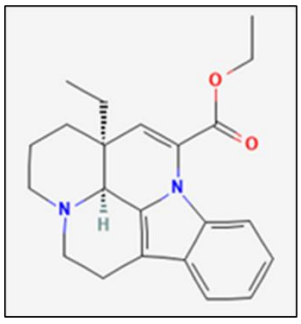
(34) Sulfapyridine



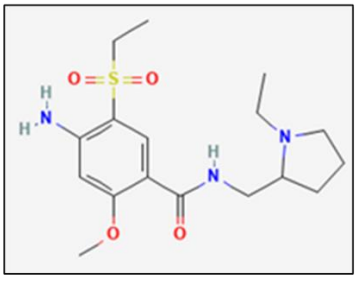
(41) Daphnoretin



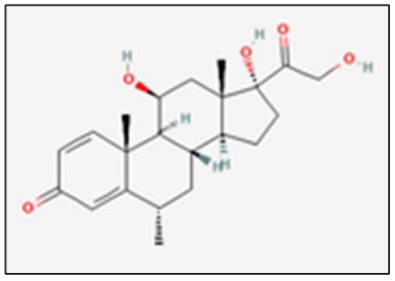
(45) Tetrahydroalstonine



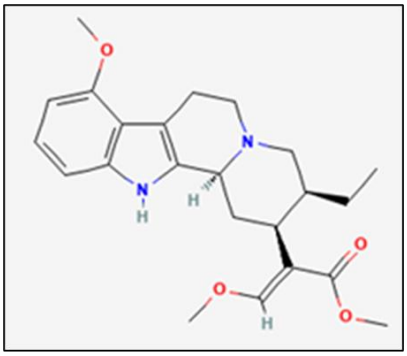
(46) Vinpocetine



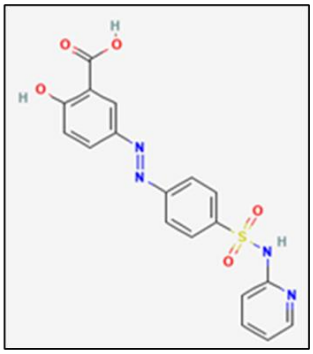
(50) Amisulpride



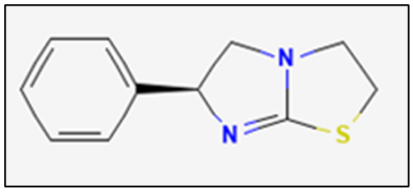
(52) Methylprednisolone



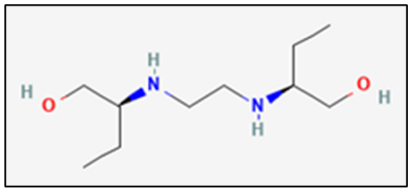
(53) Mitragynine



(55) Sulfasalazine



(63) Levamisole



(70) Ethambutol

Figure 1.6. Structure of compounds identified from aqueous extract of *M. macrophylla*.

4. DISCUSSION

In qualitative phytochemical screening, *M. macrophylla* extract was screened positive for eight phytochemicals viz. alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids (Table1.1). These compounds, belonging to the polyphenolic group, have been reported to exhibited several pharmaceutical potentials. For example, tannins as antioxidants and anti-inflammatory (Dolara et al., 2005), alkaloids as anti-hypertensive and anti-malarial (Wink et al., 1998), terpenoids as anti-ulcer and anti-microbial (Cowan, 1999; Dudareva et al., 2004), and saponins as anti-microbial and anti-inflammatory (Gangwar et al., 2014; Lacaille-Dubois and Wagner, 2000). Tumorigenesis as well as the progression of tumor cell progression were reported to be hindered by alkaloids (Sun et al., 2009; Diogo et al., 2011). Saponins are a group of structurally diverse phytochemicals found in microorganisms, higher plants and marine organisms. The anticancer activity of saponins is mediated by a variety of molecular mechanisms. It should be noted that the mechanism of anticancer action of saponins is strongly related to the nature of the structural moieties, including the length and linkage of the glycosidic chain, aglycone moiety, the presence of a functional carboxylic group on the aglycone chain, position of the hydroxyl group, the number of sugar molecules and hydroxyl group stereo-selectivity and the type of sugar molecule on the glycine chain is strongly related to the mechanism of saponin anticancer activity of saponin (Gevrenova et al., 2015; Xu et al., 2013). Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers found throughout the plant world in the form of free triterpenoids, phytosterols, triterpenic glycosides (saponins) and their precursors, all of which have potential disease preventative and treatment capabilities (Mahato et al., 1988). Because triterpenoids are becoming more popular as a source of therapy for a variety of chronic disorders, multiple studies have suggested that both natural (Laszczyk, 2009; Rabi and Bishayee, 2009) and synthesised triterpenoids (Liby et al., 2007; Petronelli et al., 2009) may have anticancer properties. The anticancer activities of tannins, such as ellagiatannin, have been reported to be involved in upregulation of cyclin E and downregulation of cyclins A and B1, obstruction of cell cycle at the S-phase and activation of apoptosis via the

intrinsic pathway through downregulation of Bcl-X_L and cytosolic mitochondrial secretion of cytochrome C, as well as caspase-3 and caspase-9 induction (Larrosa et al., 2006). Steroids are commonly used in oncology, from curative to supportive treatment. They have been shown to have an anticancer (i.e., lymphoma or leukemia management) or anti-swelling (i.e., brain metastases or brain tumor) effect, and are thought to help with refractory symptoms including dyspnea or gastrointestinal (GI) blockage (Lossignol, 2016). Certain cardiac glycosides such as digitoxin have been shown to induce significant and selective anticancer effects *in vitro* and *ex vivo*, (Haux, 1999), which may occur at concentrations typically observed in the plasma of patients treated with these pharmaceuticals (Lopez-Lazaro et al., 2005). Several cardiac glycosides (e.g., ouabain, digoxin and bufalin) have been identified as effective inhibitors of cancer cell proliferation in recent high-throughput drug library screenings (Zhang et al., 2008; Wang et al., 2014).

The genus *Mussaenda* has been reported to be a good source of phytochemicals. Flavonoids such as Aureusidin-4-glucoside, Aureusidin-6-glucoside, Aureusidin4,6-diglucoside, and Cernuoside have been isolated from *M. hirsutissima* (Harborne et al., 1983). Quercetin and hypenin were also reported to occur in *M. frondosa* (Lakshmi et al., 1985). The root bark of *M. macrophylla* has been reported to contain triterpenoid glycosides including 3-O-β-D-glucopyranosyl-28-Oα-L-rhamnopyranosyl-16α-hydroxy-23-deoxy-protobassicacid, 28-O-β-D-glucopyranosyl-16α-hydroxy-23-deoxyprotobassic acid, 3-O-β-D-glucopyranosyl-28-O-α-L-rhamnopyranosyl16α-hydroxyprotobassic acid, 3-O-acetyloleanolic acid, and 3-O-acetyldaturadiol. The triterpenoid glycosides isolated from *M. macrophylla* were reported to exhibit inhibitory action against *Porphyromonas gingivalis* (Kim et al., 1999), *Salmonella paratyphi* and *Aspergillus niger* (Chowdhury et al., 2013). Consistent to previous studies, the present study indicated the presence of significant amounts of flavonoid and phenolic compounds in *M. macrophylla* extracts. The phenolic compounds have been reported to have antioxidants property due to their conjugated ring structures and the presence of hydroxyl groups. Their scavenging activity is mostly achieved by hydrogenation and can even stabilize free radicals by complexing with oxidizing species (Amic et al., 2003; Diplock, 1997). Compounds containing phenols have been reported to exhibit cardio-protective, hepatoprotective,

anti-allergenic, anti-inflammatory, anti-microbial, antithrombotic, anti-atherogenic, and vasodilatory effects (Alpinar et al., 2009; Middleton et al., 2000; Siama et al., 2018). Similarly, flavonoids are having antioxidants, free radical scavenging, anti-inflammatory, metal chelating and lipid peroxidation inhibitory properties due to their unique structural conformations (Cook and Samman, 1996). Due to their scavenging or chelating activities, flavonoids have been reported to serve as health promoting compound (Havsteen, 1983) and protect humans against several diseases like atherogenesis, carcinogenesis, hepatotoxicity and thrombosis (Tiwari, 2001).

LC-MS analysis identified 73 compounds amongst which several of them are well known with respect to their pharmacological applications. Norcantharidin has dual activities, it can be used as a chemotherapeutic drug for cancer treatment, and it also possesses significant antiplatelet activity to treat thromboembolic disorders (Hsia et al., 2018). Chaetocin has been shown to exhibit a wide variety of anticancer effects *in vitro* and *in vivo* in numerous investigations. It has been reported to inhibit tumor angiogenesis, invasion, and migration by regulating multiple signaling pathways related to tumor initiation and progression, inducing cancer cell apoptosis (intrinsic and extrinsic), enhancing autophagy, inducing cell cycle arrest, and inhibiting tumor angiogenesis, invasion, and migration, according to several studies (Jiang et al., 2021). Several investigations have shown that daphnoretin has anticancer properties. According to these studies, the anticancer effects of daphnoretin are mediated via the reduction of cancer cell growth, the induction of G2/M-phase arrest, and apoptosis (Lee et al., 1981; Gu and He, 2012; Yang et al., 2014). In A549 lung cancer cells, daphnoretin inhibits growth while increasing apoptosis (Jiang et al., 2014). Fumonisins could be utilised as templates for therapeutic compounds to treat disorders such as Farber's disease, which are linked to sphingolipid turnover (lysosomal storage disease). It was found to be an efficient diuretic that resulted in much higher water and electrolyte excretion in patients than an equal dose of frusemide (Nair, 1998). Many Asian and European countries have clinically used vinpocetine in prevention and treatment of stroke, senile dementia, and memory problems. Vinpocetine is evidently a multi-action drug with a wide range of pharmacological targets. Its several functions, which include vasodilation, anti-oxidation, anti-inflammation, anti-thrombosis, and anti-remodeling, may act together

to produce synergistic therapeutic effects, resulting in significant advantages for multifactorial cerebrovascular and cardiovascular illnesses (Zhang et al., 2018).

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CHAPTER III

Free radical scavenging activities and antioxidative potential of various extracts of *Mussaenda macrophylla* Wall. *in vitro* and *ex vivo* systems

ABSTRACT

In this chapter, free radical scavenging activities and antioxidative potential of various extracts of *Mussaenda macrophylla* was investigated. The antioxidative potential of *M. macrophylla* extracts was measured by their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions ($O_2^{\bullet-}$), and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in a cell free system. The aqueous extract of *M. macrophylla* possessed the highest scavenging activities for DPPH, $O_2^{\bullet-}$, and ABTS with IC_{50} of 25.92 ± 0.33 μg/mL, 4.12 ± 0.94 μg/mL, and 17.20 ± 0.50 μg/mL, respectively. Furthermore, the scavenging activities of the aqueous extract of *M. macrophylla* against ABTS and $O_2^{\bullet-}$ were found to be more effective than ascorbic acid which was used as standard. The total reducing power of *M. macrophylla* extracts was also determined by measuring the transformation of Fe^{3+} into Fe^{2+} and the methanolic extract was found to exhibit the highest reducing power. The extracts were also analyzed for their anti-hemolytic and inhibitory effect on lipid peroxidation in an *ex vivo* condition using mice erythrocyte and liver, respectively. The aqueous extract of *M. macrophylla* showed the highest inhibitory activities against mice erythrocyte hemolysis and lipid peroxidation in the liver homogenate with an inhibition rate of 80.53% and 65.33%, respectively.

1. INTRODUCTION

Free radicals are essential bio-regulatory molecule required for several physiological functioning of the body such as regulation of gene expression, immune response, and cellular growth (Droge, 2002; Tochhawng et al., 2013), representing an important part of aerobic life (Tiwari, 2001). Due to their highly reactive property, over-production of the free radicals including superoxide anion radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) can hamper the body antioxidant defense systems leading to a condition called "oxidative stress". Oxidative stress is a condition wherein oxidants weigh down the protective system of antioxidant, which may result in cellular lipid peroxidation and DNA damage. It is found to be closely associated with various lifestyle-related disorders including neurodegenerative disorders, arthritis, coronary heart disease, inflammation, diabetes, and cancer (Maxwell, 1995; Statdtman, 1992; Valko et al., 2006). Superoxide ($O_2^{\bullet-}$) is the main ROS, generated via the reduction of oxygen (O_2) molecules through NADPH oxidase during mitochondrial respiratory electron transport chain. This process ends in an accumulation of ROS like hydrogen peroxide (H_2O_2), generated by the enzyme superoxide dismutase. Throughout oxidative stress, excess H_2O_2 produce toxic ROS like hydroxyl ions ($^{\bullet}OH$) via a catalytic process in the presence of reduced metals (Ni, Cu and Fe) (Reuter et al., 2010). During these conditions, the reactive molecules were shown to target nucleic acids, proteins and lipids which results in an alteration in cell structures and functions (Chen et al., 2013). Due to reduced glutathione content and high content of polyunsaturated fatty acids in membranes of neuronal cells, lipid peroxidation has been observed in patients with stroke, Alzheimer's disease (AD) and Parkinson's disease (PD). The most reactive species are the hydroxyl ions that damage DNA by producing 8-hydroxydeoxyguanosine which is a known radical attack biomarker (Reuter et al., 2010). Oxidative alterations of low-density lipoprotein (LDL) in the arterial walls were considered to induce endothelial dysfunction, the first stage of atherosclerosis (Vogiatzi et al., 2009). This increased level of ROS can be lowered to normal levels by enhancing the cellular antioxidants which promote a protective response in the cells. On the other hand, low levels of ROS play an important role in cellular signaling pathways that stimulate cell survival (Devasagayam et al., 2004). In the

presence of reduced ROS, cells produce enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) that modulate free radicals into H_2O_2 and convert them into H_2O (Reuter et al., 2010; Chen et al., 2013). Despite the presence of small antioxidant molecules (e.g., glutathione) and several antioxidant enzymes (e.g., glutathione peroxidise, glutathione reductase, catalase, superoxide dismutase) in our body, these agents are not sufficient to maintain the normal redox status during increased oxidative stress (Seifried et al., 2007). Therefore, maintenance of optimal body function and redox homeostasis in cells may require exogenous antioxidants supplementation. The most widely used synthetic antioxidants at present are butylated hydroxytoluene, butylated hydroxyanisole, tert-butylhydroquinone and propyl gallate. However, synthetic antioxidants have been shown to have harmful effects in subsequent research and as a result, their usage has been restricted. Furthermore, they are believed to cause liver damage and act as carcinogens in laboratory animals (Anagnostopoulou et al., 2006). The search for new antioxidative products with fewer adverse effects is a highly active research field. As a result, it is desirable to develop and employ more effective antioxidants of natural origin. Medicinal plants have been used for human healthcare and represent the mainstay of traditional systems of medicine. The importance of plants as natural sources of antioxidants and free radical scavengers has been reported earlier by various researchers (Auddy et al., 2003; Poullain et al., 2004; Siama et al., 2018; Shantabi et al., 2014). Many modern drugs used for the treatment of several diseases have been isolated from different medicinal plants (Cragg and Newman, 2013). Consumption of natural antioxidants, such as those derived from plants, has been reported to lower the risk of cancer and many lifestyle-related disorders (Gerber et al., 2002).

Mizoram, a state in North-East India, with its rich biodiversity offers a number of medicinal plants. One such plant is *M. macrophylla*, a member of the Rubiaceae family, locally known as Vakep. *M. macrophylla* is a flowering shrub, endemic to south-east Asia, reported to occur in India, China, and Myanmar (Manandhar, 2002). *M. macrophylla* has been traditionally used for the treatment of various health problems such as coughs, chronic ulcer, diarrhea, dysentery, indigestion, cancers, and even for the treatment of snake bites (Rosangkima and Jagetia, 2015; Sharma et

al., 2001). Among the genus *Mussaenda*, *M. frondosa* (Siju et al., 2010), *M. glabrata* (Menon and Sasikumar, 2011), and *M. roxburghii* (Aktar et al., 2014; Islam et al., 2015) have been reported to have multi-pharmaceuticals importance such as antioxidants, anti-inflammatory, and thrombolytic activity. Despite the extensive use of *M. macrophylla* as traditional medicine, there has been no scientific validation of their pharmaceutical property. Therefore, we aimed to investigate the free radical scavenging and antioxidative potentials of various solvent extracts of *M. macrophylla* both *in vitro* and *ex vivo*.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Quercetin dihydrate, gallic acid, 2,2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nicotinamide adenine dinucleotide, 2-deoxyribose, phenazine methosulfate, nitro blue tetrazolium (NBT), disodium hydrogen phosphate, ferric chloride, sodium nitrite, potassium persulfate, and hydrogen peroxide (H_2O_2) were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). Thiobarbituric acid and 1,1-diphenyl2-picrylhydrazyl (DPPH) radicals were obtained from Sigma Aldrich Inc (Louis, Germany). Folin–Ciocalteau's reagent, trichloroacetic acid (TCA), sodium hydroxide, sodium carbonate, ascorbic acid (ASA), and ferrous sulfate were purchased from SD fine–Chem Ltd. (Mumbai, India). Aluminum chloride and sodium dihydrogen phosphate were obtained from Merck Specialities Pvt., Ltd. (Mumbai, India). Ethylenediamine tetra acetic acid was obtained from Qualigens Fine Chemicals (Mumbai, India). Potassium ferricyanide was purchased from Loba Chemie Pvt., Ltd. (Mumbai, India).

2.2. Collection of plant and preparation of extracts

Collection and preparation of *M. macrophylla* was carried out as described in the previous chapter (Section 2.2) to obtain the plant extracts and the chloroform (MMCE), methanolic (MMME) and aqueous (MMAE) extracts were used for the experiment.

2.3. Determination of free radical scavenging activity *in vitro*

The ability of different extracts of *M. macrophylla* to inhibit the generation of various free radicals was carried out as described below:

2.3.1. *DPPH radical scavenging activity*

The scavenging activity of *M. macrophylla* extracts for DPPH radical was determined according to Lalhminghlui and Jagetia (2018) with minor modifications. Briefly, 0.5 mL of various extracts of *M. macrophylla* (1–400 µg/mL) was mixed with 1 mL of methanol solution of 0.1 M DPPH followed by 30 min incubation in

the dark. The absorbance of the solution at 523 nm was compared with the control. The scavenging activity of the plant extract against DPPH was expressed as IC_{50} which is the concentration (µg/mL) of extract at which 50% of the DPPH radicals were inhibited. ASA was used as the standard. The test was repeated at all concentrations of each sample in triplicate. The scavenging activity was then calculated based on the percentage of DPPH radicals scavenged using the formula:

Scavenging (%) = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$

Where A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

2.3.2. *Superoxide radical scavenging activity*

Superoxide scavenging activity was determined by the NBT reduction method (Lalhminghlui and Jagetia, 2018) with minor modifications. In brief, the reaction mixture was prepared using 0.2 mL of NBT [1 mg/mL in dimethyl sulfoxide (DMSO)] and 0.6 mL of plant extract (1–800 µg/mL). Then, the volume of the mixture was made up to 2.8 mL using 2 mL of alkaline DMSO (1 mL DMSO in 5 mM NaOH). The absorbance of the mixture was recorded at 560 nm and pure DMSO was used as blank. ASA served as the standard and the ability of *M. macrophylla* extracts to scavenge the superoxide radical was calculated.

% scavenging = $(A_e - A_o/A_e) \times 100$

Where A_o is the absorbance without plant extract and A_e is the absorbance with the plant extract.

2.3.3. *ABTS radical scavenging activity*

The scavenging activity of *M. macrophylla* against ABTS was determined using the method of Khan et al. (2012). Briefly, 5 mL each of 7 mM ABTS and 2.45 mM potassium persulfate were mixed for a stock solution. A stock solution was then incubated at room temperature in the dark for 12 h so as to yield a dark-colored solution that contains $ABTS^{\cdot+}$ radicals. A freshly prepared working solution consists of a stock solution diluted with 50% methanol having an initial absorbance of 0.70 (±0.02) at 745 nm. $ABTS^{\cdot+}$ radicals scavenging activity was then assessed by mixing 150 µL of different fractions of various extracts of *M. macrophylla* (1–200 µg/mL) with 1.5 mL of ABTS working solution. The decrease in absorbance was measured

immediately at 745 nm. The test was repeated at all concentrations of each sample in triplicate. ASA served as the standard. The scavenging activity of the plant extract was then calculated using the formula: Scavenging (%) = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$

Where A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

2.3.4. *Reducing power*

The reducing power of *M. macrophylla* extracts was estimated using the method given by Adjimani and Asare (2015) with minor modifications. Briefly, 2.5 mL each of 0.2 M phosphate buffer (pH—6.6) and 1% potassium ferricyanide solution were mixed with *M. macrophylla* extracts (1–400 µg/mL; dissolved in their respective solvent). After incubation of the mixture at 50°C for 20 min, 2.5 mL of 10% TCA was added. The mixture was then centrifuged at 3,000 rpm for 10 min. Equal volume of distilled water was added to the supernatant followed by 0.5 mL of 1% ferric chloride solution. Absorbance of the mixture was measured at 700 nm. The increase in absorbance indicated increasing reducing power of the extract.

2.4. *Ex-vivo* antioxidant assay

2.4.1. *Anti-hemolytic activity*

The inhibition of mice erythrocyte hemolysis by various extracts of *M. macrophylla* was measured to determine their antioxidative potential (Zhou et al., 2014). Blood was collected from Swiss albino mice of the same age group (10–12 weeks) and body weights (25–27 g) by heart puncture in a heparinized tube. The mice erythrocyte hemolysis was induced with H_2O_2 that serve as a free radical initiator. A mixture was prepared by adding 0.5 mL of 5% (v/v) suspension of red blood cells (RBC) in phosphate buffered saline (PBS), 0.4 mL (0.5 mg/mL) of different extracts of *M. macrophylla*, and 100 µL of 1 mol/L H_2O_2. The reaction mixture was gently mixed while being incubated at 37 °C for 3 h. It was then diluted with 4 mL of PBS and centrifuged at 2,000 rpm for 10 min. The supernatant was collected and

absorbance was recorded at 540 nm. The rate of inhibition of erythrocyte hemolysis was then calculated.

Inhibition rate (%) = $[1 - (A_1 - A_2)/A_0] \times 100$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the solution containing the plant extract, and A_2 is the absorbance without RBC.

2.4.2. *Inhibition of lipid peroxidation*

Lipid peroxidation inhibitory potential of *M. macrophylla* extracts was measured according to the method given by Rajinder et al. (2010) using mice liver. The liver was excised from Swiss albino mice and 1% liver homogenate was prepared and centrifuged at 3,000 rpm at 4°C for 10 min. 0.5 mL of supernatant was mixed with 0.5 mL (0.5 mg/mL) of *M. macrophylla* extracts, 0.25 mL each of 0.5 mol/L $FeCl_2$ and H_2O_2, and incubated at 37°C for 1 h. Absorbance was measured at 535 nm and the rate of inhibition of lipid peroxidation was calculated using the formula:

Inhibition rate (%) = $[1 - (A_1 - A_2)/A_0] \times 100$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the solution containing the plant extract, and A_2 is the absorbance without liver homogenate.

2.5. Animals

Colony of inbred Swiss albino mice is being maintained under standard environmental conditions of temperature (22°C ± 5°C) and light (12 h of light and dark, respectively) (Frontier Euro Digital Timer, Taiwan) at the Animal House, Department of Zoology, Mizoram University, India. The animals were having free access to food and water. Caring and handling of animals were carried out based on the guidelines given by WHO, Geneva, Switzerland. The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZUIAEC/2018/09) and CPCSEA, New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

2.6. Statistical analysis

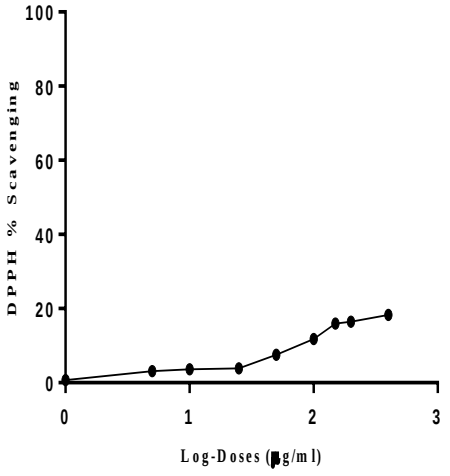
Results are expressed as mean ± standard error of mean. One-way analysis of variance was performed to test the significant variations on the antioxidative potential of various extracts followed by Tukey multiple comparisons of means. The IC_{50} values were calculated by plotting the % inhibition of free radicals against the log doses using GraphPad Prism software version 6.0. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

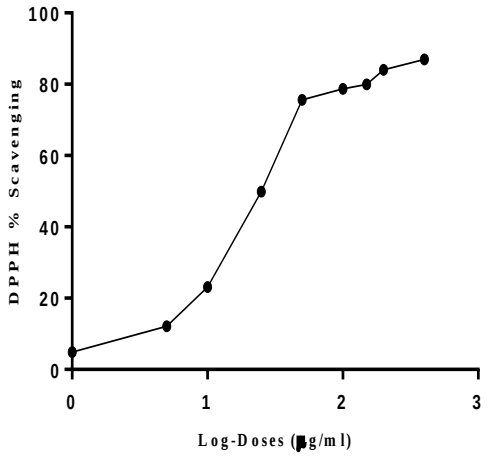
3.1. *In vitro* free radical scavenging activities of *M. macrophylla*

3.1.2. *DPPH radical scavenging activity*

The methanolic and aqueous extracts of *M. macrophylla* showed a dose-dependent increase in DPPH radicals scavenging activity as indicated by the discoloration of DPPH. Maximum scavenging activity for both methanolic and aqueous extracts was observed at a concentration of 400 µg/mL. Log-doses of various extracts of *M. macrophylla* and standard ASA were plotted against DPPH inhibition (%) for the calculation of IC_{50} (Figure 2.1). The aqueous extract (IC_{50}: 25.92 ± 0.33 µg/mL) and methanolic extract (IC_{50}: 26.43 ± 0.55 µg/mL) were found to have similar DPPH radical scavenging activity (Figure 2.2), whereas the IC_{50} of chloroform extract could not be determined within the given concentration.



'A' (MMCE)



'B' (MMME)

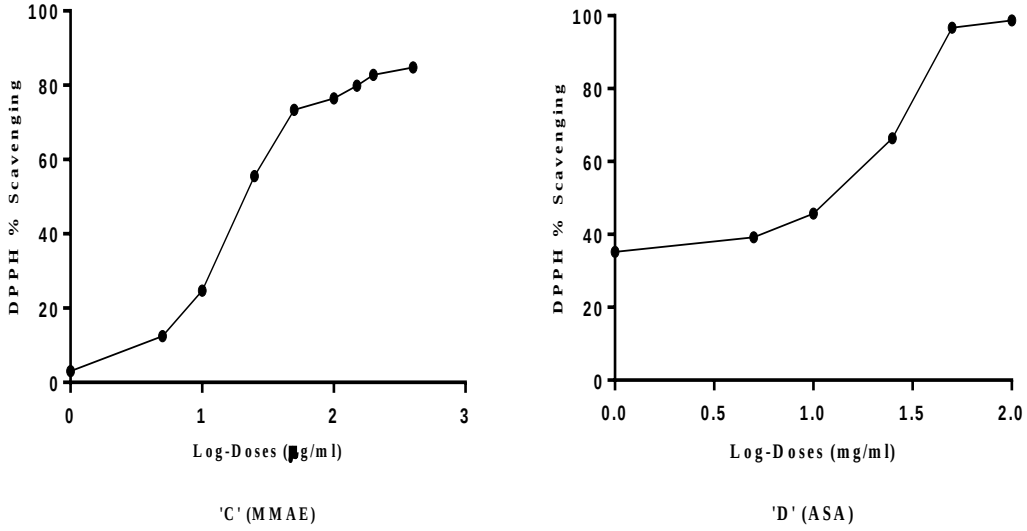


Figure 2.1. Plot of log-doses of (A) *M. macrophylla* chloroform extract (MMCE); (B) *M. macrophylla* methanolic extract (MMME); (C) *M. macrophylla* aqueous extract (MMAE); (D) Ascorbic acid (ASA) against DPPH inhibition (%) for the calculation of IC_{50}.

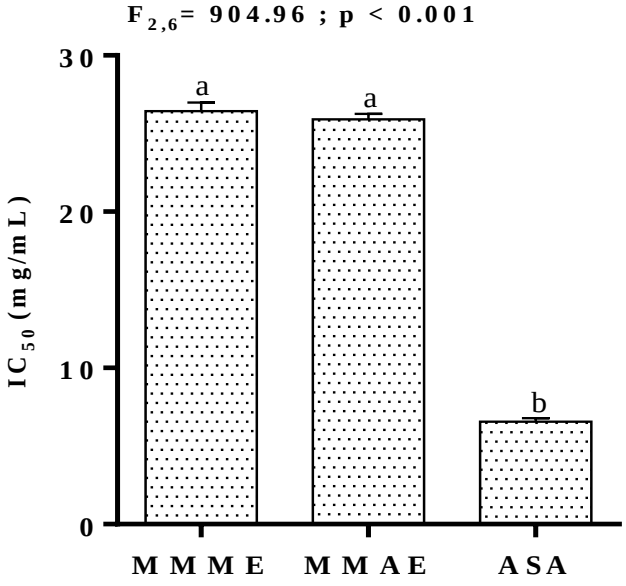


Figure 2.2. IC_{50} (µg/mL) for DPPH of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

3.1.3. *Scavenging activity of superoxide radical*

Various extracts of *M. macrophylla* showed a dose dependent increase in inhibition of superoxide radical ($O_2^{\bullet-}$) generation. Maximum $O_2^{\bullet-}$ scavenging activity was recorded at 400 µg/mL in all the extracts of *M. macrophylla*. Figure 2.3 represents the plot of log-doses of various extracts of *M. macrophylla* and standard ASA against $O_2^{\bullet-}$ scavenging activity for the calculation of IC_{50}. Aqueous extract possessed the highest scavenging activity (IC_{50}; 4.12 ± 0.94 µg/mL) followed by methanolic extract (IC_{50}; 7.83 ± 1.2 µg/mL) and chloroform extract (IC_{50}; 40.24 ± 3.5 µg/ mL). The $O_2^{\bullet-}$ scavenging activity of MMAE was even better than the ASA (IC_{50}; 8.65 ± 1.6 µg/mL) (Figure 2.4).

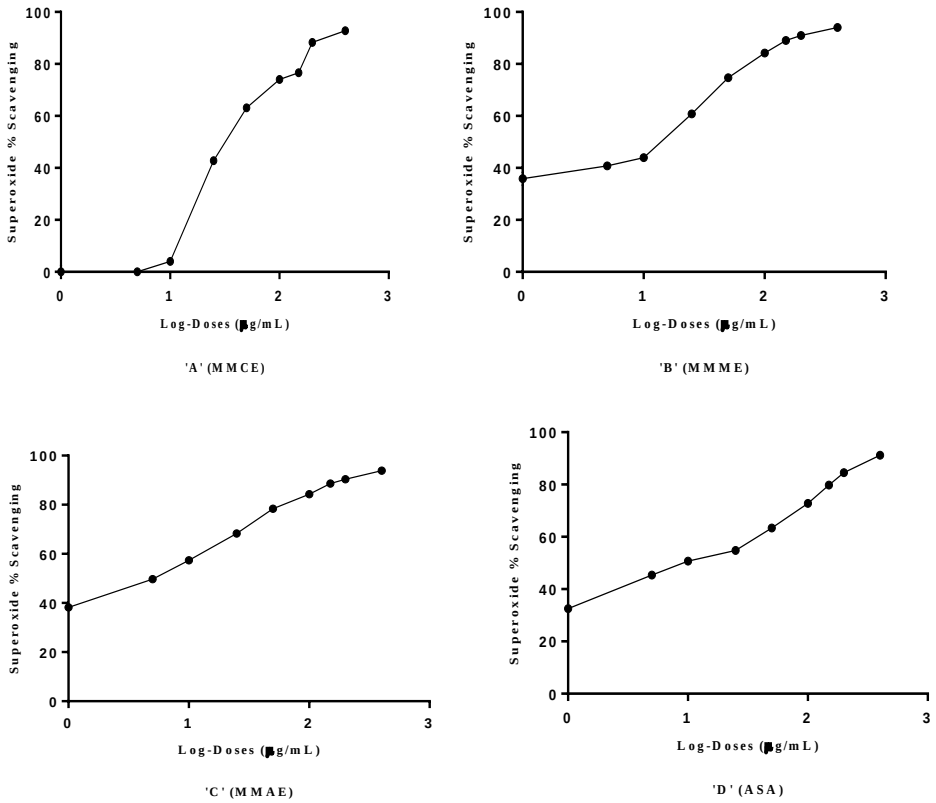


Figure 2.3. Plot of log-doses of (A) *M. macrophylla* chloroform extract (MMCE); (B) *M. macrophylla* methanolic extract (MMME); (C) *M. macrophylla* aqueous extract (MMAE); (D) Ascorbic acid (ASA) against superoxide radical inhibition (%) for the calculation of IC_{50}.

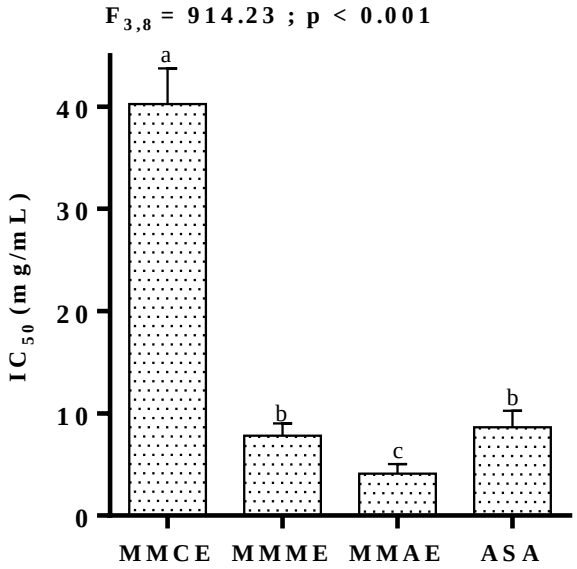


Figure 2.4. IC_{50} (µg/mL) for superoxide radical of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

3.1.4. *ABTS radical scavenging activity*

$ABTS^{\bullet+}$ radical scavenging property of *M. macrophylla* extracts increased in a concentration-dependent manner as indicated by discoloration of the $ABTS^{\bullet+}$. Maximum scavenging activity was recorded at 200 µg/mL for all the extracts of *M. macrophylla*. Log-doses of various extracts of *M. macrophylla* and standard ASA were plotted against $ABTS^{\bullet+}$ inhibition (%) for the calculation of IC_{50} (Figure 2.5). MMAE possessed the highest scavenging activity (lowest IC_{50}; 17.20 ± 1.5 µg/mL) followed by MMME (IC_{50}; 25.95 ± 1.8 µg/mL) and MMCE (IC_{50}; 197.16 ± 5.5 µg/mL). The aqueous and methanolic extracts of *M. macrophylla* were found to be more effective in scavenging $ABTS^{\bullet+}$ radical than the standard ASA (IC_{50}; 39.70 ± 1.2 µg/mL) (Figure 2.6). There was significant positive correlation between phenol (r^2 = 1.00; $p < 0.001$) and flavonoid (r^2 = 0.99; $p < 0.001$) contents of *M. macrophylla* extracts and their free radicals (DPPH, $O_2^{\bullet-}$ and ABTS) scavenging activities.

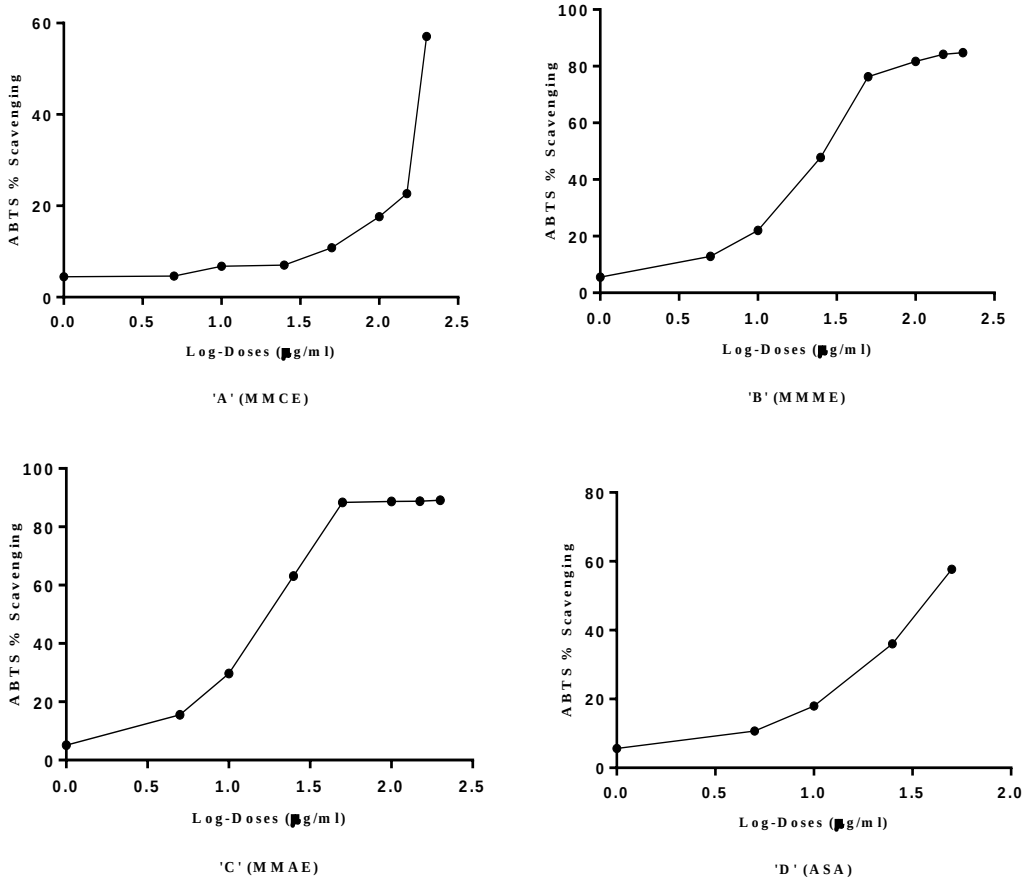


Figure 2.5. Plot of log-doses of (A) *M. macrophylla* chloroform extract (MMCE); (B) *M. macrophylla* methanolic extract (MMME); (C) *M. macrophylla* aqueous extract (MMAE); (D) Ascorbic acid (ASA) against ABTS inhibition (%) for the calculation of IC_{50}.

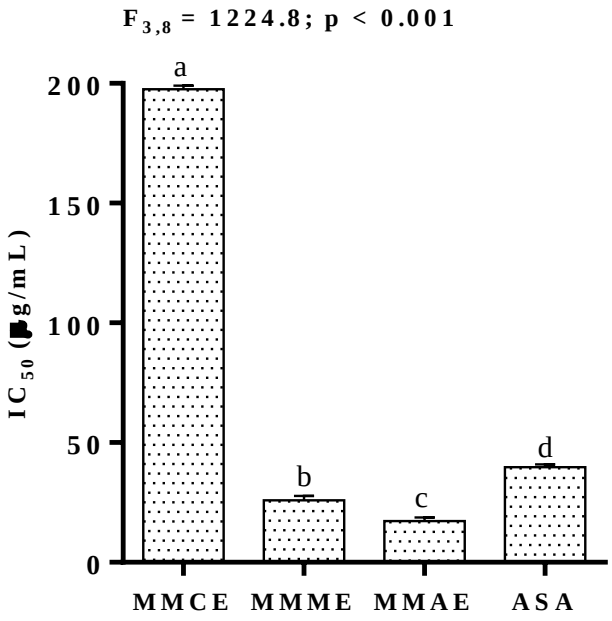


Figure 2.6. IC_{50} (µg/mL) for ABTS of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

3.2. Reducing power

The reducing power of various extracts of *M. macrophylla* was determined by measuring the transformation of Fe^{3+} to Fe^{2+}. The reducing activity of *M. macrophylla* extracts increased in a dose-dependent manner (Figure 2.7). At 400 µg/mL, the highest reducing activity was recorded for MMME (1.098 ± 0.0003) followed by MMAE (0.731 ± 0.002) and MMCE (0.527 ± 0.0008). The reducing activity of MMME was higher than the standard ASA (0.87 ± 0.02).

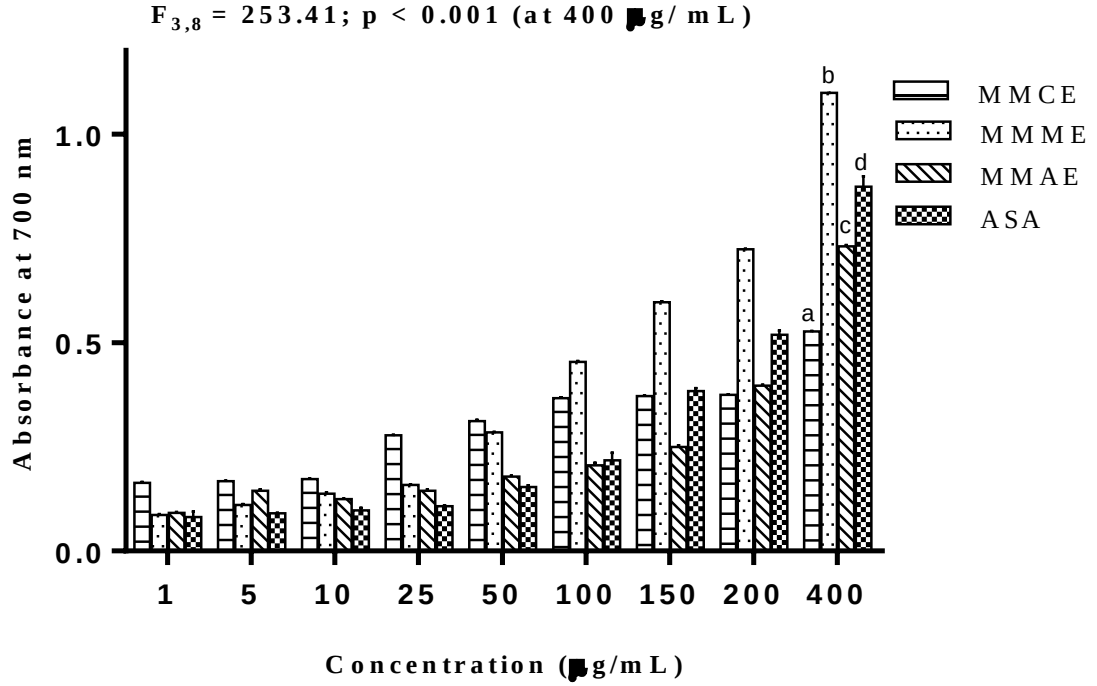


Figure 2.7. Reducing power of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

3.3. *Ex vivo* antioxidant assay

3.3.1. *Inhibition of anti-hemolytic activity*

The anti-hemolytic activity was determined using 0.5 mg/mL of various extracts of *M. macrophylla*. There was a significant variation in the anti-hemolytic activity of different extracts of *M. macrophylla* ($F_{2,6}$ = 7.19; $p < 0.05$). MMAE showed the highest ($p < 0.05$) inhibitory activity against erythrocyte hemolysis with an inhibition rate of 80.53% followed by MMCE (70.91%) and MMME (69.17%) (Figure 2.8).

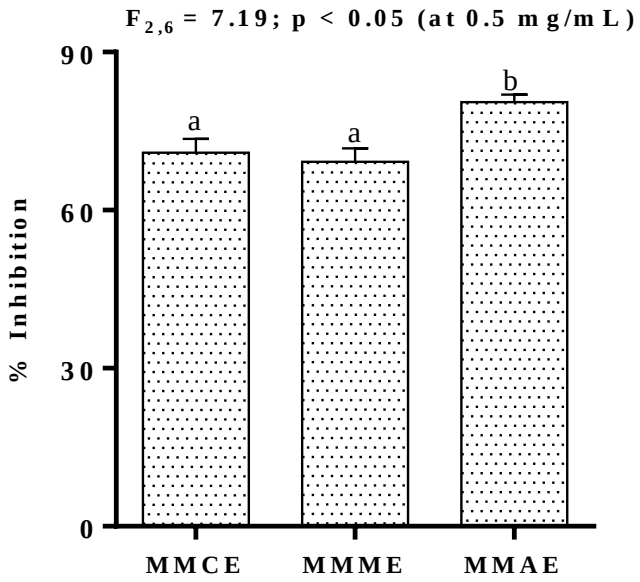


Figure 2.8. Anti-hemolytic activity of various extracts of *M. macrophylla*. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

3.3.2. *Inhibition of lipid peroxidation*

The lipid peroxidation inhibition potential of *M. macrophylla* extracts was also estimated in mice liver homogenate. Significant variation was observed among *M. macrophylla* extracts in their inhibitory activity against lipid peroxidation ($F_{2,6}$ = 8,342.13; $p < 0.001$). Highest inhibitory activity was recorded for MMAE with an inhibition rate of 65.33% followed by MMME (54.38%) and MMCE (Figure 2.9).

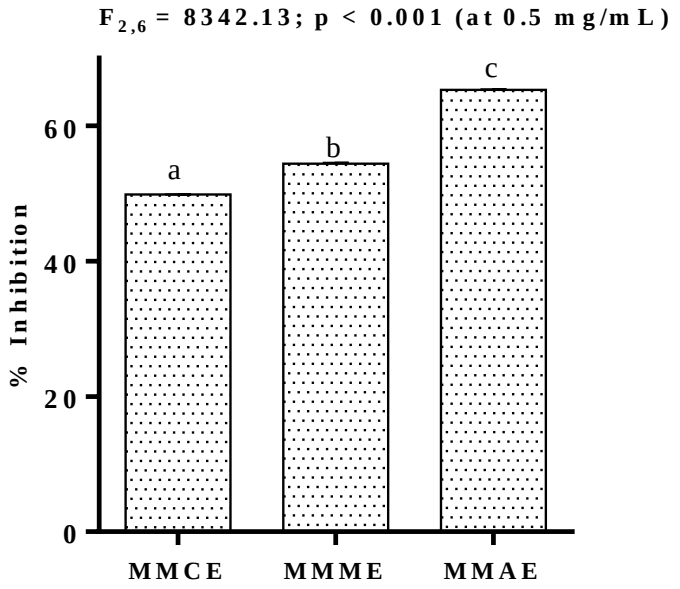


Figure 2.9. The lipid peroxidation inhibitory activity of various extracts of *M. macrophylla*. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.

4. DISCUSSION

Free radicals produced during metabolism are often harmful when they are in excess and have binding affinity to essential biomolecules including DNA, proteins, lipids, etc resulting in a variety of diseases. In order to reduce the ROS- induced oxidative damage and its associated diseases, natural antioxidants are highly suggested. Many studies support the utilization of antioxidant supplements in reducing oxidative stress and preventing or slowing down the progression of disease complications (Badmus et al., 2011). In the present study, the antioxidative potential of various extracts of *M. macrophylla* were measured by determining the scavenging activities of DPPH, ABTS and superoxide radicals and reducing power *in vitro* and inhibitory potential of hemolysis and lipid peroxidation *ex vivo*. The antioxidant property of natural compounds such as plant extracts can be evaluated through their ability to reduced methanolic DPPH solution to non-radical form DPPH-H. DPPH is a stable nitrogen-centered free radical that is widely used to assess the radical scavenging activity of a plant extract or compound. The violet color of the stable DPPH radical was reduced to yellow colored diphenylpicrylhydrazine radical when it accepted an electron from the antioxidant molecule and this was detected colorimetrically. Compounds which are capable of performing this reaction could be considered as antioxidants and therefore radical scavengers (Dehpour et al., 2009). The antioxidative potential of *M. macrophylla* extracts was determined by their ability to scavenge DPPH radicals, and *M. macrophylla* extracts were found to be active radical scavengers. Compounds such as polyhydroxyl aromatic compounds, cysteine, tocopherol, glutathione, and ASA have been known to have the ability to reduce DPPH by hydrogenation (Blois, 1958; Moon et al., 2010). Among the genus *Mussaenda*, *M. frondosa* (Siju et al., 2010), *M. glabrata* (Menon and Sasikumar, 2011), and *M. roxburghii* (Islam et al., 2015) have been reported to effectively scavenge the DPPH radicals.

Superoxide anion radical ($O_2^{\bullet-}$) is a highly reactive free radical that is generated by reduction of molecular oxygen into water and can lead to cellular damage by initiating lipid oxidation indirectly (Aruoma and Halliwell, 1987). These radicals are also generated in aerobic cells due to leakage of electrons from the electron transport chain. Activated phagocytes, such as macrophages, monocytes, eosinophils and neutrophils produce superoxide radicals, which are utilised as a defence to kill germs

by phagocytes. Superoxide radical can break down to form stronger reactive oxygen species (ROS) including hydroxyl radicals and singlet oxygen. In the presence of metals, the superoxide anion forms H_2O_2, which in turn produces hydroxyl free radicals (Halliwell, 1995; Lushchak, 2014). Thus, scavenging of $O_2^{\bullet-}$ could result in inhibition of other ROS generation and protect the cells from oxidative damage. The present study suggests that $O_2^{\bullet-}$ radical scavenging activity of *M. macrophylla* extracts increased with increases in concentration and the aqueous extract of *M. macrophylla* showed higher scavenging potential than the standard ASA. The $O_2^{\bullet-}$ scavenging activity of *M. macrophylla* extracts could be attributed to their flavonoid contents (Robak and Gryglewski, 1988).

The antioxidative activity of various extracts of *M. macrophylla* was also determined by measuring their ability to convert the blue colored $ABTS^{\bullet+}$, which is formed by the interaction of ABTS and potassium ferricyanide, to ABTS. The effectiveness of this conversion depends on a number of characteristics such as an abundance of aromatic rings, types of hydroxyl group's substitution, and the molecular weight of phenolic compounds (Hagerman et al., 1998). The study demonstrates *M. macrophylla* extracts as potent antioxidants and their $ABTS^{\bullet+}$ scavenging activity could be due to their phenolic contents.

It is evident from previous studies that reducing power is attributed to the presence of antioxidants that donate hydrogens atoms to free radicals (Fejes et al., 2000).

Reducing power is correlated with antioxidant activity and could be used as an important reflection of the antioxidant activity (Oktay et al., 2003). Compounds with reducing power are electron donors that can reduce the oxidized byproducts of lipid peroxidation processes, allowing them to function as primary and secondary antioxidants (Yen and Chen, 1995). The reduction of Fe (III) is often used as a marker for electron donating activity, which is a key mechanism of phenolic antioxidant action (Nabavi et al., 2009). Therefore, the reducing power of compounds serves as an indicator of their antioxidant property (Meir et al., 1995). The higher absorbance at higher concentrations showed the high reducing power potential of the extracts. The reducing activity of MMME being higher than the standard ASA indicates the strong redox potential of the extract. The graded increase in percentage inhibition as the concentration of the plant extracts increases indicates

that percentage scavenging effectiveness strongly depends on the extract concentration.

One of the most abundant cells within the human body are the erythrocytes, with numerous morphological and biological characteristics, and therefore have been widely used in drug transport. The erythrocytes are primarily targeted by polyunsaturated fatty acids (PUFA) and hemoglobin molecules, which are redox active oxygen transport molecules and potent promoters of activated oxygen species. Hemolysis may be caused by oxidative mutilation of erythrocyte membrane lipids and proteins in combination with other factors such as oxidative drugs, hemoglobinopathies, an excess of transition metals, insufficiencies in erythrocyte antioxidant coordination and various radiation (Ebrahimzadeh et al., 2009; Hamidi and Tajerzadeh, 2003). When red blood cells were subjected to toxicants such as hydrogen peroxide, the intensity of hemolysis appeared to be much greater (Naim et al., 1976). Hemolysis occurs due to membrane damage caused by the activity of free radicals on erythrocytes that are the major target of free radicals (Ebrahimzadeh et al., 2009; Wang and Yao, 2005).

Peroxidation of lipid moieties such as polyunsaturated fatty acids by a chain reaction of free radicals can lead to membrane damage (Klauning et al., 1998). Furthermore, LPO end-products can promote mutagenesis or protein oxidation, disrupting cellular homeostasis, and their impacts in human health have been extensively assessed (Nam, 2011; Barrera, 2012; Ramana et al., 2017). Lipid peroxidation in mice liver homogenate was induced by $FeCl_2$-H_2O_2. The formation of malonaldehyde is used as an indicator of lipid peroxidation, and subsequently oxidative stress. Various extracts of *M. macrophylla* showed significant anti-hemolytic and lipid peroxidation inhibition potential which could be due to the presence of numerous amounts of phenols and flavonoids. Certain phenolic compounds have been reported to cause partition in the cell membrane, hindering the diffusion of free radicals and consequently decreased the chain reaction of free radicals (Singh and Rajini, 2008). Flavonoids have been reported to inhibit lipid peroxidation in the erythrocytes membrane and improved their integrity against lyses by binding to the membrane (Chaudhuri et al., 2007).

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CHAPTER IV

Investigation of the anticancer activity of *Mussaenda macrophylla* Wall. in Dalton's lymphoma ascites (DLA) bearing Swiss albino mice

ABSTRACT

Mussaenda macrophylla is a shrub widely used in Mizo traditional practice for treatment of cancer, fever, cough, ulcer and dysentery. In this study, we explore the anticancer activity of the aqueous extract of *M. macrophylla* (MMAE) using Dalton's Lymphoma Ascites (DLA) bearing mice as our model. Effects of MMAE on survival, weight change and antioxidants/oxidant status were determined in DLA mice by administering different doses of MMAE for 9 consecutive days. Doxorubicin (DOX) was used as a standard drug. Effects of MMAE on cytotoxicity, serum enzymes activities and hematological parameters were also determined. Comet assay and qPCR were employed to study DNA damage and differential apoptotic gene expression induced by MMAE respectively. MMAE significantly inhibited tumor growth and increased survival time of the tumor bearing DLA mice. MMAE significantly increased the glutathione levels; and glutathione-s-transferase and superoxide dismutase activities. Consistently, MMAE decreased lipid peroxidation levels in DLA mice. Reduced RBC and hemoglobin levels were significantly reversed by MMAE treatment. MMAE also lowers the activities of aspartate aminotransferase, alanine aminotransferase, and creatinine levels that were otherwise elevated in the DLA control animals. Induction of DNA damage, up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes in DLA bearing mice following MMAE treatment provide an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our findings demonstrate the role of the aqueous extract of *M. macrophylla* as a potential anticancer agent possibly targeting the apoptotic pathway.

1. INTRODUCTION

Cancer is a disease characterized by proliferation of body cells as a result of errors in cellular modulation and restriction of cell cycle progression, and thereby leading to the formation of malignant tumor cells that are capable of becoming metastatic (Tyagi et al., 2017). Cancer is one of the main causes of death worldwide and the number of cancer-related deaths is increasing on a daily basis. Non-Hodgkin lymphoma is the 13th most frequently diagnosed cancer in the world and 11th leading cause of cancer mortality worldwide, accounting for 248,724 deaths (2.6 % of the total) each year (Bray et al., 2018). Dalton's lymphoma, a murine non-Hodgkin's transplantable T-cell lymphoma, was originated in 1947 in the thymus gland of a DBA/2 mouse at the National Cancer Institute (NCI), USA. It is then serially transplanted in the intraperitoneal cavity from mouse to mouse (Chakrabarti et al., 1984). Dalton's lymphoma has served as a convenient model for studying various parameters of cancer progression, signalling mechanisms, and ultimately for screening of drugs for effective treatment (Sriram et al., 2010; Das and Vinayak, 2015). In addition, Swiss albino mice serves as an excellent model for anti-cancer drug screening due to their easy accessibility and similarity with humans in terms of their genomic, anatomy and immunological system (Bernerdi et al., 2002).

Of the hundreds of chemicals that have been and are being evaluated for their anticancer activities, natural products derived from medicinal plants seem to be the most promising due to their safety, efficacy and lesser side effects when compared with synthetic drugs (Diantini et al., 2012; Thillaivanan and Samraj, 2014). About 80 % individuals in the economically developing countries still depend on plants to treat different diseases (Kim, 2005). Therapeutic drugs derived from medicinal plants have been reported to play an important role as anticancer agents in various experimental models of cancer and about 60% of the currently available anticancer drugs are derived from plant sources (Zasadil et al., 2014; Kamal et al., 2015). Plants derived natural products such as flavonoids, alkaloids, aldehydes, glycosides, lignans, lipids, nucleic acids, phenols and its derivatives, polysaccharides, proteins and terpenoids have received increasing attention in recent years due to their diverse pharmacological properties including cytotoxicity and cancer chemopreventive effects (Kintzios and Barberaki, 2004). There is increasing evidence for the potential

of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, indicating the importance of plant products in cancer prevention and therapy. Various plant ingredients that possess anticancer activity have been translated from pharmacology investigations to clinical applications (Man et al., 2012; Liu et al., 2014). The use of multi-compounds is preferred over the use of single drug for the treatment of several diseases including cancer due to their beneficial effects (Pan et al., 2014). Recent studies have demonstrated the anticancer properties of various plants such as *Emilia sonchifolia* (Shylesh and Padikkala, 2000), *Solanum pseudocapsicum* (Badami et al., 2003), *Astraeus hygrometricus* (Mallick et al., 2010) and *Sesbania grandiflora* (Krishna et al., 2010) in Dalton`s lymphoma ascites bearing mice. In recent years, plant-derived natural products such as glossogin (Hsu et al., 2008), lumbagin (Gomathinayagam et al., 2008), curcumin (Wu et al., 2010), celastrol (Mou et al., 2011), polydatin (Zhang et al., 2014), glycyrrhizin (Huang et al., 2014), isolinderalactone (Chang et al., 2014), embelin (Avisetti et al., 2014) and glycyrrhetinic acid (Song et al., 2014) have been reported to trigger apoptosis through the intrinsic and/or extrinsic pathway.

M. macrophylla, locally known as Vakep, is a flowering shrub belonging to Rubiaceae family. *M. macrophylla* is endemic to south-east Asia and is known to occur in India, China, and Myanmar (Manandhar, 2002). Traditionally, different health problems such as sour mouths, sour throats, oral infections, fevers, coughs, chronic ulcer, diarrhea, dysentery, indigestion, cancers and snake bites have been treated using various parts of this plant (Manandhar 1994, Rosangkima and Jagetia, 2015; Sharma et al., 2001; Kim et al., 1999). The genus *Mussaenda* consists of approximately 194 species and is an important source of phytoconstituents such as flavonoids, alkaloids, cardiac glycosides, saponins, steroids, tannins, iridoids and terpenes (Astalakshmi et al., 2017). Previous studies with the other species of the genus *Mussaenda* has revealed their anti-oxidative, anti-inflammatory, thrombolytic, anti-bacterial, anti-pyretic, anti-viral, cytotoxic and anti-diuretic properties (Siju et al., 2010, Menon and Sasikumar, 2011, Aktar et al., 2014; Islam et al., 2015, Shylaja et al., 2015). Given the present state of the scientific evidence on various pharmaceutical applications of *M. macrophylla*, high priority research is required to objectively assess the potential anticancer activity of *M. macrophylla*. Therefore, the

present study has been carried out to investigate the anticancer activity of *M. macrophylla* aqueous extract in Dalton's Lymphoma Ascites (DLA) bearing Swiss albino mice.

2. MATERIALS AND METHODS

2.1. Chemicals

Bovine serum albumin (BSA), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), Folin-ciocalteau's reagent, n-butanol, thiobarbaturic acid (TBA), phenazine methosulphate (PMS), 1-chloro-2,4 dinitrobenzene (CDNB) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Doxorubicin (Getwell Oncology Pvt., Ltd. Haryana, India) was purchased from local pharmacy. The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2. Collection of plant and preparation of extracts

Collection and preparation of *M. macrophylla* was carried out as described in Chapter 2 (Section 2.2) to obtain the plant extracts. The aqueous extract of *M. macrophylla* (MMAE), the most effective extract in the preliminary screening based on survival test, was subsequently used for further experiments.

2.3. Animals and tumor model

Swiss albino mice (10-12 weeks old) weighing 25 g to 30 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2 ºC), and light (12 h of light and dark, respectively) at the Animal Care Facility, Department of Zoology, Mizoram University. The animals were having free access to food and water. The animal care and handling was carried out according to the guidelines issued by World Health Organization, Geneva, Switzerland. Dalton's Lymphoma ascites (DLA) tumor have been maintained in 10-12 weeks old mice by serial intraperitoneal (i.p) transplantation of about 1×10^6 viable tumor cells per animal (in 0.25 mL PBS, pH 7.4) under aseptic condition. The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZU-IAEC/2018/09.) and CPCSEA (Committee for the Purpose of Control & Supervision of Experiments on Animals), New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

2.4. Preparation of drug and mode of administration

The aqueous extract of *M. macrophylla* (MMAE) and doxorubicin (a standard drug) were dissolved in distilled water. Animal from each group received different dose of treatments according to body weight intraperitoneally (i.p).

2.5. Acute toxicity study

The acute toxicity study (Prieur et al., 1972) of MMAE was carried out in Swiss albino mice according to the OECD guidelines 420-425. Animals were divided into four groups of ten animals each, and treated with aliquot doses of MMAE intraperitoneally (1.2, 1.4, 1.6 and 1.8 g/kg b. wt) and monitored for toxic symptoms and mortality up to 14 days post treatment. The LD_{50} value of MMAE was calculated using probit analysis (Miller and Tainter, 1944). The mortality percentage for '0' and '100' are corrected before determination of probits as under:

For 0% dead: 100(0.25/n)

For 100% dead: 100(n-0.25/n)

The probit values were plotted against log-doses and then the dose corresponding to probit 5 i.e., 50%, was calculated. The standard error of the mean (SEM) of LD_{50} was calculated using the following formula:

Approx. SEM of LD_{50} = $\frac{\text{(Log LD84-Log LD16)}}{\sqrt{2N}}$

where N is number of animals in each group.

2.6. Experimental design

For the assessment of survival time and weight change, Swiss albino mice were randomly assigned to five equal groups (n = 6). All mice were inoculated (i.p) with 1×10^6 cells in 0.25 mL of PBS on day '0'. Group I was treated as the control group, which received 0.25 mL of distilled water. Group II-IV were injected (i.p) with MMAE at the dose of 50, 100 and 150 mg/kg b.wt, respectively. Group V received doxorubicin (DOX) at the dose of 0.5 mg/kg b.wt as a standard drug. After 72 h of tumor inoculation, treatment was given to each group for 7 consecutive days.

A separate experiment was conducted to estimate antioxidant status, lipid peroxidation, cytotoxicity, activities of serum enzymes, and hematological

parameters where the grouping of DLA bearing mice was same as described earlier. The expression of both pro-apoptotic and anti-apoptotic genes, and the level of DNA damage were also compared between the control group and MMAE (100 mg/kg b.wt) treated group.

2.7. Estimation of survival time and weight change

The deaths, if any, of the tumor bearing mice were recorded daily and survival time was determined for all the groups. The tumor response was assessed by calculating median survival time (MST) and average survival time (AST). The increase in median life span (% IMLS) and increase in average life span (% IALS) were also calculated using the formulae given by Gupta et al. (2000).

T/C value, which is the MST of the treated group of animals (T) divided by that of control group (C) was also computed. The T/C ratio is given as a percentage and a compound is considered active if it shows T/C value $\geq$ 120% (National Cancer Institute Protocols). The animals of all the experimental groups were also monitored regularly for alteration in body weight. The body weight of animals was recorded every 3 days up to 18 days post tumor inoculation.

2.8. Processing of liver and tumor cell for biochemical assays

After seven consecutive days of treatment with MMAE or DOX, animals from each group were euthanized through an overdose of ketamine followed by immediate excision of liver (Al Batran et al., 2013). 5% (w/v) tissue homogenate was prepared with ice-cold buffer (5mM EDTA, 0.15M NaCl, pH 7.4) in a glass homogenizer. The homogenates were centrifuged for 30 min at 13,000 rpm at 4 °C and the supernatants were immediately used for biochemical assays. The tumor cells were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. The tumor cells were pelleted, sonicated (PCi Analytics) and homogenized with ice cold buffer to produce 5% (w/v) homogenate. The homogenates were centrifuged for 30 min at 10,000 rpm at 4 °C and the supernatants obtained were immediately used for the estimation of antioxidant enzyme activities and lipid peroxidation.

2.9. Antioxidants assays

2.9.1. *Protein estimation*

The protein content of the samples was determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard.

2.9.2. *Glutathione (GSH)*

Glutathione (GSH) levels were measured using the method of Moron et al. (1979). GSH was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. Briefly, 80µL of the sample was mixed with 900µL of 0.02M sodium phosphate buffer and 20 µL of 10 mM DTNB and incubated at room temperature for 2 min. Blank consisted of distilled water instead of the sample. The absorbance of the sample was read against blank at 412 nm in a UV-visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). GSH concentration was calculated from the standard graph and expressed in µ mol /mg protein.

2.9.3. *Glutathione-s-transferase (GST)*

Glutathione-s-transferase (GST) was measured according to the method of Beutler (1984). Briefly, a mixture of 50 µL 20mM CDNB and 850 µL of 0.1 M phosphate buffer (pH 6.5) was incubated for 10 min at 37°C. Then, 50 µL each of 20 mM GSH and sample were added to the mixture. For blank, distilled water was added instead of the sample. The absorbance of the sample was measured at 1 min interval for 6 min at 340 nm. GST activity was calculated as:

GST activity = (OD of test – OD of blank/9.6×vol. of test sample) ×1000

where, 9.6 is the molar extinction coefficient for GST.

2.9.4. *Superoxide dismutase (SOD)*

Superoxide dismutase (SOD) activity was determined by the nitroblue tetrazolium (NBT) reduction method (Fried, 1975). Briefly, 100 µL each of the sample and 186 µM PMS were mixed with 300 µL of 3 mM NBT and 200 µL of 780 µM NADH. The mixture was incubated for 90 sec at 30 °C and 1 mL of acetic acid and 4 mL of n-butanol were added to stop the reaction. The blank consisted of all the reagents, and distilled water was added instead of sample. The absorbance of test and blank

were measured at 560 nm and the enzyme activity was expressed in units (1 unit = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank – OD of test/OD of blank) x 100

SOD unit = 1/50 x % inhibition.

2.9.5. *Lipid peroxidation (LPO)*

Lipid peroxidation (LPO) was estimated by the method of Beuege and Aust (1978). Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LPO) that reacts with thiobarbituric acid (TBA) to give a red species absorbing at 535 nm. Briefly, the sample was added to a mixture of 10% TCA, 0.8% TBA and 0.02N HCl in 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath, cooled immediately at room temperature and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank. The blank contained all the reagents minus the sample substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

2.10. Effect of MMAE on cell toxicity and hematological parameters

The cytotoxic effect of MMAE was examined by studying tumor cell volume and the percentage of the viable and non-viable cell count using trypan blue dye exclusion test, in a hemocytometer. Blood was collected from each animal by heart puncture using heparin coated 1 mL syringe. Red blood cell count (RBC), white blood cells count (WBC) and hemoglobin (Hb) content were measured using standard protocols (D'Amour et al., 1965).

2.11. Measurement of serum ALT, AST and CRE

The blood obtained from heart puncture was centrifuged at 3,000 rpm for 15 min at 4°C and serum was collected for enzyme assays. Activities of aspartate amino-transferase (AST) (EC 2.6.1.1), alanine amino-transferase (ALT) (EC 2.6.1.2) and the level of creatinine (CRE) were determined in serum using commercially available kits (Coral Clinical Systems, Uttarakhand, India).

2.12. Assessment of DNA damage using Comet assay

The alkaline single cell gel electrophoresis (Comet assay) was performed as described by Singh et al. (1988) with minor modifications. The tumor cells from both control and treatment groups were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. Briefly, 2 x 10^4 tumor cells were suspended in 75 µL of 0.5 % low-melting point agarose prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose. Slides were immersed for 2 h in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10). After lysis, slides were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA. The slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralization, slides were washed with distilled water and then stained with ethidium bromide (EtBr) solution (2 µg/mL) for 5 min. Two slides were prepared for each animal and 100 randomly selected cells from each slide were examined using fluorescence microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615) with a magnification of 200x. Image capture and analysis were performed with Image J software.

2.13. qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression

The tumor cells from both control and treatment groups were aspirated in an aseptic condition and washed with NH_4Cl and 1X PBS twice. The cells were pelleted and total RNA was extracted using Tri reagent (BR Biochem, Life Science Pvt. Ltd, R1022). Extracted RNA was quantified using Nanodrop Spectrophotometer (Nanodrop One C, Thermo Fisher Scientific) and RQ1 DNase kit (Promega, M198A, Madison, WI, USA) was used to remove the genomic contamination. cDNA was synthesized from 2 µg of total RNA using first-strand cDNA synthesis kit (Thermoscientific, K1621; Lithuania, Europe). Gene-specific primers (Table 3.1) were designed using Primer 3, Boston, MA, USA. qPCR was performed using Quant-Studio 5 (ThermoFisher Scientific, Foster City, CA, USA). PCR reaction volume of 7 µL for each gene comprised of 1 µL each of cDNA, gene-specific

forward and reverse primers, 3 µL PowerUp^{TM} SYBR^{TM} Green Master Mix (Thermo Fisher Scientific, A25742, Lithuania, Europe) and 1 µL of nuclease-free water (ThermoFisher Scientific, A19938, Bangalore, India). The cycling condition of qPCR were 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s) (Renthlei et al., 2018). Afterwards, melting curves were generated to confirm a single uniform peak. GAPDH gene was used as a reference gene for determining the relative expression levels of specific target genes. Each sample was run in duplicate along with non-template and negative RT controls. The relative expression of genes was determined using ΔΔCt method (Livak and Schmittgen, 2001).

Table 3.1. Primer sequences and their product size used in qRT-PCR analyses of Dalton`s lymphoma ascites (DLA) bearing mice treated with aqueous extract of *M. macrophylla* (100 mg/kg b.wt).

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product Size(bp)
p53	GTATTTCACCCTCAAGATCCGC	AGACTCCTCTGTAGCATGGG	100
Bax	CACCTGAGCTGACCTTGGAG	CAATCATCCTCTGCAGCTCCA	117
Apaf-1	ATGGAATTGGCAGACAGGGG	TTCCACACCTTCACCGTTCC	126
Bcl-2	GACTTCTCTCGTCGCTACCG	CTCTCCACACACATGACCCC	176
Bcl-X_L	AGGGGCTTAGCTGCTGAAAG	GTGGACAAGGATCTTGGGGG	81
GAPDH	AAAGGGTCATCATCTCCGCC	AGTGATGGCATGGACTGTGG	197

2.14. Statistical analysis

All data were expressed as mean ± standard error of mean of pooled results obtained from three independent experiments. One-way ANOVA followed by Tukey's test was performed to test significant variations on survival time, change in body weight, antioxidants status, lipid peroxidation, tumor volume, cytotoxicity, hematological

parameters, and activities of serum enzymes. Significance variation on relative gene expression and DNA damage were calculated using Student's t-test between control and treatment groups. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 6.0 were used for statistical and graphical analyses. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Acute toxicity test

In order to assess the acute toxicity of MMAE, the approximate LD_{50} was determined as a pilot study by a so called 'staircase method'. Four doses were chosen for the determination of LD_{50} starting from 0 % mortality to 100 % mortality. Thus, four doses were given intraperitoneally to 4 groups of 10 mice each (Table 3.2). The probit values were plotted against log-doses and then the dose corresponding to probit 5, i.e., 50 %, was found to be 0.182 (log LD_{50}) with LD_{50} of 1.52 ± 0.117 g/kg b. wt.

Table 3.2. Results of the lethal doses of aqueous extract of *M. macrophylla* (MMAE) for the determination of LD_{50} in Swiss Albino mice.

Groups	Dose (g/kg b.wt)	Log dose	% dead	Corrected %	Probits
1	1.2	0.08	0%	2.5%	3.03
2	1.4	0.14	20%	20%	4.16
3	1.6	0.2	60%	60%	5.25
4	1.8	0.26	100%	97.5%	6.96

3.2. Effects of MMAE on survival time

To understand the anticancer activity of MMAE on DLA bearing mice, the tumor bearing mice were treated with different doses of MMAE. All untreated tumor bearing mice died within 19 days with MST and AST of 15.0 ± 0.57 days and 15.2 ± 0.46 days, respectively. Interestingly, treatment of DLA bearing mice with MMAE resulted in a dose dependent increase in MST, AST, % IMLS and % IALS up to 100 mg/kg. However, reduction in life span of DLA mice was observed with the treatment of 150 mg/kg MMAE which may indicate that prolonged treatment with higher dose could have other effects. The results of the *in vivo* anticancer activity were also expressed as ratio of the median survival days of the treatment and control

group (T/C) of DLA bearing mice. Treatment of DLA mice with MMAE at 50 and 100 mg/kg b.wt showed T/C values of 140.0% and 196.6% respectively indicating the effectiveness of MMAE as a potential anticancer agent. Treatment of DLA mice with MMAE at 150 mg/kg b.wt, however, reduced the T/C value to 160.0 %, which is consistent with the MST, AST, % IMLS and % IALS results. DOX treatment also increased MST to 22.5 ± 0.50 days and AST to 24.2 ± 0.56 days, respectively. Consequently, DOX treatment caused an increase in % IMLS and % IALS to 63.3% and 59.4%, respectively. The DOX treatment also caused a significant increase in life span of DLA mice with T/C ratio of 163.3 % (Table 3.3). Taking together, MMAE at the dose up to 100 mg/kg could increase the survival time of the animals showing the prospective of the plant extract for future therapeutic use. Summary of the effects of MMAE and DOX on the survival of DLA bearing mice are given in Figure 3.1.

Table 3.3. Effect of MMAE and DOX treatment on DLA bearing mice on the tumor response assessment based on MST, AST, % IMLS, % IALS and T/C ratio. DLA Control: DLA bearing mice without treatment; DLA + $MMAE_{50,}$ DLA + $MMAE_{100,}$ DLA + $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100 and 150 mg/kg, respectively. $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg). The results were expressed as percent (%) ± SEM, n=10.

Dose (mg/kg b.wt)	MST	AST	% IMLS	% IALS	% T/C ratio
DLA Control	15.0 ± 1.57	15.2 ± 1.46	-	-	-
DLA + $MMAE_{50}$	21.0 ± 1.50**	21.3 ± 1.34**	40.0 ± 1.57^{b}	40.7 ± 2.35^{b}	140.0 ± 2.57*
DLA + $MMAE_{100}$	29.5 ± 1.28***	27.3 ± 1.91***	96.6 ± 1.88^{c}	80.1 ± 1.96^{c}	196.6 ± 3.33*
DLA + $MMAE_{150}$	24.0 ± 1.52***	22.5 ± 1.04**	60.0 ± 2.00^{a}	46.4 ± 2.56^{b}	160.0 ± 2.80*
DLA + $DOX_{0.5}$	22.5 ± 1.50**	24.2 ± 1.56***	63.3 ± 2.35^{a}	59.4 ± 1.86^{a}	163.3 ± 2.48*

$^{**}p \leq 0.01$; $^{***}p \leq 0.001$ between the control and treatment groups.

Different letters indicate significant variation between different treatment groups.

'*' indicates a T/C ratio > 120

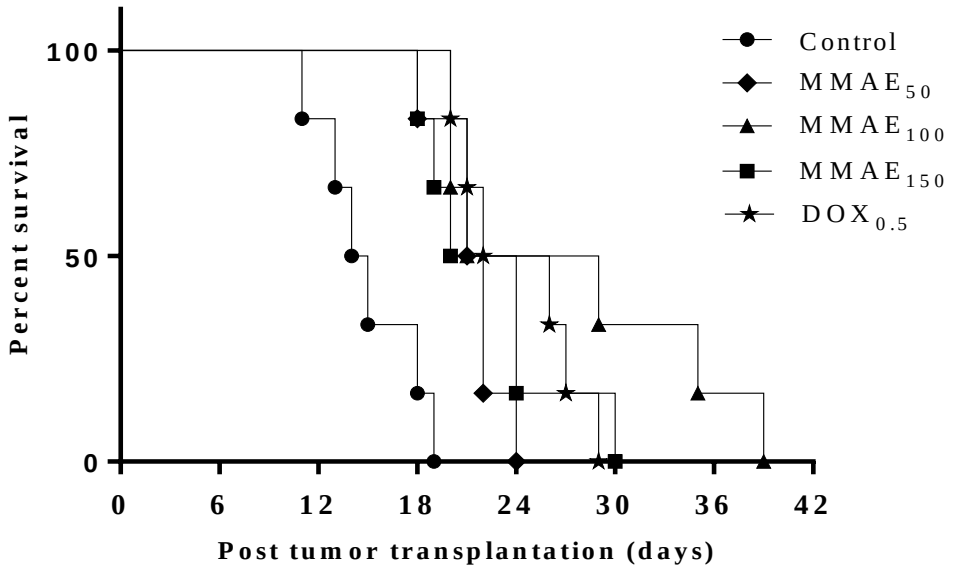


Figure 3.1. Kaplan Meier's estimate of survival time of DLA bearing mice. Control: DLA bearing mice without treatment; $MMAE_{50,}$ $MMAE_{100,}$ $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100 and 150 mg/kg b.wt, respectively; $DOX_{0.5}$: DLA bearing mice treated with doxorubicin (0.5 mg/kg).

3.3. Effects of MMAE on weight change

The weights of the animals in all the groups were recorded every 3rd day starting from the day of tumor transplant in order to determine the change in body weight. Due to the proliferation of the tumor cells, the untreated DLA bearing mice exhibited continuous weight gain until their survival. The treatment of DLA mice with 100 mg/kg MMAE after 72 h of tumor inoculation arrested the weight gain indicating inhibition of tumor cell proliferation and growth (Table 3.4; Figure 3.2).

Table 3.4. Average increased in weight of DLA mice treated with different doses of MMAE and DOX at 3 days interval.

Dose (mg/kg b.wt)	Weight gain (g) (Mean ± SEM)
Control	1.64 ± 0.07
$MMAE_{50}$	0.888± 0.11**
$MMAE_{100}$	0.863± 0.032***
$MMAE_{150}$	0.927± 0.009**
$DOX_{0.5}$	0.52 ± 0.01*

**$p \leq 0.01$; ***$p \leq 0.001$ between the control and treatment groups.

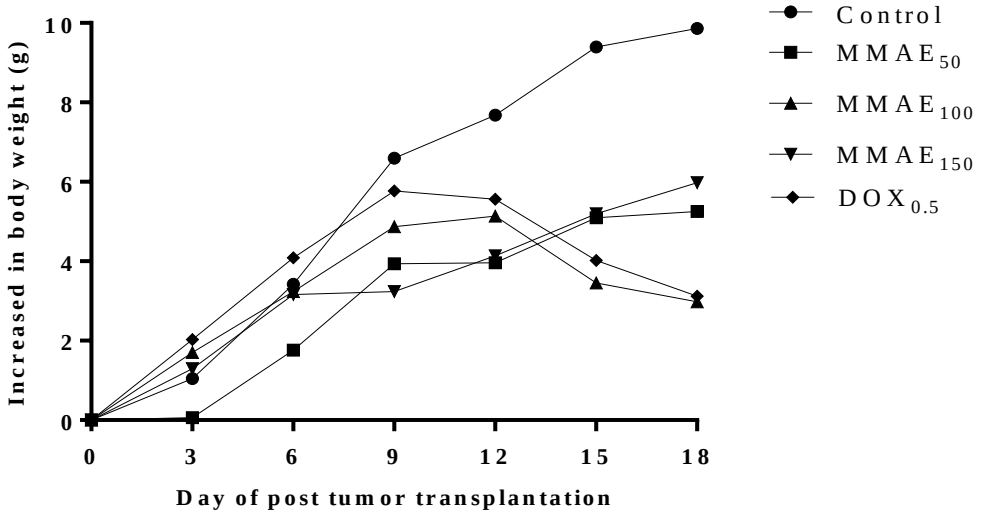


Figure 3.2. Average increase in body weight of DLA mice treated with different doses of MMAE (50, 100, and 150 mg/kg b.wt) and DOX (0.5 mg/kg b.wt) in three days intervals (The initial weight of all the animals was taken as '0').

3.4. Effect of MMAE on tumor volume and cell toxicity

The tumor volume and cell viability were assessed in order to determine the effect of MMAE on tumor load and cytotoxicity. Treatment of DLA mice with MMAE caused a significant reduction in the tumor volume in a dose dependent manner when compared with the DLA control group. The reduction observed in the tumor volume following MMAE treatment was comparable to the reduction of tumor volume when DLA mice were treated with DOX. Similarly, percentage of non-viable cells increases significantly in both MMAE and DOX treated DLA mice suggesting the cytotoxic effect of MMAE (Table 3.5).

Table 3.5. Effects of MMAE and DOX treatment on tumor volume and cytotoxicity in DLA bearing mice. Values are mean ± SEM (n = 6).

Groups	Tumor volume (mL)	Viable cell (%)	Non-viable cell (%)
DLA Control	5.50 ± 0.28^{a}	98.80 ± 0.42^{a}	1.19 ± 0.42^{a}
DLA + $MMAE_{50}$	3.40 ± 0.32^{b}	76.84 ± 0.52^{b}	23.16 ± 0.52^{b}
DLA + $MMAE_{100}$	1.65 ± 0.39^{c}	64.57 ± 2.91^{c}	35.43 ± 2.91^{c}
DLA + $MMAE_{150}$	1.70 ± 0.38^{c}	61.90 ± 3.90^{c}	38.10 ± 3.90^{c}
DLA + $DOX_{0.5}$	1.55 ± 0.86^{c}	62.77 ± 2.13^{c}	37.23 ± 2.13^{c}

DLA Control: DLA bearing mice without treatment; DLA + $MMAE_{50}$, DLA + $MMAE_{100}$, DLA + $MMAE_{150}$: DLA bearing mice treated with 50, 100 and 150 mg/kg b.wt of aqueous extract of *M. macrophylla*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg b.wt of doxorubicin. Different letters indicate significant variation.

3.5. Effect of MMAE on hematological parameters

The DLA bearing mice had reduced RBC and hemoglobin levels as compared to the normal animals. Interestingly, the decreased RBC and hemoglobin levels were reversed significantly by MMAE and DOX treatment when compared with the DLA control mice. The level of reversal of RBC and hemoglobin contents by MMAE treatment is comparable to the reversal by treatment with the standard DOX treatment. Elevated levels of WBC were observed in the DLA bearing mice as compared to the normal control animals. However, with MMAE and DOX treatment, the WBC levels were significantly reduced to the level close to the normal control group (Table 3.6).

Table 3.6. Effects of MMAE and DOX on hematological parameters of DLA bearing mice. Values are mean ± SEM (n = 6).

Groups	RBC (million/mm^3)	WBC (thousand/mm)	Hb (g%)
Normal Control	5.47 ± 0.07^a	10.20 ± 1.23^a	20.20 ± 2.34^a
DLA Control	2.14 ± 0.42^b	29.04 ± 2.57^b	9.08 ± 1.37^b
DLA + $MMAE_{50}$	2.87 ± 0.74^b	26.55 ± 1.15^b	10.60 ± 1.18^b
DLA + $MMAE_{100}$	4.52 ± 0.89^c	14.48 ± 1.07^c	15.40 ± 1.60^c
DLA + $MMAE_{150}$	4.27 ± 1.07^c	15.46 ± 1.08^c	14.08 ± 1.36^c
DLA + $DOX_{0.5}$	4.40 ± 0.57^c	12.77 ± 1.78^a	16.93 ± 2.02^c

Normal Control: Healthy mice without treatment; DLA Control: DLA bearing mice without treatment; DLA + $MMAE_{50}$, DLA + $MMAE_{100}$, DLA + $MMAE_{150}$: DLA bearing mice treated with 50, 100 and 150 mg/kg b.wt of aqueous extract of *M. macrophylla*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg b.wt of doxorubicin. Different letters indicate significant variation.

3.6. Effect of MMAE on serum biochemical parameters

Serum biochemical parameters including the enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine (CRE) level were found to be significantly increased in the DLA control mice as compared to the normal control group. Treatment with 100 and 150 mg/kg b.wt MMAE and DOX (0.5 mg/kg) significantly lowers the activities of ALT, AST and CRE level near to the levels as that of the normal control animals (Table 3.7).

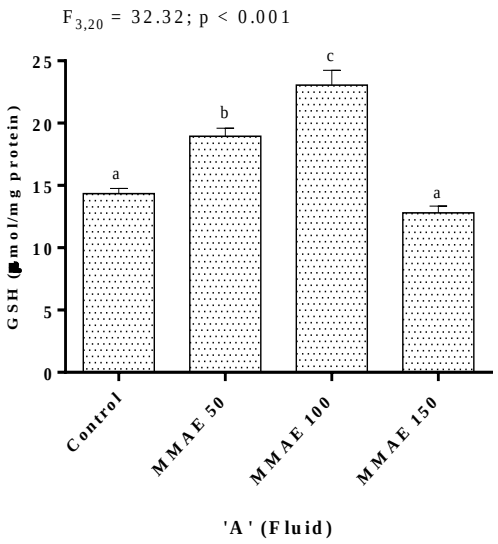
Table 3.7. Effects of MMAE and DOX on serum ezymes of DLA bearing mice. Values are mean ± SEM (n = 6).

Groups	**ALT (U/L)**	**AST (U/L)**	**CRE (mg/dL)**
Normal Control	18.45 ± 1.78^{a}	90.41 ± 2.46^{a}	0.44 ± 0.03^{a}
DLA Control	29.41 ± 1.77^{b}	131.93 ± 3.66^{b}	0.97 ± 0.04^{b}
DLA + $MMAE_{50}$	29.03 ± 2.68^{b}	126.94 ± 3.88^{b}	0.72 ± 0.05^{c}
DLA + $MMAE_{100}$	20.86 ± 1.04^{c}	104.90 ± 2.65^{c}	$0.59 \pm 0.05^{c, d}$
DLA + $MMAE_{150}$	21.49 ± 2.29^{c}	109.44 ± 3.87^{c}	0.51 ± 0.04^{d}
DLA + $DOX_{0.5}$	$25.94 \pm 1.09^{b,c}$	113.91 ± 2.68^{c}	$0.49 \pm 0.04^{c,d}$

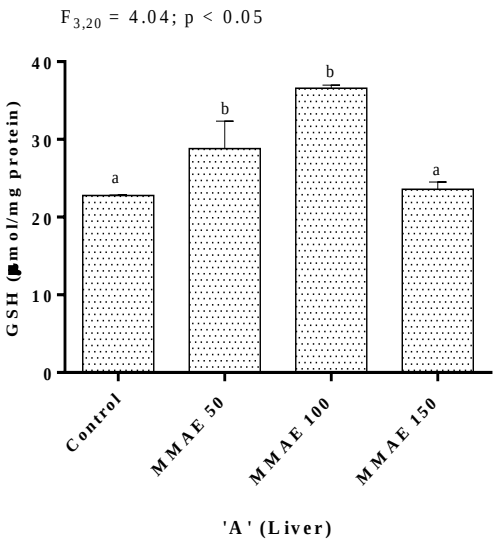
Normal Control: Healthy mice without treatment; DLA Control: DLA bearing mice without treatment; DLA + $MMAE_{50}$, DLA + $MMAE_{100}$, DLA + $MMAE_{150}$: DLA bearing mice treated with 50, 100 and 150 mg/kg b.wt of aqueous extract of *M. macrophylla*; DLA + $DOX_{0.5}$: DLA bearing mice treated with 0.5 mg/kg b.wt of doxorubicin. Different letters indicate significant variation.

3.7. Effect of MMAE on Antioxidants/Oxidant status

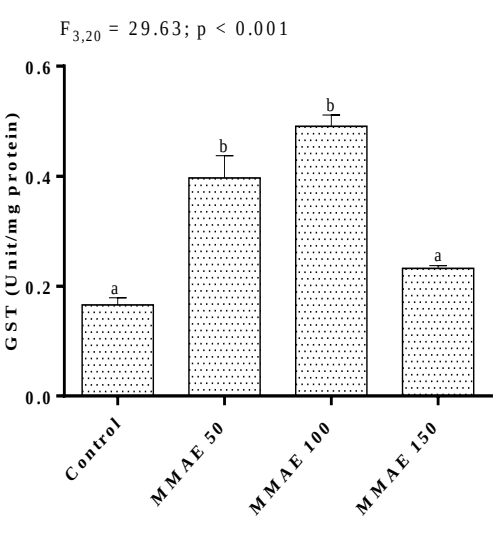
The level and activities of antioxidants and oxidants in DLA bearing mice were measured in the tumor cells and liver after treatment with MMAE. Treatment of DLA mice with MMAE results in increased antioxidant levels and activities both in tumor cells and liver when compared with the untreated control. MMAE treatment significantly increased glutathione content in a dose dependent manner up to 100 mg/kg b.wt when compared to the control group (Figure 3.3A). To determine the effect of MMAE on antioxidant enzymes, the activities of GST and SOD were assessed. In response to MMAE treatment, the antioxidant enzyme activities were significantly increased when compared with the control (Figure 3.3 B&C). In an effort to investigate whether MMAE treatment affect intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was assessed. Consistent to the increased antioxidant enzyme activities, the oxidative stress levels were significantly lower in both liver and tumor cells after treatment of DLA mice with MMAE. The decreased in LPO was found to correspond to MMAE treatment in a dose dependent manner up to 100 mg/kg b.wt (Figure 3.3D).



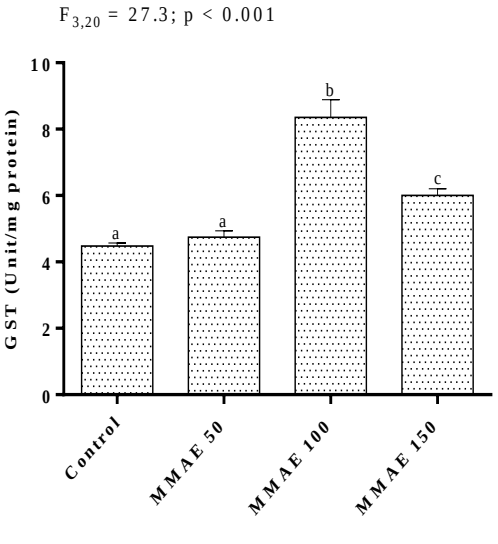
'A' (Fluid)



'A' (Liver)



'B' (Fluid)



'B' (Liver)

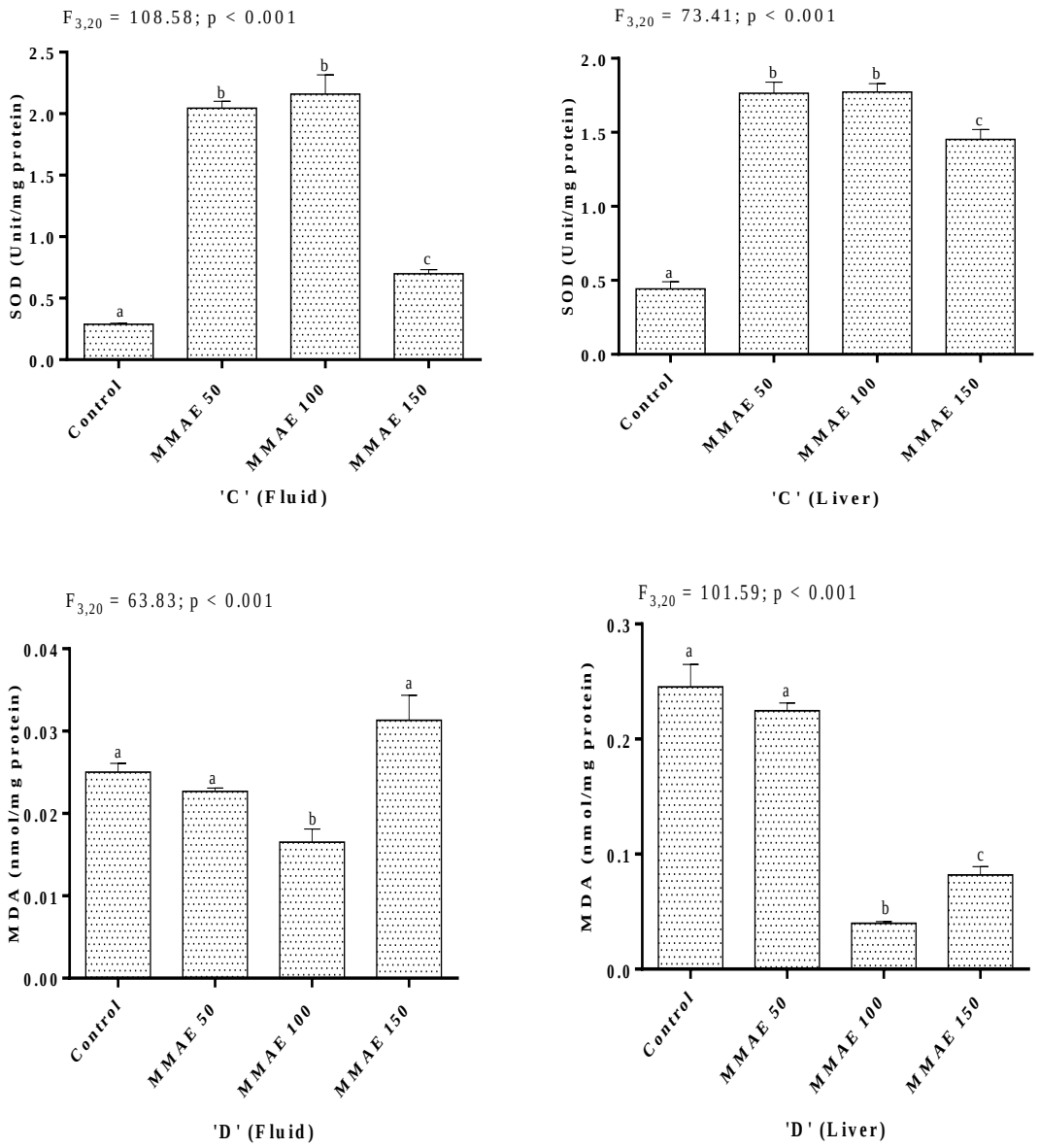


Figure 3.3. Effects of aqueous extract of *M. macrophylla* on (A) glutathione level (GSH); (B) glutathione-s-transferase activity (GST); (C) superoxide dismutase activity (SOD); (D) lipid peroxidation (LPO) expressed in malondialdehyde (nmol/mg protein) in the fluid and liver of DLA bearing mice. Control: Dalton's Lymphoma Ascites bearing mice without treatment; $MMAE_{50}$, $MMAE_{100}$, $MMAE_{150}$: DLA bearing mice treated with aqueous extract of *M. macrophylla* at the dose of 50, 100, 150 mg/kg b.wt, respectively. Means not sharing the same letter are significantly different.

3.8. Induction of DNA strand breaks by MMAE

The alkaline Comet assay was used to assess the level of DNA damage in DLA bearing mice after 7 consecutive days of treatment with MMAE (100 mg/kg). Our results showed that MMAE induced DNA damage in ascites tumor cells as indicated by significant increased tail length and olive moment in MMAE treated group when compared to untreated control (Figure 3.4).

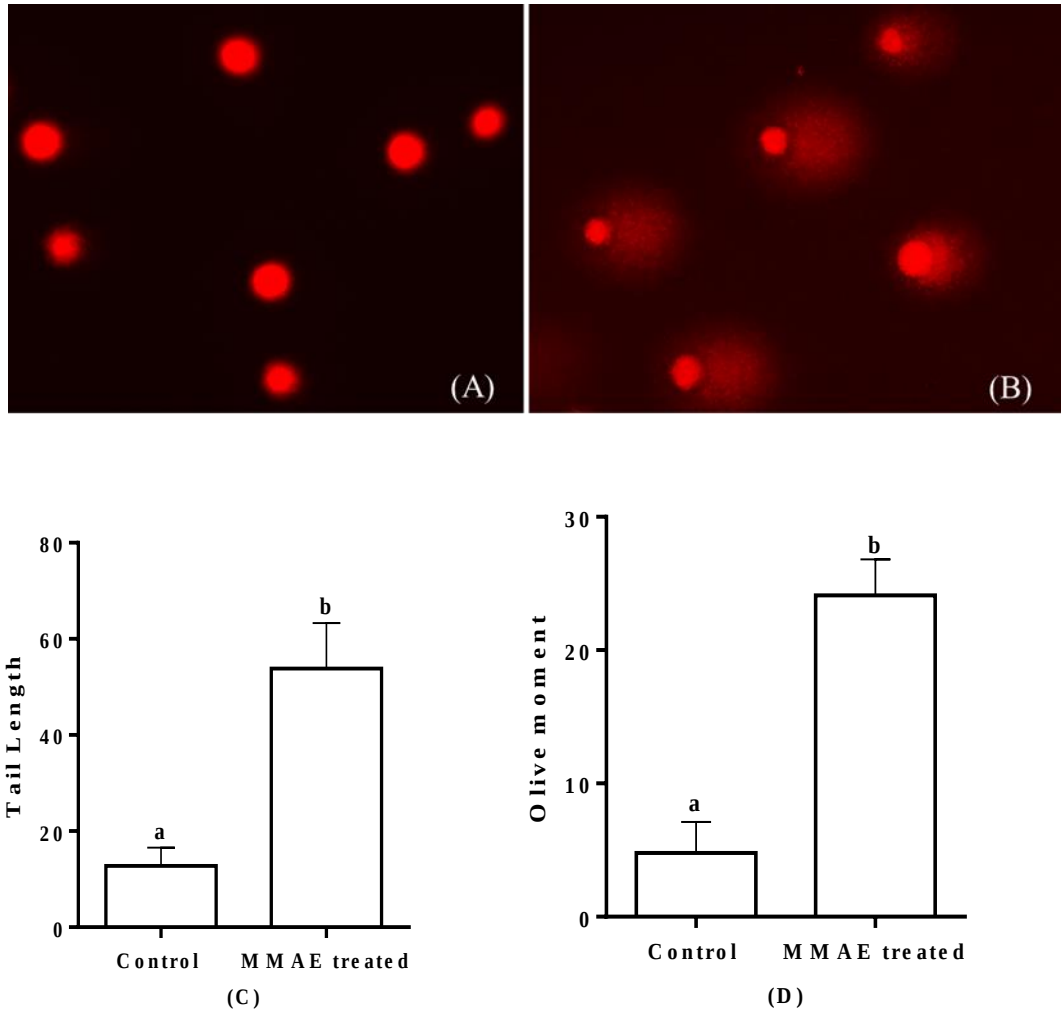
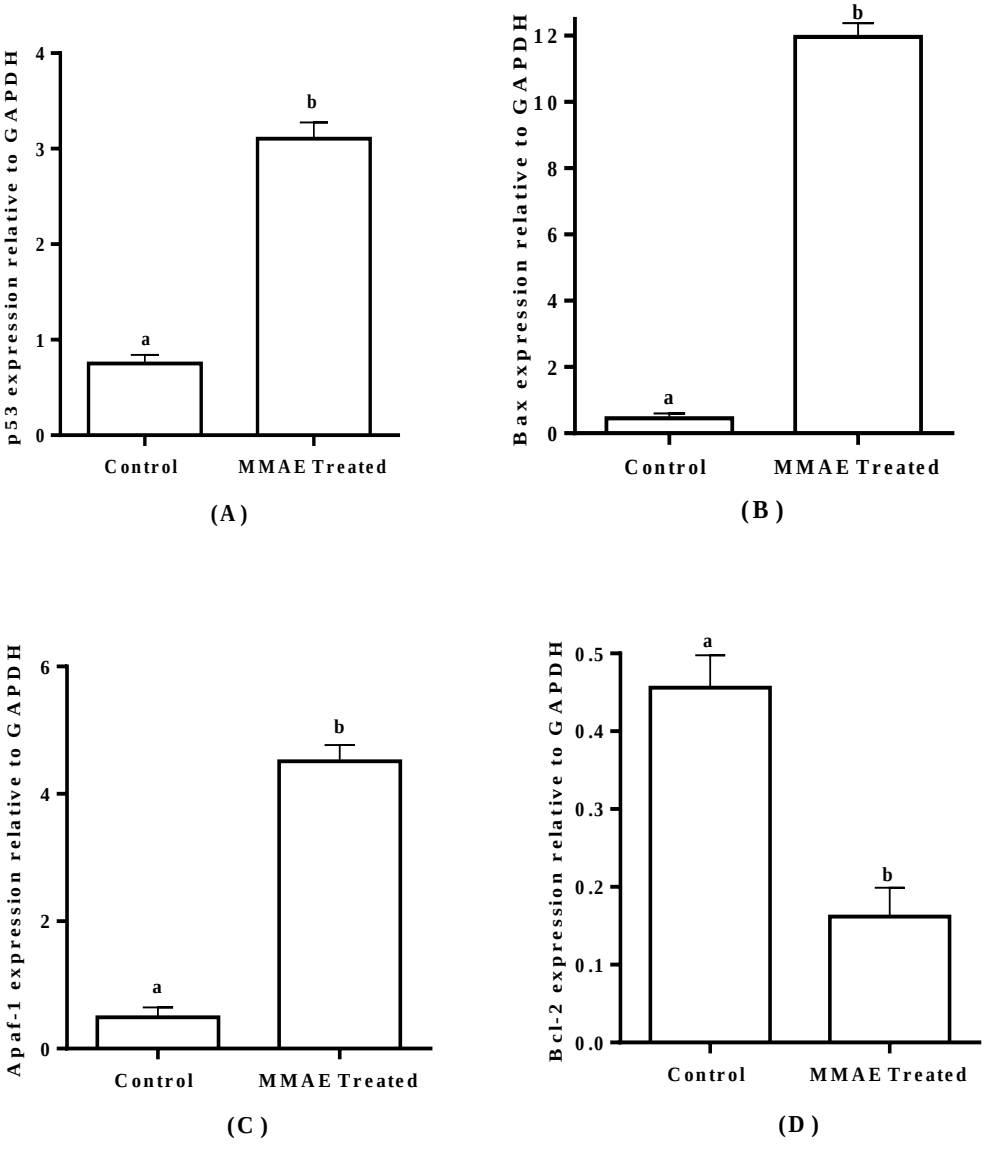


Figure 3.4. Fluorescence images of Comets observed in control (A) and MMAE treated (B), and the extent of DNA damage expressed in terms of Tail length (C) and Olive moment (D). Control: DLA bearing mice without treatment. MMAE treated: DLA bearing mice treated with 100 mg/kg b.wt of aqueous extract of *M. macrophylla*. Different letters indicate significant variation.

3.9. Effect of MMAE on the expression of p53, Bax, Apaf-1, Bcl-2 and Bcl- X_L

The mRNA expression levels of both pro-apoptotic and anti-apoptotic genes were also investigated in DLA bearing mice using qPCR techniques. We found that MMAE induced up-regulation of pro-apoptotic genes including p53, Bax and Apaf-1 by 4.12 folds, 26.57 folds and 4.51 folds, respectively, and down-regulation of Bcl-2 and Bcl-X_L by 2.81 folds and 2.24 folds, respectively, when compared to untreated control. The relative mRNA expression levels of pro-apoptotic genes (Bax, p53 and Apaf1) and anti-apoptotic genes (Bcl-2 and Bcl-X_L) in control and MMAE (100 mg/kg) treated DLA bearing mice is given in Figure 3.5(A-E).



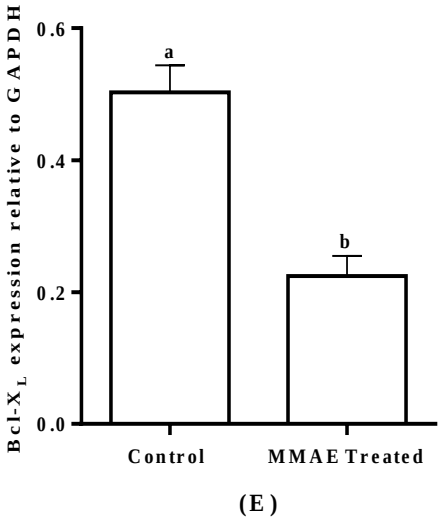


Figure 3.5. Effects of aqueous extract of *M. macrophylla* on mRNA expression levels of (A) p53; (B) Bax; (C) Apaf-1; (D) Bcl-2; (E) Bcl- X_L in Dalton's Lymphoma Ascites (DLA) bearing mice. Control: DLA bearing mice without treatment. MMAE treated: DLA bearing mice treated with 100 mg/kg b.wt of aqueous extract of *M. macrophylla*. Different letters indicate significant variation.

4. DISCUSSION

In order to increase the efficacy of cancer treatment, studies have recently been focused on drugs that have been used in traditional medicine (Singh et al., 2016). Study of the medicinal property of *M. macrophylla* has mostly been focused on its antioxidant, anti-microbial, thrombolytic and anti-diabetic activities with no report on its anticancer activity. Therefore, in this study, we investigate the anticancer activities of the aqueous extract of *M. macrophylla* using DLA bearing mice as our model. Following the standard method of drug administration in DLA mice, the MMAE was also injected intraperitoneally. The dose used in this study was carefully selected after performing the acute toxicity that gave us the approximate LD_{50} which was found to be 1.52 ± 0.117 g/kg b.wt. In all the subsequent analyses, MMAE dose between 50-150 mg/kg b.wt were used which are all below the LD_{50}.

The different plants of the genus *Mussaenda* has been known for exhibiting anticancer activities. Mussaenin A, a compound isolated from *M. glabrata* has been shown to induce apoptosis in Hep G2 via up-regulation of pro-apoptotic genes (Bax, Bak and Bad) and down-regulation of anti-apoptotic genes (Bcl-2 and Cox-2) (Lipin and Darsan, 2017). Sanshiside D, an iridoid glycoside isolated from *M. dona aurora* displayed cytotoxicity and inhibition of cell growth in various cancer cells including Vero, HeLa and SMMC-7721 (Vidyalakshmi and Rajamanickam, 2009). The sepals of *M. phillipica* were shown to exhibit antitumor effects by triggering the antioxidant defence system in Caco-2 and MCF-7 bearing mice (Renilda and Fleming, 2016). *M. roxburghii* (Chowdury et al., 2015) and *M. luteola* (Shylaja and Sathiavelu, 2017) have also shown to have anticancer activities. Interestingly, similar to the other members of the *Mussaenda* genus, *M. macrophylla* shows anticancer potential as evidenced from our current study. Our results indicate that MMAE increased the life span of DLA bearing mice as shown by the increase in MST, AST, % IMLS and % IALS (Table 3.3). Increase in life span of animals is an important criterion for the efficacy of an anticancer agent (Gupta et al., 2004). The body weight of DLA bearing mice generally increases due to the increased cell proliferation of the cancer cells. The potential of MMAE as an anticancer agent was also shown by its ability to suppress the weight gain in DLA bearing mice possibly by inhibiting proliferation of cancer cells *in vivo*. Cytotoxicity of plant extract is another important feature for

consideration as anticancer agent. MMAE treatment was found to exhibit cytotoxicity against ascitic tumors as the treatment reduced tumor volume and increased the percentage of non-viable cells (Table 3.5). Therefore, our findings suggest that the chemoprotective effects of MMAE could be linked to its role in inhibiting cancer cell proliferation, reduction of tumor load and cytotoxicity which account to increase in life span of the cancer bearing animals.

Majority of cancer cells are marked with alteration in hematological parameters (Dongreet al., 2008; Thavamani et al., 2014) as is observed in the case of DLA bearing mice. The haematological changes include reduced levels of RBCs and hemoglobin content accompanied by increased levels of WBC count. Treatment of DLA bearing mice with MMAE showed a pronounced effect in restoring the levels of RBC, hemoglobin and WBC close to that of the normal control levels (Table 3.6). Inflammation of liver is another feature generally observed in ascitic tumors which could be assessed by determining the levels and activities of key liver enzymes such as the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine (CRE). In the DLA bearing mice, all these enzymes were highly elevated which was however significantly lowered with MMAE treatment (Table 3.7). Taking together, MMAE could influence and restore the altered hematological and biochemical parameters in ascitic tumors. The aspect that MMAE could reinstate the altered biochemical and hematological profiles of the DLA bearing mice suggest a promising prospect of *M. macrophylla* as an anticancer agent.

The balance between oxidants and antioxidants in the cell of many cancer types remain key to disease progression or improvement. This is accounted to the known role of accumulation of intracellular ROS and its association with cancer progression. Many cancer cells are shown to have higher ROS levels as compared to their normal counterpart (Tafani et al., 2016). Therefore, to counterbalance the increased ROS levels in the cancer cell, the cells antioxidant mechanisms have to be efficient. Unfortunately, in many cancer types the activities of the antioxidant system are overwhelmed by the high ROS levels. Therefore, many cancer treatments require external agent to assist the cellular antioxidants. Since high ROS ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$) levels have close association with tumor initiation, angiogenesis, cell invasion,

metastasis and chemoresistance in different cancer models (Galadari et al., 2017) use of antioxidants or agents that enhance antioxidant system may provide an opportunity to reduce intracellular ROS-mediated tumorigenesis and cancer progression. In fact, natural products including plant extract have demonstrated antioxidant efficacy such as sesamol, curcumin, ascorbic acid and vitamin E for cancer treatment both *in vitro* and *in vivo* (Galadari et al., 2017). Furthermore, a large variety of antioxidants, either alone or in combination with conventional anticancer agents, have been carried out clinically for their use towards anticancer therapeutics in different types of cancer including Acute lymphoblastic leukemia (Al-Tonbary et al., 2009), breast cancer (Zhang et al., 2012) and ovarian cancer (Ma et al., 2014). Consistently, in the present study, augmentation of GSH level and activities of GST and SOD, and decreased lipid peroxidation as evidenced by the significant decrease in MDA levels after MMAE treatment clearly demonstrates its antioxidant nature which may be responsible for its anticancer activity in DLA bearing mice. Over-expression of antioxidant enzymes such as SOD1, SOD2, SOD3, GPx3 and Prx6 have been reported to induce cell death, decrease survival time and suppression of metastasis in various cancer cells (Galadari et al., 2017). Plants such as *Hypericum hookerianum* (Dongre et al., 2008), *Aegle marmelos* (Chockalingam et al., 2012), *Cyathula prostrate (*Mayakrishnan et al., 2014) and *Cocculus hirsutus* (Thavamani et al., 2014) have been reported to possess anticancer activities in DLA bearing mice via elevation of antioxidant defence system and reduction of lipid peroxidation. An intravenous injection of SOD or its pegylated-SOD derivative have also been reported to significantly inhibit the peroxidation, metastatic tumor growth and extended the survival period of mice inoculated with B16-BL6 cells (Hyoudou et al., 2008).

DNA damage and induction of apoptosis in response to anticancer agents is an important factor in anticancer therapy. In our study we observe that MMAE treatment induces significant DNA damage in the ascites tumor cells (Figure 3.4). Several plant derived anti-cancer drug have been reported to show similar effects in various cancer types including Dalton's lymphoma (Fattahi et al., 2013; Madunić et al., 2018). The Bcl-2 (B-cell lymphoma/leukemia-2) family of both pro- and anti-apoptotic proteins, through their interactions, play central roles in regulation of

diverse cell death mechanisms including apoptosis (Reed, 2008). Alterations in the expression of these genes contribute to the pathogenesis and progression of cancers, thus providing targets for anticancer drug discovery. Altered expression of anti-apoptotic genes such as Bcl-2 and Bcl-X_L and pro-apoptotic genes such as Bax, Bid and Apaf-1 have been documented in several human cancers (Sung et al., 2016; Shang et al., 2018). Pharmacological inhibition of anti-apoptotic gene expression in cancer has emerged as major strategies for inducing apoptosis and ultimately causing tumor regression (Fesik, 2005). In order to assess the effect of MMAE in inducing apoptosis the expression levels of apoptotic genes including p53, Bax, and Apaf-1 and anti-apoptotic genes such as Bcl-2 and Bcl-X_L were determined. Our result shows up-regulation of pro-apoptotic gene expression whilst the anti-apoptotic gene expressions were down-regulated (Figure 3.5). It is thus possible that MMAE triggers apoptotic response in DLA mice and offers protective effects in the animals.

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CHAPTER V

In vitro anticancer activity of *Mussaenda macrophylla* Wall. extracts on human lung adenocarcinoma cell line (A549 cells)

ABSTRACT

The incidence of lung cancer has increased globally along with the increased in the mortality rate due to lung cancer. In this chapter, we explore the anticancer activity of *Mussaenda macrophylla* extracts against Type-II human lung adenocarcinoma cells (A549). MTT assay was used to investigate the cytotoxicity of chloroform (MMCE), methanol (MMME), and aqueous (MMAE) extracts of *M. macrophylla*, and MMAE was shown to be the most effective extract, with an IC_{50} of 49.6 ± 1.12 µg/mL. As a result, MMAE was used for the subsequent tests. Effects of MMAE on reproductive integrity and colony-forming ability of A549 cells was assessed using clonogenic assay and MMAE was found to significantly inhibit cancer cell proliferation in a dose-dependent manner. Effects of MMAE on antioxidants/oxidant status were also determined and MMAE decreased the glutathione (GSH) levels and glutathione-s-transferase (GST) and superoxide dismutase (SOD) activities while consistently increasing the level of lipid peroxidation (LPO) in A549 cells. Fluorescent dual staining, comet assay, qPCR and caspase-3/6 assay were employed for the analysis of apoptotic cell morphology, DNA damage, differential apoptotic gene expression and execution of apoptotic pathway induced by MMAE, respectively. Induction of cell death, DNA damage, up-regulation of pro-apoptotic genes (Bax and p53) and elevation of caspase 3/6 activities in A549 cells following MMAE treatment provide an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our findings demonstrate the role of the aqueous extract of *M. macrophylla* as a potential anticancer agent targeting the apoptotic pathway.

1. INTRODUCTION

Lung cancer has been a major cause of cancer-related deaths for many years, with the numbers of incidence and mortality varying greatly around the world (Barta et al., 2019). In 2020, lung cancer became the 2nd most frequently diagnosed cancer and the leading cause of cancer-related mortality, affecting nearly 2.22 million people with about 1.8 million deaths (Ferlay et al., 2020). Although factors such as tobacco smoke, genetic susceptibility, occupational exposures, alcohol consumption and viral infection are known to be a serious risk factor for lung cancer, the mechanisms involved in development of lung cancer remain largely unknown (Wang et al., 2010; Malhotra et al., 2016). Based on the histology of the cells, lung cancer is classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) and about 85% of all lung cancers are NSCLC that includes adenocarcinoma, squamous cell and large cell carcinoma. Despite recent advances in the treatment of lung cancer including surgery, chemotherapy, radiotherapy and immunotherapy, the prognosis for patients with lung cancer is still poor, with 5-year survival rate of less than 15% demonstrating that current therapy is still inefficient (Collins et al., 2007; Dempke et al., 2010). Combination of chemotherapy drugs with platinum compounds is the most frequently used therapy and optimal treatment options for patients having NSCLC. However, the efficacy of this treatment is still limited due to medication resistance and toxic side effects of the chemotherapeutic drugs (Ramalingam et al., 2011; Reck et al., 2013). Therefore, in order to increase the efficacy of chemotherapy, the search for novel anticancer agents with an enhanced specificity and lesser side effect for cancer remains an urgent need. Moreover, current medications or other strategies to combat chemotherapy-induced side effects are often ineffective, do not address potential long-term consequences, and may even cause additional side effects that merely add to the patient's discomfort. Several synthetic chemotherapeutic treatments have been known to possess both beneficial and detrimental side effects, such as cardiotoxicity and hair loss associated with vomiting. In contrast, in plants, highly active chemicals coexist with compounds that are antagonistic to one another. As a result, research centering on the use of natural products has provided potential alternatives for anticancer therapy.

Over the last few years, the use of plants in primary health care and phytotherapeutic research has expanded in the wake of identification of bioactive compounds in medicinal plants. Research centering on the use of natural products has provided potential alternatives for anticancer therapy and more than half of the anticancer medications tested in clinical trials are derived from plant-based natural sources (Desai et al., 2008). A549 cells are human alveolar basal squamous epithelial cells adenocarcinoma obtained from a 58-year-old Caucasian male (Giard et al., 1972) that grows adherently as a monolayer *in vitro*. Recent studies have reported the anticancer properties of different plants such as *Bridelia ovata*, *Croton oblongifolius*, *Erythrophleum succirubrum* (Poofery et al., 2020), *Sapindus mukorossi* (Liu et al., 2018) and *Ebenus boissieri* (Aydemir et al., 2015) in A549 cells.

The genus *Mussaenda* has been reported to be a remarkable source of natural products in the field of pharmacology (Vidyalakshmi et al., 2008). *M. macrophylla* Wall. is a flowering shrub belonging to the Rubiaceae family and is endemic to south-east Asia (Arunachalam et al., 2015). Traditionally, different parts of *M. macrophylla* has been used for the treatment of various health problems such as cough, sore mouth, fever, chronic ulcer, diarrhea, dysentery, indigestion, cancer, and even for the treatment of snake bites (Manandhar, 1994; Sharma et al., 2001; Rosangkima and Jagetia, 2015). *M. macrophylla* have also been reported to exhibit multi-pharmaceutical properties including antioxidant (Islam et al., 2012), anti-microbial (Kim et al., 1999; Chowdhury et al., 2013), anti-diabetic (Bhandari et al., 2020) and thrombolytic (Islam et al., 2013). Research prioritized towards objectively evaluating the potential anticancer activity of *M. macrophylla* is crucial given the existing state of scientific evidence on various pharmaceutical applications. Therefore, the present study was undertaken to examine growth inhibitory and cytotoxic effects of *M. macrophylla* in human lung adenocarcinoma A549 cells and the underlying mechanism by which *M. macrophylla* exerts apoptosis-based anticancer activity was evaluated.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA, Eagle's Minimal Essential Medium (MEM), fetal bovine serum (FBS), sodium bicarbonate, bovine serum albumin (BSA), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), Folin-ciocalteau's reagent, n-butanol, thiobarbaturic acid (TBA), potassium chloride (KCl), sodium Chloride (NaCl), Triton X-100, acridine orange, ethidium bromide, phenazine methosulphate (PMS), dimethyl sulphoxide (DMSO), 1-chloro-2,4 dinitrobenzene (CDNB) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). L-Glutamine, phenol red, agarose (low gelling temperature), ethylenediamine tetra-acetic acid (EDTA), Trizma base, Trizma hydrochloride and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., Bangalore, India. Fluorouracil (5FU) was obtained from GLS Pharma Ltd. (Hyderabad, India). The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2. Preparation of *M. macrophylla* extracts

Collection and preparation of *M. macrophylla* was carried out as described in Chapter 2 (Section 2.2) to obtain the plant extracts.

2.3. Cell lines and Culture

Type II human lung adenocarcinoma cell line (A549 cells) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in MEM supplemented with 10% FBS, 1% L-Glutamine and 50 µg/mL gentamicin sulfate in a humidified incubator containing 5% CO_2 at 37^o C (Eppendorf AG, Hamburg, Germany).

2.4. Cytotoxicity assay

The cytotoxicity of *M. macrophylla* was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Mossman, 1983). Briefly,

1×10^4 cells were seeded into flat-bottomed 96 well plates (Himedia Laboratories Pvt. Ltd., Mumbai, India) containing 100 µL of MEM. The cells were allowed to adhere for 24 h at 37 ºC with 5 % CO_2 and treated with different concentrations (25-300 µg/mL) of various solvent extracts of *M. macrophylla* for 12, 24 and 48 h along with a control sample. At the end of treatments, drug containing media were removed and cells were washed with FBS free media. Then, 10 µL of MTT (5 mg/mL) was added to each well and incubated for another 2 h at 37 ºC in a CO_2 incubator. The insoluble purple formazan crystals formed were then dissolved in 100 µL of DMSO and incubated for 30 min. The absorbance of the solution was measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). Three independent experiments consisting of three replicates were carried out for each treatment. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

% inhibition = Control-Treatment/Control X 100.

The aqueous extract of *M. macrophylla* (MMAE), the most effective extract in the preliminary cytotoxicity screening using MTT assay was subsequently used for further experiments.

2.5. Clonogenic Assay

The effect of aqueous extract of *M. macrophylla* (MMAE) on the reproductive integrity of A549 cells was assessed using clonogenic assay (Franken et al., 2006). Exponentially growing cells were harvested from a stock culture by trypsinization and 200 cells were seeded into several individual petri-dish containing 5 mL of media. After overnight adherence, cells were treated with different concentrations of MMAE (25-100 µg/mL) or 5FU (100 µg/mL) for 24 h. After treatment with MMAE or 5FU, cells were washed with sterile 1X PBS and cultured in a fresh medium for another 11 days. The resultant colonies were then stained with 1% crystal violet in methanol (w/v) for 30 min at room temperature and colonies with more than 50 cells were counted using an inverted microscope. Plating efficiency (PE) and surviving fraction (SF) of A549 cells were calculated by the following formula:

PE = (Number of colonies counted × 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) × (Mean plating efficiency)

2.6. Cell morphology analysis by fluorescent staining (Apoptotic assay)

The ability of MMAE to induce apoptosis was studied by acridine orange/ethidium bromide (AO/EtBr) staining. Briefly, 1×10^5 A549 cells were seeded in several six-well plates containing 5 mL of media. Cells were allowed to adhere overnight and treatment was given for 24 h with different concentrations of MMAE (25-100 µg/mL) or 5FU (100 µg/mL). Untreated controls were also maintained in culture medium alone. After treatments, cells were washed with sterile 1X PBS and detached with 1X trypsin EDTA. The cells were pelleted and resuspended in 100 µL of FBS free media. Subsequently, 25 µL cell suspension was stained with 2.5 µL each of acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL) in a ratio of 1:1 for 2 min followed by gentle mixing. The morphology of apoptotic cells was then examined on a slide under a fluorescent microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615). Acridine orange is a nucleic acid fluorescent cationic dye that permeates both live and dead cells, intercalating in the double-stranded DNA, and makes the nuclei appear green. Ethidium bromide is taken up only by dead cells whose cytoplasmic membrane integrity is lost and stains the nuclei yellowish orange. For this reason, live cells have green nuclei whereas apoptotic cells that incorporated ethidium bromide exhibit condensed and fragmented orange chromatin. Conversely, necrotic cells have structurally normal orange nucleus (Kasibhatla et al., 2006). At least 300 cells were scored and the apoptotic index was determined as follows:

Apoptotic index (%) = Number of apoptotic cells scored × 100/Total number of cells counted

2.7. Assessment of genotoxicity of MMAE using Comet assay

The Comet assay was performed using the method described earlier with minor modifications (Singh et al., 1988). Briefly, 2×10^4 A549 cells treated with different concentrations of MMAE or 5FU for 24 h along with the untreated control were suspended in 75 µL of 0.5% low-melting point agarose (LMPA) prepared in 1X PBS

and spread onto a frosted slide precoated with 1% normal-melting point agarose (NMPA) and covered with a coverslip. Once the gel got solidified following incubation of the slide at 4°C, the coverslip was gently removed and the third layer of 90 µL 0.5% LMPA was added. The slides were then incubated for 2 h in a freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10). After lysis, slides were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA. The slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralization, slides were washed with distilled water and then stained with ethidium bromide (EtBr) solution (2 µg/mL) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using florescence microscope with a magnification of 200x. Image capture and analysis were performed with Image J software.

2.8. Antioxidants/oxidant assays

For the estimation of antioxidant enzyme activities and lipid peroxidation level, 1×10^6 cells were seeded in a T-25 flask containing 5 mL media. At the end of 24 h treatments with 100 µg/mL MMAE or 5FU, drug containing media was discarded and the cells were washed with sterile 1X PBS and harvested. The cancer cells were pelleted, sonicated (PCI Analytics Pvt. Ltd., Mumbai, India) and 5% homogenate was prepared using cold sterile PBS (pH-7.4) and used for biochemical estimations. Total protein contents were determined using standard protocol (Lowry et al., 1951) using bovine serum albumin as standard.

2.8.1. *Glutathione (GSH)*

Glutathione (GSH) levels were measured by its reaction with DTNB in Ellman's reaction to give a compound that absorbs light at 412 nm (Moron et al., 1979). Briefly, 80 µL of the cell homogenate was mixed with 900 µL of 0.02M sodium phosphate buffer and 20 µL of 10 mM DTNB and incubated for 2 min at room temperature. The blank consisted of distilled water instead of cell homogenate. The absorbance of the sample was read against blank at 412 nm in a UV-visible

spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). GSH concentration was calculated from the standard graph and expressed in µmol/mg protein.

2.8.2. *Glutathione-s-transferase (GST)*

Glutathione-s-transferase (GST) activity was measured using the standard method (Beutler, 1984). Briefly, 50 µL of 20 mM CDNB was added to 850 µL of 0.1M phosphate buffer (pH 6.5) and incubated for 10 min at 37 °C. Then, 50 µL each of 20 mM GSH and cell homogenate was added to the mixture. For blank, distilled water was added instead of cell homogenate. The absorbance of the sample was measured at 1 min interval for 5 min at 340 nm. GST activity was measured as: GST activity = (OD of test–OD of blank/9.6 ×vol. of test sample) × 1000; where, 9.6 is the molar extinction coefficient for GST.

2.8.3. *Superoxide dismutase (SOD)*

Superoxide dismutase (SOD) activity was measured by the NBT reduction method (Fried, 1975). Briefly, 100 µL each of cell homogenate and 186 µM PMS was mixed with 300 µL of 3 mM NBT and 200 µL of 780 µM NADH. The mixture was incubated for 90 sec at 30 ºC and 1 mL of acetic acid and 4 mL of n-butanol were added to stop the reaction. The blank consisted of all the reagents, except the cell homogenate. The absorbance of test and blank was measured at 560 nm and the enzyme activity was expressed in unit (1 unit = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank - OD of sample/OD of blank) × 100

2.8.4. *Lipid peroxidation (LPO)*

Lipid peroxidation (LPO) was measured by the standard method (Beuege and Aust, 1978). Malondialdehyde (MDA), one of the toxic products formed from the oxidation of fatty acids such as polyunsaturated fatty acids and phospholipids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA derived from LPO reacts with TBA to give a red fluorescent adduct absorbing at 535 nm. Briefly, cell homogenate was added to a mixture of 10% TCA, 0.8% TBA and 0.02 N HCl in 1:2 ratio. The mixture was boiled for 10 min, cooled immediately at room temperature and centrifuged at 1,000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank. The

MDA concentration of the sample was calculated using the extinction coefficient of 1.56 x 106/M/cm.

2.9. qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression

1×10^6 A549 cells were seeded in six-well plates containing 5 mL of media. Cells were allowed to adhere overnight at 37 ^oC with 5% CO_2 and treated with 100 µg/mL MMAE or 5FU for 24 h along with a control sample. After treatment, cells were washed and detached. The cells were then pelleted and total RNA was extracted using Tri reagent (BR Biochem, Life Science Pvt. Ltd, R1022). Extracted RNA was quantified using Nanodrop Spectrophotometer (Eppendorf Biophotometer Plus, Hamburg, Germany) and RQ1 DNase kit (Promega, M198A, Madison, WI, USA) was used to remove the genomic contamination. cDNA was synthesized from 2 µg of total RNA using first-strand cDNA synthesis kit (Thermoscientific, K1621; Lithuania, Europe). Gene-specific primers were designed using Primer 3, Boston, MA, USA. The primer sequences used in qRT-PCR analyses are given in Table 4.1. qPCR was performed using Quant-Studio 5 (ThermoFisher Scientific, Foster City, CA, USA). PCR reaction volume of 7 µL for each gene comprised of 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25742, Lithuania, Europe) and 1 µL of nuclease-free water (ThermoFisher Scientific, A19938, Bangalore, India). The cycling condition of qPCR were 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s). Afterwards, melting curves were generated to confirm a single uniform peak. GAPDH gene was used as a reference gene for determining the relative expression levels of specific target genes. Each sample was run in duplicate along with non-template and negative RT controls. The relative expression of genes was determined using ΔΔCt method (Livak and Schmittgen, 2001).

Table 4.1. Primer sequences and their product size used in qRT-PCR analyses of A549 cells treated with aqueous extract of *M. macrophylla* (100 µg/mL).

Genes	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
Bax	TCCCCCCGAGAGGTCTTTT	CGGCCCCAGTTGAAGTTG	68
p53	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTGT	159
Bid	CCTTGCTCCGTGATGTCTTTC	GTAGGTGCGTAGGTTCTGGT	62
DR-5	CAGAGGGATGGTCAAGGTCG	TGATGATGCCTGATTCTTTGTGG	77
Apaf-1	AAGGTGGAGTACCACAGAGG	TCCATGTATGGTGACCCATCC	116
Bcl-X_L	GGCCACTTACCTGAATGACC	AAGAGTGAGCCCAGCAGAAC	180
PARP	CCAGATGCTTGTCTTCCTGAGAG	AGTGACAGCAGGGTTGGCATGA	133
Survivin	AGAACTGGCCCTTCTTGGAGG	CTTTTTATGTTCCTCTATGGGGTC	153
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185

2.10. Caspase-3/6 activity assay

A quantitative enzymatic activity assay was carried out for caspase 3 and caspase 6 according to the manufacturer protocols (BioVision Incorporated, USA). Briefly, 5×10^5 A549 cells were treated with 100 µg/mL of MMAE or 5FU for 24 h in a six-well plate along with the untreated control. After treatment, cells were washed and lysed in 50 µL of chilled lysis buffer followed by 10 min incubation on ice. The cell lysates were centrifuged at 15,000 × g for 1 min at 4°C, and the supernatant was collected. Total amount of protein was determined by Bradford assay (Bradford, 1976). The assay was performed in a total volume of 100 µL in 96-well plates. 100 µg of protein from each sample was assayed for caspase-3/6 activity against their specific colorimetric substrate DEVD-*p*NA for caspase-3, and VEID-*p*NA for

caspase-6. The mixture was incubated for another 2 h at 37 °C and absorbance of free *p*-nitroanilide (*p*NA) produced via cleavage from their specific substrates by activated caspase-3 and caspase- 6 was measured at 405 nm using microplate reader.

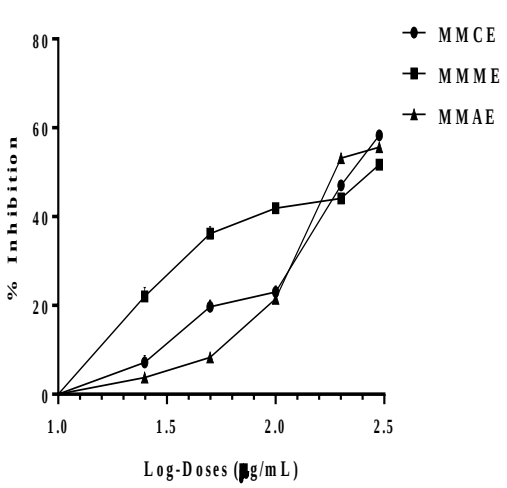
2.11. Statistical analysis

All data were expressed as mean ± standard error of mean of pooled results obtained from three independent experiments. One-way ANOVA followed by Tukey's test was performed to test significant variations on cytotoxic, clonogenic, apoptotic assays, antioxidants/oxidant status, comet assay, differential gene expression and caspase activities. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 6.0 were used for statistical and graphical analyses. A p-value of less than 0.05 was considered statistically significant.

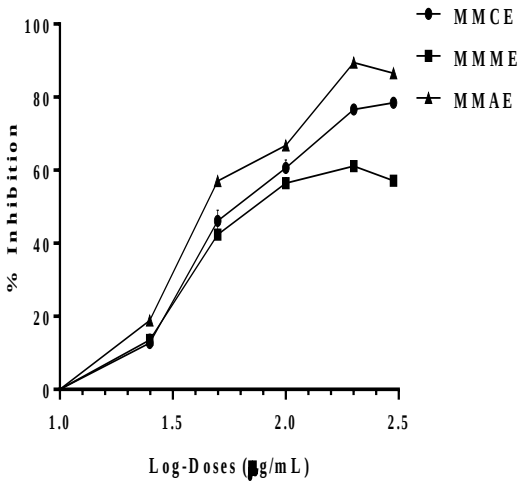
3. RESULTS

3.1. The anti-proliferative and cytotoxic effects of *M. macrophylla* extracts in A549 cells

To determine the cytotoxic effect of different extracts of *M. macrophylla*, A549 cells were treated with different doses for 12, 24 and 48 h. The inhibition (%) of A549 cells by different extracts of *M. macrophylla* was plotted against log-doses for the calculation of IC_{50} (Figure 4.1A-C). Aqueous extract of *M. macrophylla* showed higher cytotoxic effect compared to chloroform and methanol extracts at all time points and its cytotoxicity was found to be highest at 24 h treatment duration with an IC_{50} of 49.6 ± 1.12 (Figure 4.1D). Therefore, only aqueous extract was used in the subsequent experiments.



A (12 h)



B (24 h)

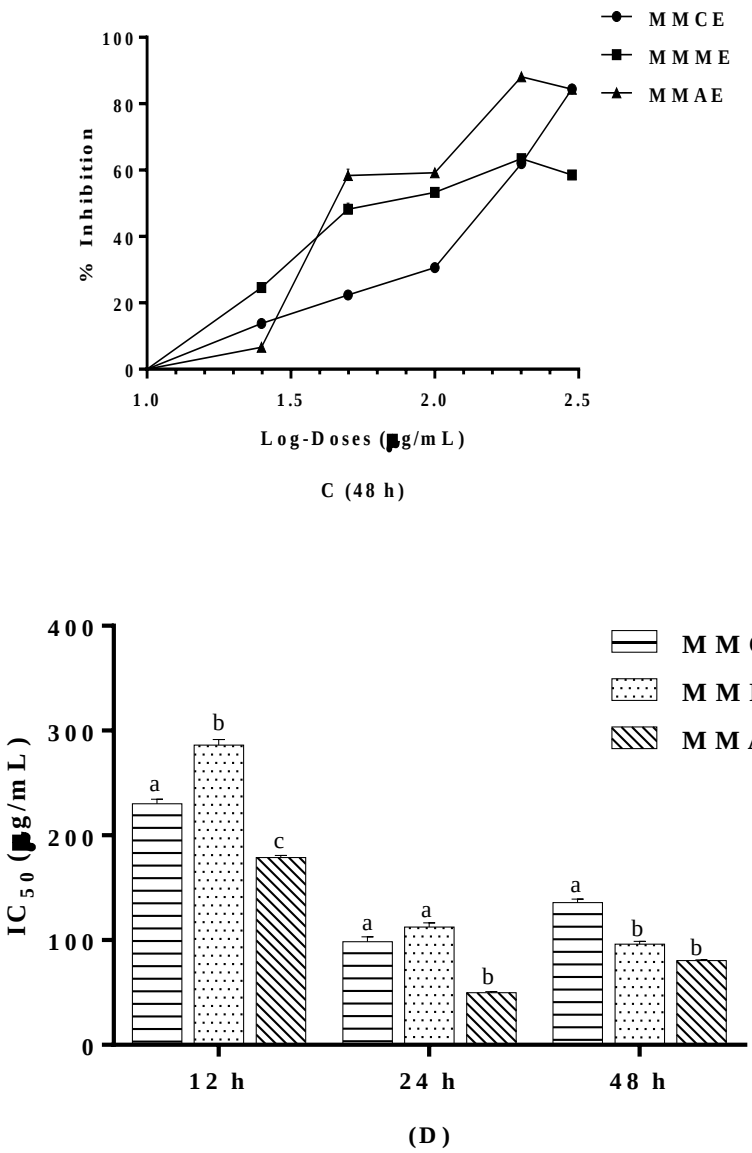
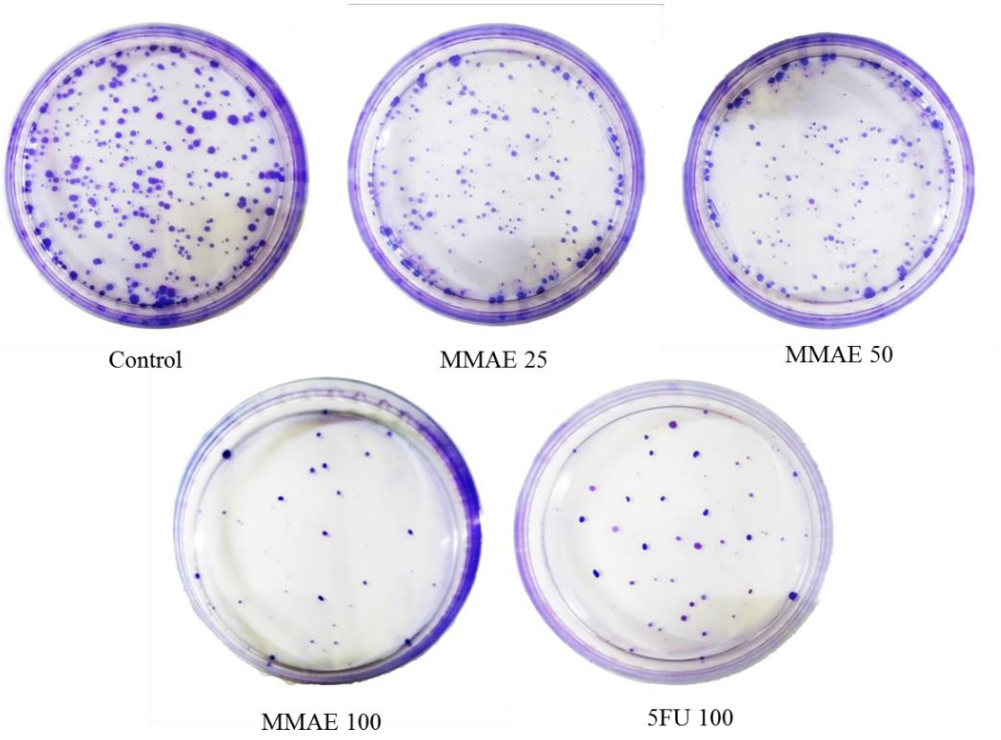


Figure 4.1. (A-C) Plots of log-doses of various extracts of *M. macrophylla* against inhibition (%) of A549 cells after 12, 24 and 48 h treatment for the calculation of IC_{50}. (D) Cytotoxic effects (IC_{50}) of different extracts of *M. macrophylla* on A549 cells after 12, 24 and 48 h treatment. MMCE: *M. macrophylla* chloroform extract; MMME: *M. macrophylla* methanolic extract; MMAE: *M. macrophylla* aqueous extract. Values are expressed as Mean ± SEM. Different letters indicate significant variation between extracts at each treatment duration.

3.2. The inhibitory effect of MMAE on the clonogenicity of A549 cells

In order to determine the effect of MMAE on reproductive viability, low concentration of A549 cells plated on petri-dishes were treated with different doses of MMAE and allowed to grow for 11 days. Only single cells that were resilient enough to divide and form colonies were assessed. We found that MMAE treatment effectively reduced the clonogenicity of A549 cells in a dose dependent manner when compared with the untreated control (Figure 4.2A). A549 cells treated with 25, 50 and 100 µg/mL of MMAE led to a significant inhibition of colony forming capability of the cells with surviving fraction of 51.5%, 40.3% and 2.6% respectively (Figure 4.2B). MMAE at the dose of 100 µg/mL showed comparable colony inhibiting activity with the standard drug 5FU.



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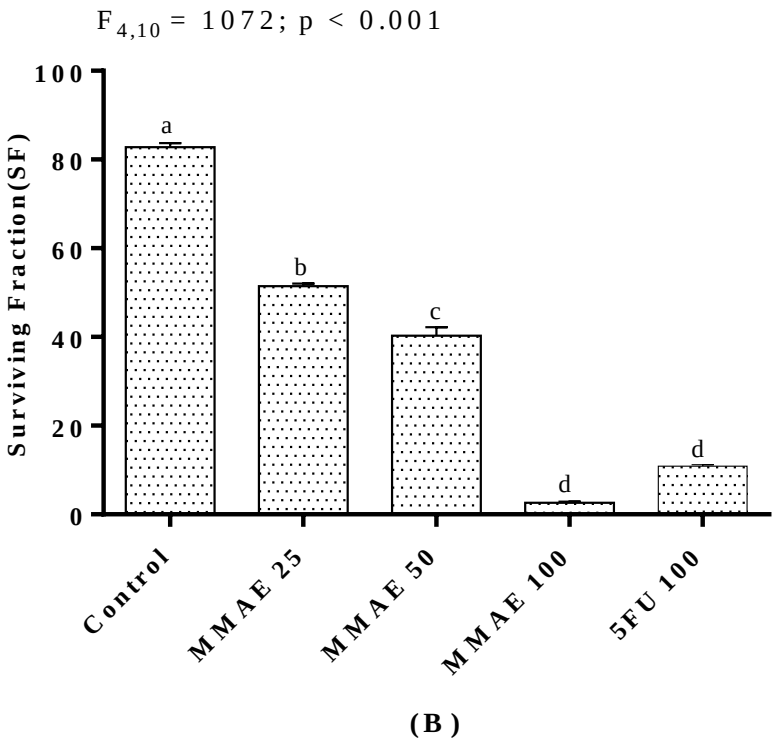
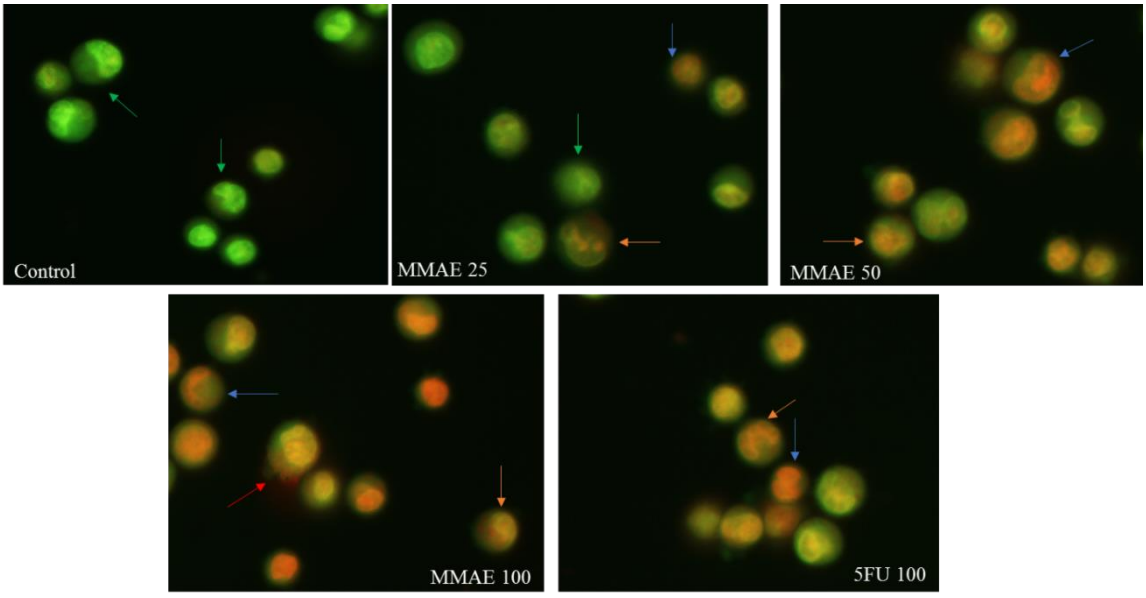


Figure 4.2. (A) Inhibition of colony formation of A549 cells mediated by aqueous extract of *M. macrophylla.* (B) Effect of aqueous extract of *M. macrophylla* on the reproductive capability of A549 cells after 24 h treatment, expressed as surviving fraction (SF). Control: A549 cells without treatment; MMAE 25, MMAE 50 and MMAE 100: A549 cells treated with 25, 50 and 100 µg/mL of aqueous extract of *M. macrophylla* respectively. 5FU 100: A549 cells treated with 100 µg/mL of 5FU (positive control). Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.3. Morphological evidence of apoptosis induced by MMAE

Treatment of A549 cells with different concentrations of MMAE for 24 h resulted in a dose dependent increase in the number of apoptotic cells. Fluorescence microscopic images of the MMAE treated cells (Figure 4.3A) clearly revealed morphological alterations such as membrane blebbing, nuclear condensation and nuclear fragmentation which are the distinct characteristics of apoptotic cells. The apoptotic index increased in a concentration dependent manner in cells treated with MMAE, where the percentage of dead cells were found to be 16.8%, 60.2% and 80.3% after treatment with MMAE at the dose of 25, 50 and 100 µg/mL respectively (Figure 4.3B). The apoptotic index of MMAE at the dose of 100 µg/mL was comparable to that of the standard 5FU.



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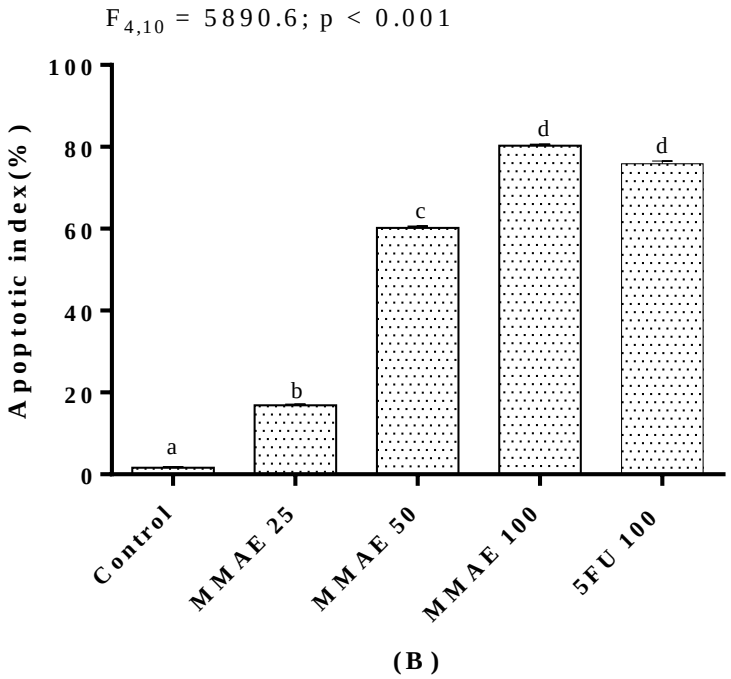
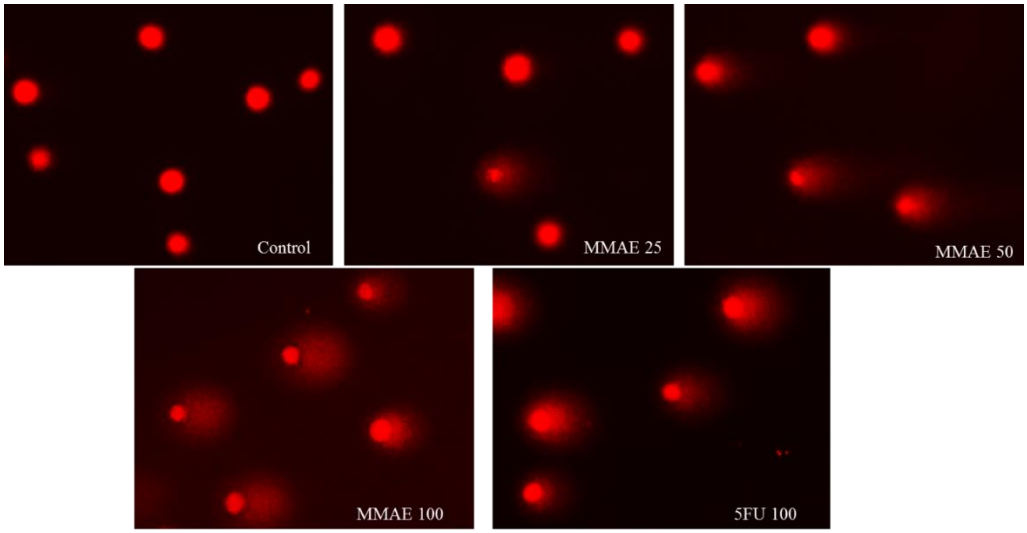


Figure 4.3. (A) Acridine orange/Ethidium bromide (AO/EtBr) dual staining of A549 cells after treatment with different doses of aqueous extract of *M. macrophylla* for 24 h (green arrow shows the live cells, blue arrow shows apoptotic cells with nuclear condensation, orange arrow shows apoptotic cells with nuclear fragmentation, and red arrow shows apoptotic cells with membrane blebs). (B) Percentage of dead cells after treatment of A549 with aqueous extract of *M. macrophylla*. Control: A549 cells without treatment; MMAE 25, MMAE 50 and MMAE 100: A549 cells treated with 25, 50 and 100 µg/mL of aqueous extract of *M. macrophylla* respectively. 5FU 100: A549 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.4. Induction of DNA strand breaks by MMAE

The alkaline Comet assay was used to assess DNA damage in A549 cells after treatment with different doses of MMAE (25, 50 and 100 µg/mL) for 24 h. Our results showed that MMAE induced significant DNA damage in A549 cells in a dose dependent manner as indicated by increased tail length and olive moment in MMAE treated groups when compared to untreated control (Figure 4.4A-C). The induction of DNA damage by MMAE treatment at the dose of 100 µg/mLwas comparable to the induction by treatment with the standard 5FU.



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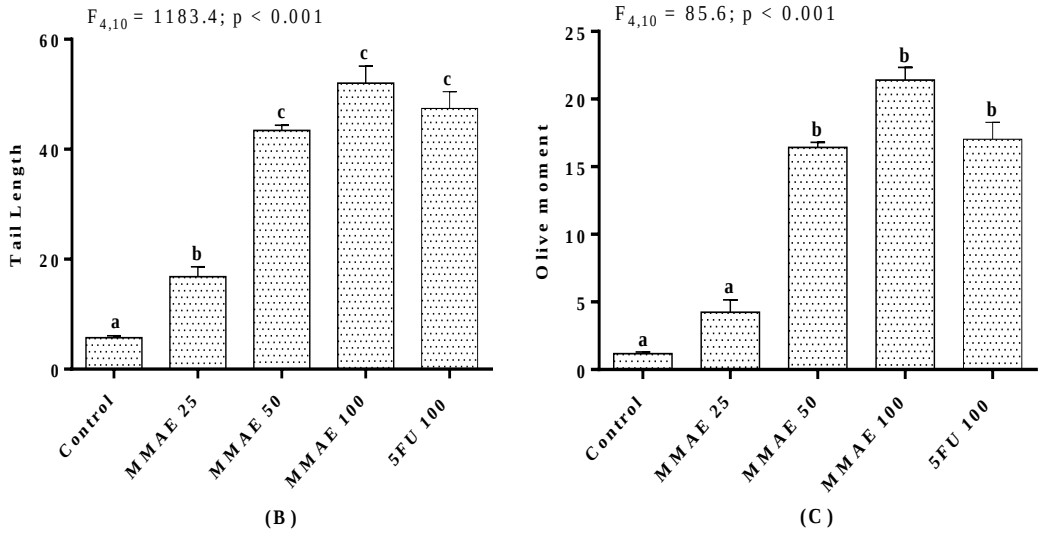


Figure 4.4. (A) Fluorescence images of Comets observed in control and A549 cells treated with different concentration of MMAE. (B & C) The extent of DNA damage expressed in terms of Tail length and Olive moment. Control: A549 cells without treatment; MMAE 25, MMAE 50 and MMAE 100: A549 cells treated with 25, 50 and 100 µg/mL of aqueous extract of *M. macrophylla* respectively. 5FU 100: A549 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.5. Antioxidants/Oxidant Status

Treatment of A549 cells with MMAE (100 µg/mL) significantly decreased glutathione (GSH) concentration and the activities of glutathione-s-transferase (GST) and superoxide dismutase (SOD) when compared to the untreated control (Figure 4.5A-C). In an effort to investigate whether MMAE treatment affect intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was also assessed. Our result shows that MMAE treatment significantly enhanced MDA level in A549 cells (Figure 4.5D), indicating that MMAE might lead to increase in the intracellular ROS levels via disruption of the antioxidant system. MMAE treatment was found to show similar effectiveness with the standard drug 5FU.

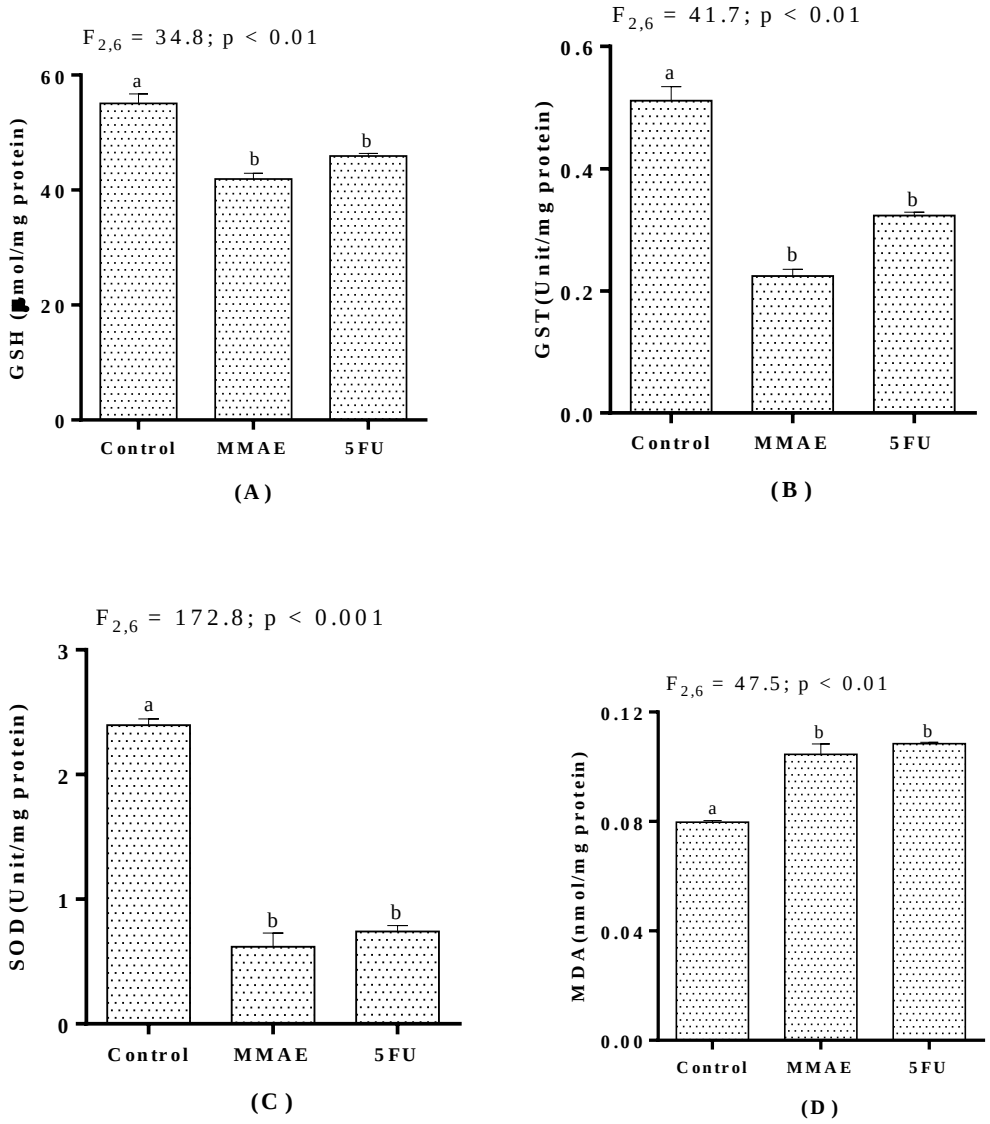
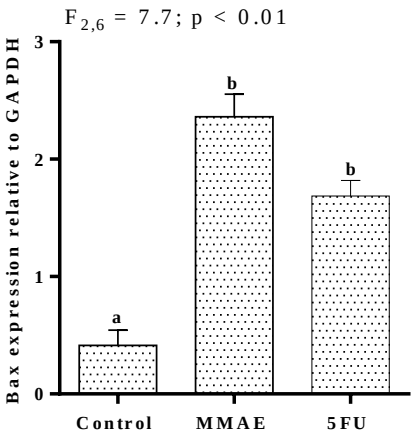


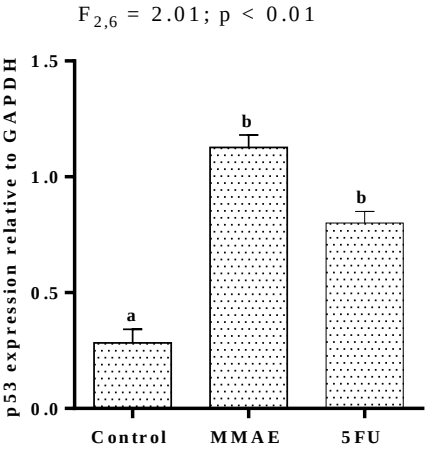
Figure 4.5. Effects of the aqueous extract of *M. macrophylla* on (A) glutathione level (GSH); (B) glutathione-s-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; and (D) lipid peroxidation (LPO) expressed in malondialdehyde (nmol/mg protein) in A549 cells after 24 h treatment. Control: A549 cells without treatment; MMAE: A549 cells treated with 100 µg/mL of aqueous extract of *M. macrophylla*. 5FU: A549 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.6. Effect of MMAE on the relative expression of pro-apoptotic and anti-apoptotic genes

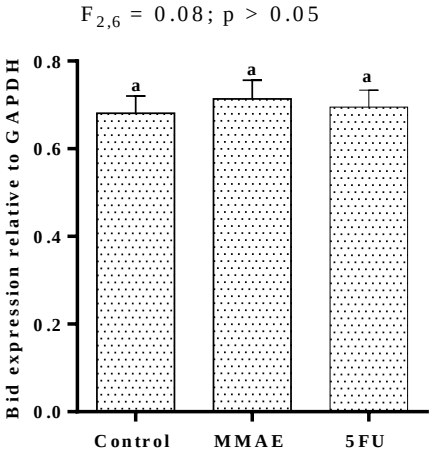
The mRNA expression levels of both pro-apoptotic and anti-apoptotic genes were also investigated in A549 cells using qPCR techniques. We found that treatment of A549 cells with 100 µg/mL MMAE for 24 h induced up-regulation of the pro-apoptotic genes such as Bax and p53 by 5.71 and 3.98 folds respectively when compared to the untreated control. The capability of MMAE to up-regulate pro-apoptotic genes (Bax and p53) was found to be comparable to that of the standard 5FU treatment. However, no significant change was observed in the level of mRNA expression of pro-apoptotic genes (Bid, DR-5 and Apaf-1) and anti-apoptotic genes (PARP, Bcl-X_L and Survivin) between the control and MMAE treated groups (Figure 4.6).


$F_{2,6}$ = 7.7; p < 0.01
Bax expression relative to GAPDH
3
2
1
0
a
b
b
Control
MMAE
5FU

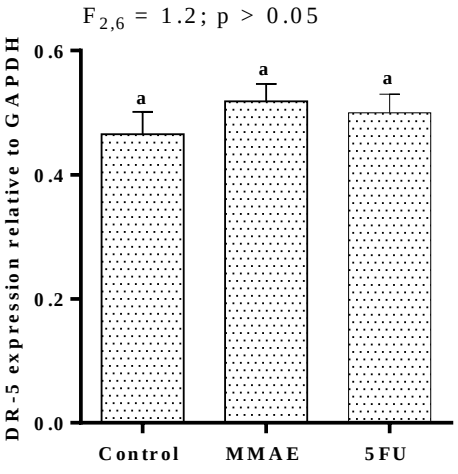
(A)


$F_{2,6}$ = 2.01; p < 0.01
p53 expression relative to GAPDH
1.5
1.0
0.5
0.0
a
b
b
Control
MMAE
5FU

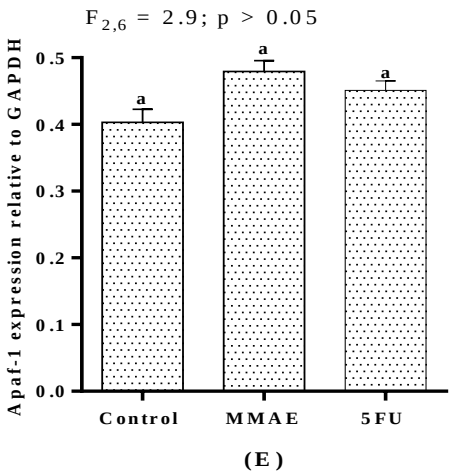
(B)


$F_{2,6}$ = 0.08; p > 0.05
Bid expression relative to GAPDH
0.8
0.6
0.4
0.2
0.0
a
a
a
Control
MMAE
5FU

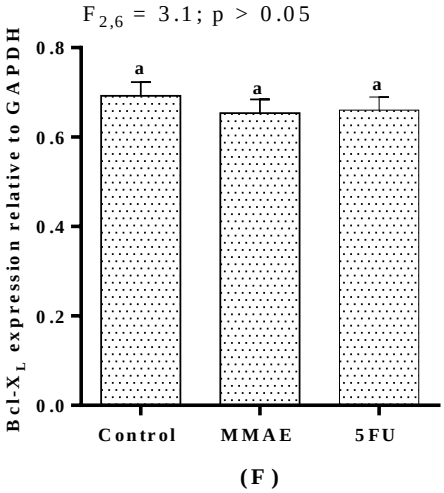
(C)


$F_{2,6}$ = 1.2; p > 0.05
DR-5 expression relative to GAPDH
0.6
0.4
0.2
0.0
a
a
a
Control
MMAE
5FU

(D)


$F_{2,6}$ = 2.9; p > 0.05
Apaf-1 expression relative to GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
Control
MMAE
5FU

(E)


$F_{2,6}$ = 3.1; p > 0.05
Bcl-X_L expression relative to GAPDH
0.8
0.6
0.4
0.2
0.0
a
a
a
Control
MMAE
5FU

(F)

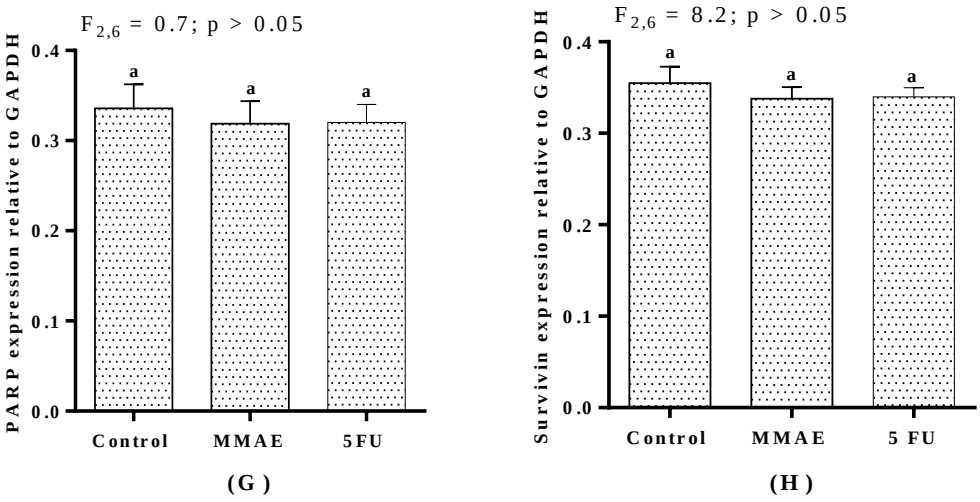


Figure 4.6. Effects of the aqueous extract of *M. macrophylla* on mRNA expression levels of (A) Bax; (B) p53; (C) Bid; (D) DR-5; (E) Apaf-1; (F) Bcl- X_L; (G)PARP; and (H) Survivin in A549 cells after 24 h treatment. Control: A549 cells without treatment; MMAE: A549 cells treated with 100 µg/mL of aqueous extract of *M. macrophylla*. 5FU: A549 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.7. Activation of caspase-3/6 by MMAE on A549 cells

Caspase-3/6 plays an important role in execution of apoptosis in cancer cells. Effect of MMAE on A549 cell apoptosis was assessed by caspase-3/6 activity. Treatment of A549 cells with 100 µg/mL of MMAE for 24 h shows significant increase in the activation of the effector caspases, caspase-3 and 6. Our result shows that MMAE treatment increases the activities of caspase-3 and caspase-6 in A549 cells by 1.88 and 3.21 folds respectively, compared to the untreated control (Figure 4.7). The efficacy of MMAE to induce caspase-6 activity in A549 cells was even better than the standard drug 5FU which increase the caspase-6 activity by 2.07 folds.

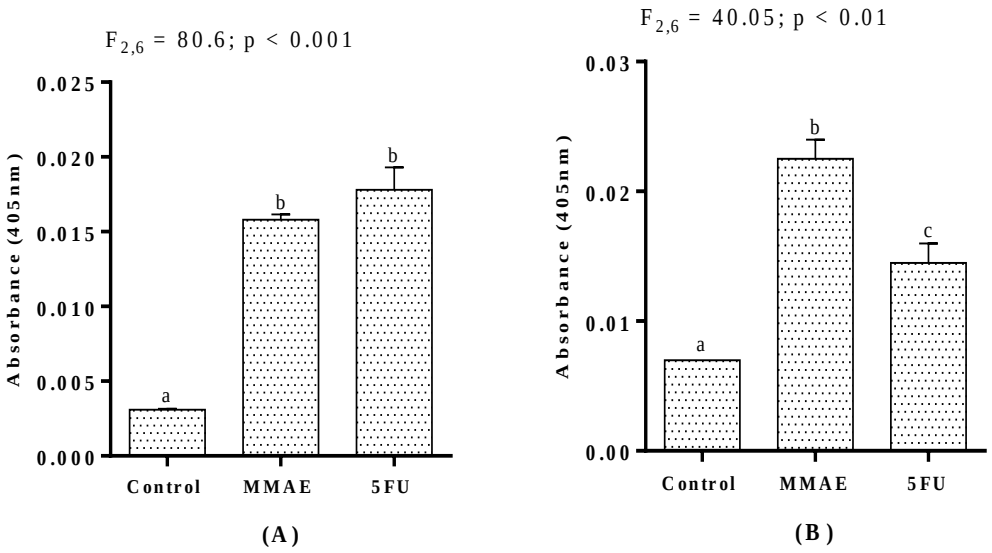


Figure 4.7. Effects of the aqueous extract of *M. macrophylla* on activities of (A) Caspase-3 and (B) Caspase-6 in A549 cells after 24 h treatment. Control: A549 cells without treatment; MMAE: A549 cells treated with 100 µg/mL of aqueous extract of *M. macrophylla*. 5FU: A549 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

4. DISCUSSION

Due to the toxic side effects posed by the chemotherapy drugs, medicinal plants have been extensively screened to explore their potential for the development of new anticancer drugs (Singh et al., 2016). Among the genus *Mussaenda*, species such as *M. dona aurora* (Vidyalakshmi and Rajamanickam, 2009), *M. roxburghii* (Chowdury et al., 2015), *M. phillipica* (Renilda and Fleming, 2016), *M. glabrata* (Lipin and Darsan, 2017), and *M. luteola* (Shylaja and Sathiavelu, 2017) have been known for exhibiting anticancer activities through different molecular mechanisms. In this study, we examined growth inhibitory and cytotoxic effects of *M. macrophylla* and the underlying mechanism by which *M. macrophylla* exerts apoptosis-based anticancer activity was investigated in human lung adenocarcinoma A549 cells.

The goal of targeting cell proliferation in cancer cell is to induce cell death or arrest the cell cycle using cytotoxic compounds. Calorimetric MTT assay is a rapid and standard method for testing cytotoxicity of drugs in various cultured cells wherein reduction of MTT can occur only in metabolically active cells. Treatment of A549 cells with different concentrations of various extracts of *M. macrophylla* resulted in a dose dependent increase in cytotoxicity. Among the various extracts of *M. macrophylla*, aqueous extract of *M. macrophylla* (MMAE) was found to possess the highest cytotoxic effect at 24 h treatment duration with an IC_{50} of 49.6 ± 1.12 µg/mL (Figure 4.1D). Plants such as *Curcuma longa* (Mohammad et al., 2010), *Urginea maritima* (Bozcuk et al., 2010), *Tecoma stans* (Robinson et al., 2017), *Clerodendrum inerme* (Chouhan et al., 2018), *Momordica charantia* (Gunes et al., 2019), *Lactifluus rugatus* (Sevindik, 2020) and *Seseli petraeum* (Cinar et al., 2020) have recently been found to exert cytotoxic effect on A549 cells. The ability of cancer cells to form colonies enables them to communicate and build into solid tumor. Thus, a significant parameter to assess the effectiveness of treatment on cancer cells is to determine their reproductive integrity and colony-forming capability. MMAE treatment was found to reduce the clonogenicity of A549 cells in a concentration dependent manner which were correlated well with our MTT assay result, and the surviving fraction of A549 cells after treatment with 100 µg/mL MMAE was only 2.6 % (Figure 4.2) indicating the effectiveness of the plant extract in inhibition of colony formation in cancer cell.

Exploring the precise mechanisms by which anticancer agent exerted their actions has become an important approach for the evaluation and development of anticancer drug. Apoptosis is an essential and highly regulated cell death mechanism that serves to eliminate ailing cells without causing injury to the normal cells, and loss of its regulation underlies numerous pathologies including cancer (Elmore, 2007). Any compound that induces apoptosis is considered to be a promising cancer chemotherapeutic treatment (Tor et al., 2015). To investigate whether MMAE-induced inhibition of A549 cell growth is via apoptosis, acridine orange/ethidium bromide (AO/EtBr) dual staining was used to identify and quantify the apoptotic morphology. A549 cells treated with different concentrations of MMAE exhibited characteristic apoptotic morphology with brightly orange red and condensed nuclei (intact or fragmented) compared to the untreated control cells which showed round and intact green nucleus representing the live cells (Figure 4.3A). A progressive rise in apoptotic index (%) was observed in a concentration-dependent manner in the treated cells compared to the control (Figure 4.3B). Many studies have revealed that various anticancer drugs work by inducing apoptosis in cancer cells, and several compounds isolated from medicinal plants have been proven to exhibit anticancer activity through induction of apoptosis (Wang et al., 2009). According to recent clinical data, anticancer drugs that can restore deregulated apoptotic pathways in cancer cells prolonged the life of patients in various advanced cancer diseases (Ocker and Hopfner, 2012).

Anticancer therapy relies heavily on DNA damage. To check for DNA strand breaks as a result of MMAE treatment, single-cell gel electrophoresis assay (Comet assay) was performed. Our results showed that MMAE possessed DNA-damaging effect in a dose-dependent manner which was evident from the increased comet tail length and olive moment in A549 cells treated with MMAE (Figure 4.4). The genotoxic effects of MMAE coincides with the lipid peroxidation results, as malondialdehyde, the end product of LPO, is able to form mutagenic adducts that consequently led to DNA damage (Niedernhofer et al., 2003). Several plant-derived anticancer drugs have also been reported to show similar effects in various cancer types (Madunic et al., 2018; Moorthy et al., 2018).

Excessive ROS production is often associated with pathophysiology of various diseases including cancer (Dickinson and Chang, 2011; Weidinger and Kozlov, 2015; Reczek and Chandel, 2017). Therefore, redox balance is maintained in a cell by an impressive repertoire of antioxidant system both enzymatic (such as CAT, SOD, GPx and GST) and non-enzymatic (such as GSH, ascorbic acid and lipoic acid) (Hanschmann et al., 2013). In most cancer cells, high levels of ROS have been observed, that are responsible for cancer cell dissemination and promoting disease progression via alteration of several signalling pathways (Tochhawng et al., 2012). Interestingly, elevated levels of ROS in cancer cells also leads to cell death. Thus, agents that can either elevate ROS production to highly toxic level, or inhibit antioxidant defense system may provide an opportunity to eliminate cancer cells. Indeed, a large variety of anti-cancer drugs including doxorubicin, paclitaxel, vinblastine, and some of those that are being under clinical trials, effectively eliminate cancer cells and sensitize cancer cells to chemotherapeutic agents by enhancing ROS production and/or reducing the antioxidant defense mechanism (Yokoyama et al., 2017; Galadari et al., 2017). Treatment of A549 cells with 100 µg/mL MMAE showed reduced glutathione level and the activities of antioxidant enzymes including GST and SOD (Figure 4.5A-C). Consistently, MMAE treatment showed significant increase in the levels of lipid peroxidation which could be due to the decreased antioxidant activities resulting in accumulation of cellular ROS (Figure 4.5D). The increase in ROS levels may in turn be responsible for the cytotoxic effects of MMAE on A549 cells in the present study. Similar is the case in many anti-cancer agents such as doxorubicin whereby the main mechanism of its cytotoxicity is via induction of ROS (Mai et al., 2016). Although efficient antioxidants are required to maintain redox homeostasis, increased levels of antioxidants have been shown to be involved in cancer progression and chemoresistance. For example, high level of glutathione has been reported in cancer cells (Godwin et al., 1992) leading to increase neoplastic transformation, drug resistance and cancer treatment failure (Traverso et al., 2013; Ramsay and Dilda, 2014). Similarly, increased activities of GST and SOD have also been reported in several human cancer cells resulting in resistance to therapeutic intervention (Jagetia and Venkatesha, 2012; Che et al., 2016; Tew, 2016; Allocati et al., 2018; Glorieux et

al., 2018). Our study shows that MMAE treatment may lead to an increase in intracellular ROS levels via disrupting the antioxidant system of A549 cells and further implicates that cell death induced by MMAE may be linked to down regulation of antioxidant activities. Plants such as *Momordica charantia* (Thiagarajan et al., 2019), *Rhynchosia rufescens* (Khader et al., 2019), *Malvapseudo lavatera* (Khoury et al., 2020) have been reported to induce cytotoxic effects in cancer cells by increasing ROS production. The present study also showed a significant lipid-damaging effect of MMAE in A549 cells. Lipid peroxidation is an important event associated with cell death and has been reported to induce severe impairment in the membrane function through increased membrane permeability, membrane protein oxidation and DNA damage that eventually result in cell death (Chen and Niki, 2011).

The pro- and anti-apoptotic proteins in the Bcl-2 (B-cell lymphoma/leukemia-2) family, through their interactions, play central roles in mitochondria-mediated intrinsic apoptosis via regulation of mitochondrial membrane permeabilization (Xiong et al. 2014). The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 proteins. An excess of pro-apoptotic Bcl-2 proteins at the surface of the mitochondria causes the formation of the permeability transition (PT) pore, leading to a hierarchical release of the pro-apoptotic proteins such as cytochrome c, Smac/Diablo and apoptosis inducing factor (AIF), and subsequently leading to downstream activation of caspase-3 and caspase-6 (Arnoult et al., 2003). Thus, alterations in the expression of these genes contribute to the pathogenesis and progression of cancers, thus providing targets for anticancer drug discovery. To determine whether Bcl-2 family proteins are involved in MMAE-induced apoptosis, the relative mRNA expression of pro-apoptotic genes (Bax, p53 and Bid) and anti-apoptotic genes (DR-5, Apaf-1, Bcl-X_L, PARP and Survivin) were assessed by qPCR techniques. Up-regulation of pro-apoptotic genes in the present study suggest that MMAE-induced apoptosis in A549 cells could be modulated, at least in part, via the mitochondrial pathway. Altered expression of anti-apoptotic genes such as PARP, DR-5 and Bcl-X_L and pro-apoptotic genes such as Bax, Bid and p53 by several plant extracts have been documented earlier in A549 cells (Hansakul et al., 2014; Sharifi et al., 2018; Thiagarajan et al., 2019; Panicker et al., 2020).

Caspase-3 and caspase-6 plays a central role in the execution of both the intrinsic (the mitochondrial mediated) and extrinsic (the death receptor mediated) apoptotic pathways by cleaving several key proteins, such as inhibitor of caspase-activated DNase (ICAD), poly (ADP ribose) polymerase (PARP), and intra-nuclear proteins (Porter and Janicke, 1999). This cleavage mediates disassembly of the cell into the apoptotic morphological changes including cell shrinkage, chromatin condensation and nuclear fragmentation (Elmore, 2007). Thus, activation of caspase-3 and caspase-6 are a strong biomarker for cells undergoing apoptosis. Increased caspase-3 and caspase-6 activities in A549 cells following MMAE treatment strongly indicated that MMAE-induced apoptosis was executed through a caspase-dependent pathway. The mechanisms of MMAE anticancer activities involve induction of mitochondria-mediated apoptosis via increased activities of caspase-3 and caspase- 6, and upregulation of pro-apoptotic genes including Bax and p53 suggesting that MMAE could be regarded as a new candidate for cancer treatment.

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CHAPTER VI

Anti-carcinogenic activities of *Mussaenda macrophylla* Wall. leaf extracts on human colon cancer cells, HCT 116 cells

ABSTRACT

Mussaenda macrophylla is a traditionally widely used shrub for treatment of various ailments including cancer, ulcer, dysentery, fever and cough. In this chapter, we determine the anticancer effects of *M. macrophylla* using colon cancer cell line (HCT 116). The cytotoxicity of various extracts of *M. macrophylla* such as chloroform (MMCE), methanol (MMME) and aqueous (MMAE) extracts were examined using MTT assay and the inhibitory concentration (IC_{50}) was measured wherein MMAE was found to be most effective extract with an IC_{50} of 29.03 ± 2.46 µg/mL. Thus, only MMAE was used for the subsequent tests. The extract was screened for their inhibitory effects on HCT 116 cells using clonogenic assay. Additionally, acridine orange/ethidium bromide (AO/EtBr) staining verified apoptotic features in HCT 116 cells following MMAE treatment through fluorescent microscopic analysis. Antioxidants/oxidant assay, comet assay, qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression and caspase-3/6 activity assay were performed to investigate the anticancer effects and possible mechanism of actions leading to apoptosis in HCT 116. Our results showed that MMAE inhibited the cell proliferation of HCT 116 cells and significantly increased the level and activities of antioxidants including glutathione (GSH), glutathione-s-transferase (GST) and superoxide dismutase (SOD), while decreasing the lipid peroxidation (LPO) level in HCT 116 cells. Treatment of HCT 116 cells with MMAE also resulted in DNA damage, up-regulation of pro-apoptotic genes (Bax and p53), down-regulation of anti-apoptotic genes (Bcl-X_L and PARP) and elevation of caspase-3 and caspase-6 activities providing an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our study showed that MMAE could be a promising agent for colon cancer therapy targeting the mitochondrial apoptotic pathway.

1. INTRODUCTION

Colon cancer is the 3rd most common cancer worldwide and ranks 2nd in terms of cancer-related mortality. In recent years, a rapid rise in colon cancer incidence and mortality has been observed in several developing countries. An increase of 60% of this malignancy incidence is expected by 2030 (Sung et al., 2021). Colon cancer is the result of a progressive accumulation of genetic and epigenetic alterations, leading to a marked genomic instability. The disease occurs as a result of tumor suppressor and/or oncogenic gene mutations that in turn leads to polyp's generation and eventually adenocarcinomas. Indeed, colon cancer has been associated with mutational inactivation of tumor-suppressor genes such as p53, TGF-β and APC and activation of oncogene pathways such as BRAF, RAS and PI3K (Markowitz and Bertagnolli, 2009; Semlali et al., 2018). Colon cancer is classified genetically into three categories: sporadic (60%) comprising patients with no blood relative with colon cancer or an adenoma, familial (30%) referring to patients with at least one family history and hereditary (10%) referring to colon cancers resulting from germline inheritance of mutations (Kheireldeid et al., 2013). With the application of advanced technologies like deep deoxyribonucleic acid (DNA) sequencing, recent researches have revealed that colon cancer is the most complex cancer at a genetic level (Fearon, 2011; Vogelstein et al., 2013). Currently available treatments for colon cancer are chemotherapy with the single medication fluoropyrimidine, as well as multiple agent regimens such as irinotecan, oxaliplatin and capecitabine. Furthermore, the optimum colon cancer treatment is to remove the tumor and all of its metastases completely, which usually involves surgical intervention. However, the existing treatment options for colon cancer are complex and associated with toxic side effects. It results in the damage of many healthy cells and vital organs of patients owing to a decrease in quality of life (Xie et al., 2020). Therefore, researchers are in search of novel drug candidates with minimal toxicity towards the normal cells. Due to unique structural nature, vast diversity in their chemical properties and minimal toxicity, secondary metabolites available in medicinal plants are considered an appealing target for screening of novel drug candidates against cancer.

Medicinal plants used by about 70% of the world population are a promising source of anticancer bioactive molecules (Benarba et al., 2015). Searching for anticancer drugs from plants started in the 1950′s when vinca alkaloids were discovered (Moudi et al., 2013). Several anticancer drugs currently in clinical use are plant-derived products and include taxol, vinblastine, or vincristine, irinotecan, camptothecin and their derivatives or analogs (Shukla and Mehta, 2013). Natural phytochemicals are multiple-target molecules found in plants and microorganisms, and they exert strong anticancer activity. Phytochemicals isolated from natural sources also exhibit various beneficial effects against inflammation, cancer, and neurodegenerative disorders (Rahman and Chung, 2011; Choudhari et al., 2020). This broad spectrum of biological and pharmacological activities has made natural compounds suitable candidates for treating multifactorial diseases, such as colon cancer. HCT 116 is a human colon cancer cell line that is commonly used to study cancer biology. It is a growth factor independent cell line that has been shown to be invasive and highly motile in *in vitro* studies (Awwad et al., 2003) and it has been commonly used for the screening of anticancer properties of various plants and plant products *in vitro* such as *Pistacia atlantica* and *Pistacia lentiscus var. Chia.* (Balan et al., 2005), Baicelein (Chen et al., 2018), *Sesuvium portulacastrum* (Chintalapani et al., 2019) and *Eclipta alba* (Nelson et al., 2020).

Traditionally, different parts of *M. macrophylla* has been used to treat persons with sore throats and sore mouths in different parts of Nepal. Fever is treated with root juice and is also used for diabetic patients and helps with stomach acidity (Manandhar, 1994). In addition to its traditional medicinal use, *M. macrophylla* has been reported to exhibit antioxidant activity (Islam et al., 2012), anti-microbial activity (Kim et al., 1999; Chowdhury et al., 2013), thrombolytic activity, anti-diabetic activity (Bhandari et al., 2020) and is also found to be effective against oral pathogens (Kim et al., 1999). Despite the current state of scientific knowledge on numerous medicinal applications, there has been no scientific validation for its anticancer activities. Hence, the present research has been carried out to study the anticancer activity of *M. macrophylla* extract in the human colon cancer cell line HCT 116.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Eagle's Minimal Essential Medium (MEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazole-2-yl)-2, Trypsin-EDTA, 5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), sodium bicarbonate, nitroblue tetrazolium (NBT), Folin-ciocalteau's reagent, thiobarbaturic acid (TBA), potassium chloride (KCl), n-butanol, sodium Chloride (NaCl), Triton X-100, ethidium bromide, acridine orange, phenazine methosulphate (PMS), 1-chloro-2,4 dinitrobenzene (CDNB), dimethyl sulphoxide (DMSO) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ethylenediamine tetra-acetic acid (EDTA), L-Glutamine, phenol red, agarose (low gelling temperature), Trizma hydrochloride, Trizma base and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., Bangalore, India. Fluorouracil (5FU) was obtained from GLS Pharma Ltd. (Hyderabad, India). The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

2.2. Preparation of *M. macrophylla* extracts

Collection and preparation of *M. macrophylla* was carried out as described in Chapter 2 (Section 2.2) to obtain the plant extracts.

2.3. Cell lines and Culture

Human colon carcinoma cell line (HCT 116 cells) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in MEM supplemented with 10% FBS in a humidified incubator containing 5% CO_2 at 37 ^{o}C (Eppendorf AG, Hamburg, Germany).

2.4. Cytotoxicity assay

The cytotoxicity of *M. macrophylla* was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (Mossman, 1983). Briefly, 1×10^4 cells were seeded into flat-bottomed 96 well plates (Himedia Laboratories Pvt.

Ltd., Mumbai, India) containing 100 µL of MEM. The cells were allowed to adhere for 24 h at 37 ºC with 5% CO_2 and treated with different concentrations (25-300 µg/mL) of various solvent extracts of *M. macrophylla* for 12, 24 and 48 h along with a control sample. At the end of treatments, drug containing media were removed and cells were washed with FBS free media. Then, 10 µL of MTT (5 mg/mL) was added to each well and incubated for another 2 h at 37 ºC in a CO_2 incubator. The insoluble purple formazan crystals formed were then dissolved in 100 µL of DMSO and incubated for 30 min. The absorbance of the solution was measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices). Three independent experiments consisting of three replicates were carried out for each treatment. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

% inhibition = Control-Treatment/Control X 100.

The aqueous extract of *M. macrophylla* (MMAE), the most effective extract in the preliminary cytotoxicity screening using MTT assay was subsequently used for further experiments.

2.5. Clonogenic Assay

The effect of aqueous extract of *M. macrophylla* (MMAE) on the reproductive integrity of HCT 116 cells was assessed using clonogenic assay (Franken et al., 2006). Exponentially growing cells were harvested from a stock culture by trypsinization and 200 cells were seeded into several individual petri dish containing 5 mL of media. After overnight adherence, cells were treated with different concentrations of MMAE (25-100 µg/mL) or 5FU (100 µg/mL) for 48 h. After treatment with MMAE, cells were washed with sterile 1X PBS and cultured in a fresh medium for another 11 days. The resultant colonies were then stained with 1% crystal violet in methanol (w/v) for 30 min at room temperature and colonies with more than 50 cells were counted using an inverted microscope. Plating efficiency (PE) and surviving fraction (SF) of HCT 116 cells were calculated by the following formula:

PE = (Number of colonies counted × 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) × (Mean plating efficiency)

2.6. Cell morphology analysis by fluorescent staining (Apoptotic assay)

The ability of MMAE to induce apoptosis was studied by AO/EtBr staining. Briefly, 1×10^5 HCT 116 cells were seeded in several six-well plates containing 5 mL of media. Cells were allowed to adhere overnight and treatment was given for 48 h with different concentrations of MMAE (25-100 µg/mL) or 5FU. Untreated controls were also maintained in culture medium alone. After treatments, cells were washed with sterile 1X PBS and detached with 1X trypsin EDTA. The cells were pelleted and resuspended in 100 µL of FBS free media. Subsequently, 25 µL cell suspension was stained with 2.5 µL each of acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL) in a ratio of 1:1 for 2 min followed by gentle mixing. The morphology of apoptotic cells was then examined on a slide under a fluorescent microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615). Acridine orange is a nucleic acid fluorescent cationic dye that permeates both live and dead cells, intercalating in the double-stranded DNA, and makes the nuclei appear green. Ethidium bromide is taken up only by dead cells whose cytoplasmic membrane integrity is lost and stains the nuclei yellowish orange. For this reason, live cells have green nuclei whereas apoptotic cells that incorporated ethidium bromide exhibit condensed and fragmented orange chromatin. Conversely, necrotic cells have structurally normal orange nucleus (Kasibhatla et al., 2006). At least 300 cells were scored and the apoptotic index was determined as follows:

Apoptotic index (%) = Number of apoptotic cells scored × 100/Total number of cells counted

2.7. Assessment of genotoxicity of MMAE using Comet assay

The alkaline single cell gel electrophoresis (Comet assay) is a simple method for detection of DNA strand breaks in eukaryotic cells. The assay was performed using the method described earlier with minor modifications (Singh et al., 1988). Briefly, 2×10^4 HCT 116 cells treated with different concentrations of MMAE or 5FU for 48 h along with the untreated control were suspended in 75 µL of 0.5% low-melting point

agarose (LMPA) prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose (NMPA) and covered with a coverslip. Once the gel got solidified following incubation of the slide at 4°C, the coverslip was gently removed and the third layer of 90 µL 0.5% LMPA was added. The slides were then incubated for 2 h in a freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10). After lysis, slides were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA. The slides were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralization, slides were washed with distilled water and then stained with ethidium bromide (EtBr) solution (2 µg/mL) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using fluorescence microscope with a magnification of 200x. Image capture and analysis were performed with Image J software.

2.8. Antioxidants/Oxidant assays

For the estimation of antioxidant enzyme activities and lipid peroxidation level, 1×10^6 cells were seeded in a T-25 flask containing 5 mL media. At the end of 48 h treatments with 50 µg/mL of MMAE or 5FU, drug containing media was discarded and the cells were washed with sterile 1X PBS and harvested. The cancer cells were pelleted, sonicated (PCI Analytics Pvt. Ltd., Mumbai, India) and 5% homogenate was prepared using cold sterile PBS (pH-7.4), which was used for biochemical estimations. Total protein contents were determined using standard protocol (Lowry et al., 1951) using bovine serum albumin as standard.

2.8.1. *Glutathione (GSH)*

Glutathione (GSH) levels were measured by its reaction with DTNB in Ellman's reaction to give a compound that absorbs light at 412 nm (Moron et al., 1979). Briefly, 80 µL of the cell homogenate was mixed with 900 µL of 0.02M sodium phosphate buffer and 20 µL of 10 mM DTNB and incubated for 2 min at room

temperature. The blank consisted of distilled water instead of cell homogenate. The absorbance of the sample was read against blank at 412 nm in a UV-visible spectrophotometer (SW 3.5.1.0. Biospectrometer, Eppendorf India Ltd., Chennai). GSH concentration was calculated from the standard graph and expressed in µmol/mg protein.

2.8.2. *Glutathione-s-transferase (GST)*

Glutathione-s-transferase (GST) activity was measured using the standard method (Beutler, 1984). Briefly, 50 µL of 20 mM CDNB was added to 850 µL of 0.1M phosphate buffer (pH 6.5) and incubated for 10 min at 37 °C. Then, 50 µL each of 20 mM GSH and cell homogenate was added to the mixture. For blank, distilled water was added instead of cell homogenate. The absorbance of the sample was measured at 1 min interval for 5 min at 340 nm. GST activity was measured as: GST activity = (OD of test–OD of blank/9.6 ×vol. of test sample) × 1000; where, 9.6 is the molar extinction coefficient for GST.

2.8.3. *Superoxide dismutase (SOD)*

Superoxide dismutase (SOD) activity was measured by the NBT reduction method (Fried, 1975). Briefly, 100 µL each of cell homogenate and 186 µM PMS was mixed with 300 µL of 3 mM NBT and 200 µL of 780 µM NADH. The mixture was incubated for 90 sec at 30ºC and 1 mL of acetic acid and 4 mL of n-butanol were added to stop the reaction. The blank consisted of all the reagents, except the cell homogenate. The absorbance of test and blank was measured at 560 nm and the enzyme activity was expressed in unit (1 unit = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank - OD of sample/OD of blank) × 100

2.8.4. *Lipid peroxidation (LPO) assay*

Lipid peroxidation (LPO) was measured by the standard method (Beuege and Aust, 1978). Malondialdehyde (MDA), one of the toxic products formed from the oxidation of fatty acids such as polyunsaturated fatty acids and phospholipids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA derived from LPO reacts with TBA to give a red fluorescent adduct absorbing at 535 nm. Briefly, cell homogenate was added to a mixture of 10% TCA, 0.8% TBA and 0.02 N HCl in 1:2 ratio. The mixture was boiled for 10 min, cooled

immediately at room temperature and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank. The MDA concentration of the sample was calculated using the extinction coefficient of 1.56 x 106/M/cm.

2.9. qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression

1×10^6 HCT 116 cells were seeded in six-well plates containing 5 mL of media. Cells were allowed to adhere overnight at 37 ^oC with 5 % CO_2 and treated with 50 µg/mL MMAE or 5FU for 48 h along with a control sample. After treatment, cells were washed and detached. The cells were then pelleted and total RNA was extracted using Tri reagent (BR Biochem, Life Science Pvt. Ltd, R1022). Extracted RNA was quantified using Nanodrop Spectrophotometer (Eppendorf Biophotometer Plus, Hamburg, Germany) and RQ1 DNase kit (Promega, M198A, Madison, WI, USA) was used to remove the genomic contamination. cDNA was synthesized from 2 µg of total RNA using first-strand cDNA synthesis kit (Thermoscientific, K1621; Lithuania, Europe). Gene-specific primers were designed using Primer 3, Boston, MA, USA. The primer sequences used in qRT-PCR analyses were given in Table 5.1. qPCR was performed using Quant-Studio 5 (ThermoFisher Scientific, Foster City, CA, USA). PCR reaction volume of 7 µL for each gene comprised of 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25742, Lithuania, Europe) and 1 µL of nuclease-free water (ThermoFisher Scientific, A19938, Bangalore, India). The cycling condition of qPCR were 1 cycle at 95°C (20 s), 35 cycles at 95°C (01 s), 60°C (20 s) and 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and 1 cycle of 95°C (01 s). Afterwards, melting curves were generated to confirm a single uniform peak. GAPDH gene was used as a reference gene for determining the relative expression levels of specific target genes. Each sample was run in duplicate along with non-template and negative RT controls. The relative expression of genes was determined using ΔΔCt method (Livak and Schmittgen, 2001).

Table 5.1. Primer sequences and their product size used in qRT-PCR analyses of HCT 116 cells treated with aqueous extract of *M. macrophylla* (50 µg/mL).

Genes	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
Bax	TCCCCCCGAGAGGTCTTTT	CGGCCCCAGTTGAAGTTG	68
p53	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTGT	159
Bid	CCTTGCTCCGTGATGTCTTTC	GTAGGTGCGTAGGTTCTGGT	62
Apaf-1	AAGGTGGAGTACCACAGAGG	TCCATGTATGGTGACCCATCC	116
Bcl-X_L	GGCCACTTACCTGAATGACC	AAGAGTGAGCCCAGCAGAAC	180
PARP	CCAGATGCTTGTCTTCCTGAGAG	AGTGACAGCAGGGTTGGCATGA	133
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185

2.10. Caspase-3/6 activity assay

A quantitative enzymatic activity assay was carried out for caspase-3 and caspase-6 according to the manufacturer protocols (BioVision Incorporated, USA). Briefly, 5×10^5 HCT 116 cells were treated with 50 µg/mL of MMAE or 5FU for 48 h in a six-well plate along with the untreated control. After treatment, cells were washed and lysed in 50 µL of chilled lysis buffer followed by 10 min incubation on ice. The cell lysates were centrifuged at 15,000 × g for 1 min at 4 °C, and the supernatant was collected. Total amount of protein was determined by Bradford assay (Bradford, 1976). The assay was performed in a total volume of 100 µL in 96-well plates. 100 µg of protein from each sample was assayed for caspase-3/6 activity against their specific colorimetric substrate DEVD-*p*NA for caspase-3, and VEID-*p*NA for caspase-6. The mixture was incubated for another 2 h at 37 °C and absorbance of free *p*-nitroanilide (*p*NA) produced via cleavage from their specific substrates by activated caspase-3 and caspase-6 was measured at 405 nm using microplate reader.

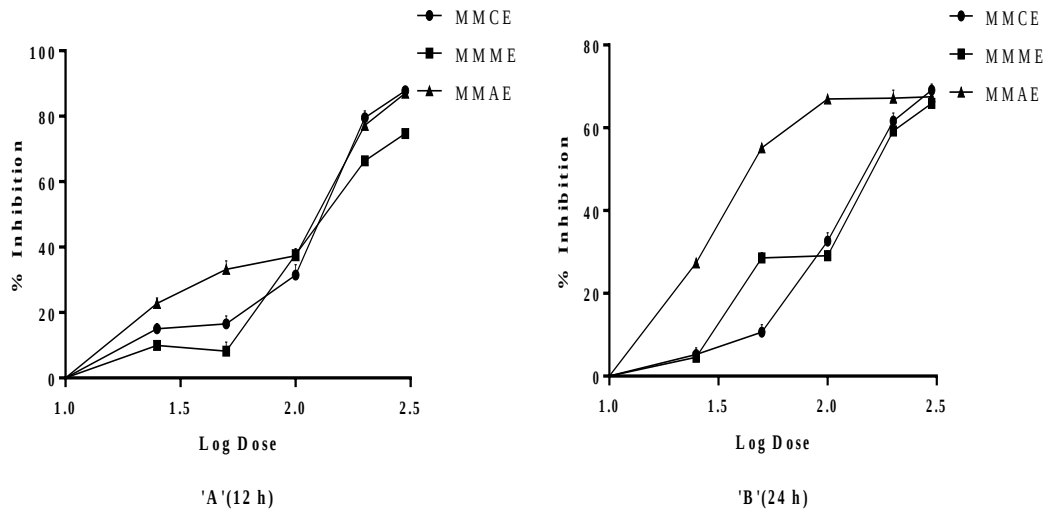
2.11. Statistical analysis

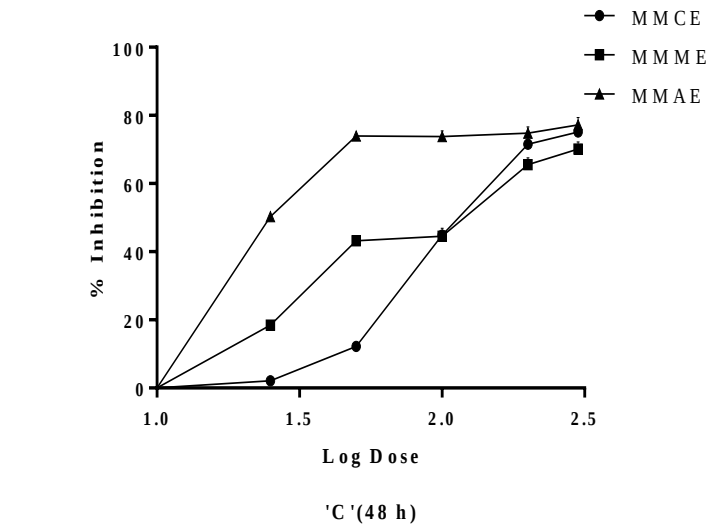
All data were expressed as mean ± standard error of mean of pooled results obtained from three independent experiments. One-way ANOVA followed by Tukey's test was performed to test significant variations on cytotoxic, clonogenic, apoptotic assays, antioxidants/oxidant status, comet assay, differential gene expression and caspase activities. SPSS ver.16.0 software (SPSS Inc, Chicago, Illinois, USA) and Graph Pad Prism ver. 6.0 were used for statistical and graphical analyses. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. The anti-proliferative and cytotoxic effects of *M. macrophylla* extracts in HCT 116 cells

To determine the cytotoxic effect of different extracts of *M. macrophylla*, HCT 116 cells were treated with different doses of various solvent extracts of *M. macrophylla* for 12, 24 and 48 h. The inhibition (%) of HCT 116 cells by different extracts of *M. macrophylla* was plotted against log-doses for the calculation of IC_{50} (Figure 5.1A-C). Aqueous extract of *M. macrophylla* showed higher cytotoxic effect compared to chloroform and methanol extracts at all time points and its cytotoxicity was found to be highest at 48 h treatment duration with an IC_{50} of 29.03 ± 2.46 µg/mL (Figure 5.1D). Therefore, only aqueous extract was used in the subsequent experiments.





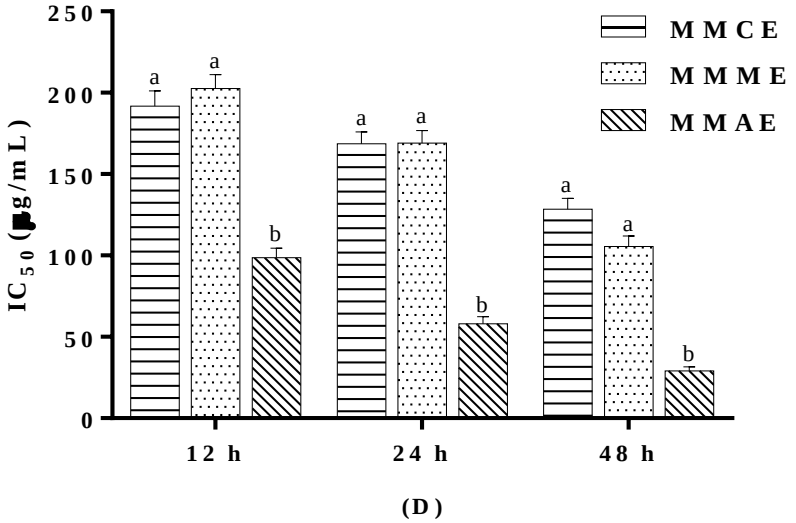
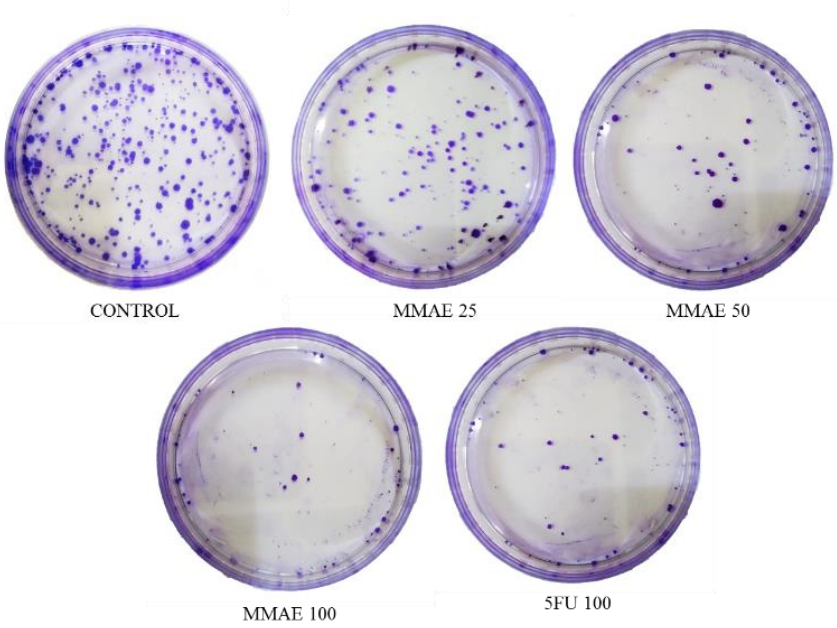


Figure 5.1. (A-C) Plots of log-doses of various extracts of *M. macrophylla* against inhibition (%) of HCT 116 cells after 12, 24 and 48 h treatment for the calculation of IC_{50}. (D) Cytotoxic effects (IC_{50}) of different extracts of *M. macrophylla* on HCT 116 cells after 12, 24 and 48 h treatment. MMCE: *M. macrophylla* chloroform extract; MMME: *M. macrophylla* methanolic extract; MMAE: *M. macrophylla* aqueous extract. Values are expressed as Mean ± SEM. Different letters indicate significant variation between extracts at each treatment duration.

3.2. The inhibitory effect of MMAE on the clonogenicity of HCT 116 cells

In order to determine the effect of MMAE on reproductive viability, low concentration of HCT 116 cells plated on petri-dishes were treated with different doses of MMAE and allowed to grow for 11 days. Only single cells that were resilient enough to divide and form colonies survived the treatment were assessed. We found that MMAE treatment effectively reduced the clonogenicity of HCT 116 cells in a dose dependent manner when compared with the untreated control (Figure 5.2A). HCT 116 cells treated with 25, 50 and 100 µg/mL of MMAE led to a significant inhibition of colony forming capability of the cells with surviving fraction values of 72.8%, 30% and 1.21% respectively (Figure 5.2B). MMAE at the dose of 100 µg/mL showed comparable colony inhibiting activity with the standard drug 5FU.



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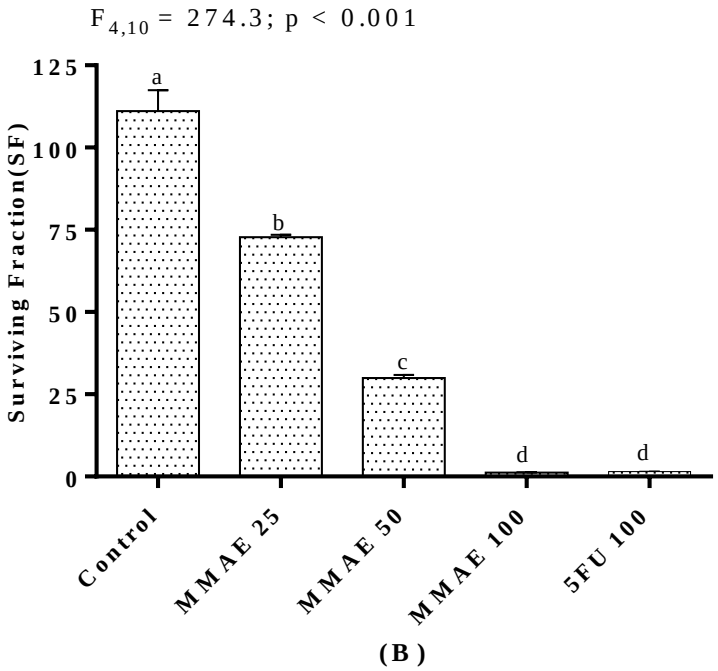
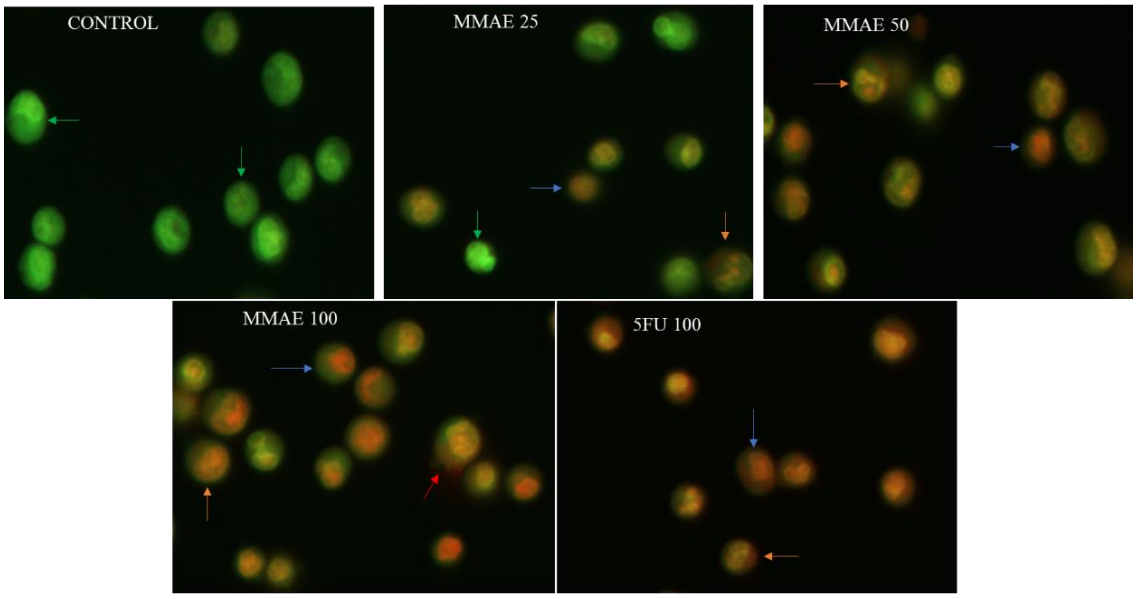


Figure 5.2. (A) Inhibition of colony formation of HCT 116 cells mediated by aqueous extract of *M. macrophylla.* (B) Effect of aqueous extract of *M. macrophylla* on the reproductive capability of HCT 116 cells after 48 h treatment. Control: HCT 116 cells without treatment; MMAE 25, MMAE 50 and MMAE 100: HCT 116 cells treated with 25, 50 and 100 µg/mL of aqueous extract of *M. macrophylla* respectively. 5FU 100: HCT 116 cells treated with 100 µg/mL of 5FU (Positive control). Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.3. Morphological evidence of apoptosis induced by MMAE

Treatment of HCT 116 cells with different concentrations of MMAE (25-100 µg/mL) for 48 h resulted in a dose dependent increase in the number of apoptotic cells. Fluorescence microscopic images of the MMAE treated cells (Figure 5.3A) clearly revealed morphological alterations such as membrane blebbing, nuclear condensation and nuclear fragmentation which are the distinct characteristics of apoptotic cells. The apoptotic index increased in a concentration dependent manner in cells treated with MMAE, where the percentage of dead cells were found to be 51.7%, 89.1% and 96.6% after treatment with MMAE at the dose of 25, 50 and 100 µg/mL respectively (Figure 5.3B). The apoptotic index of MMAE at the doses of 50 and 100 µg/mL was similar to that of the standard 5FU.



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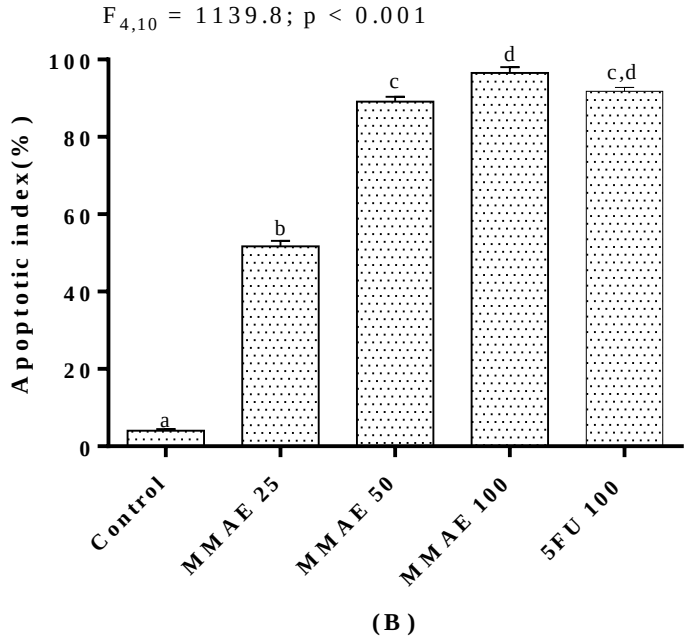
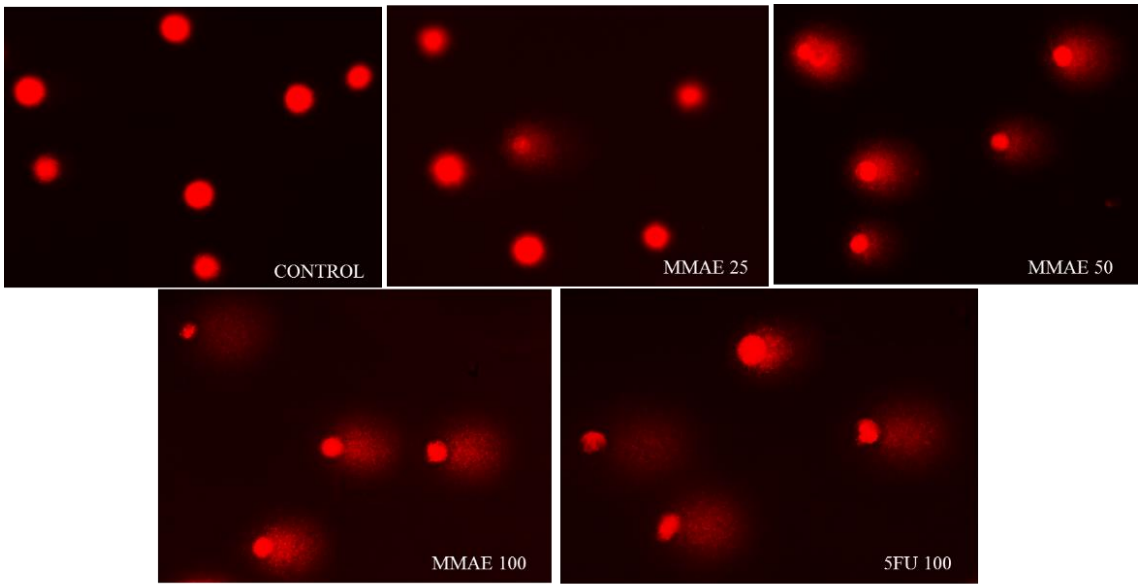


Figure 5.3. (A) Acridine orange/Ethidium bromide (AO/EtBr) dual staining of HCT 116 cells after treatment with different doses of aqueous extract of *M. macrophylla* for 48 h (green arrow shows the live cells, blue arrow shows apoptotic cells with nuclear condensation, orange arrow shows apoptotic cells with nuclear fragmentation, and red arrow shows apoptotic cells with membrane blebs). (B) Percentage of dead cells after treatment of HCT 116 with aqueous extract of *M. macrophylla.* Control: HCT 116 cells without treatment; MMAE 25, MMAE 50 and MMAE 100: HCT 116 cells treated with 25, 50 and 100 µg/mL of aqueous extract of *M. macrophylla* respectively. 5FU 100: HCT 116 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.4. Induction of DNA strand breaks by MMAE

The alkaline Comet assay was used to assess DNA damage in HCT 116 cells after treatment with different doses of MMAE (25, 50 and 100 µg/mL) for 48 h. Our results showed that MMAE induced significant DNA damage in HCT 116 cells in a dose dependent manner as indicated by increased tail length and olive moment in MMAE treated groups when compared to untreated control (Figure 5.4A-C). Moreover, increased tail length and olive moment induced by MMAE treatment at the dose of 100 µg/mL was higher than that of the standard drug 5FU (Figure 5.4B &C).



(A)

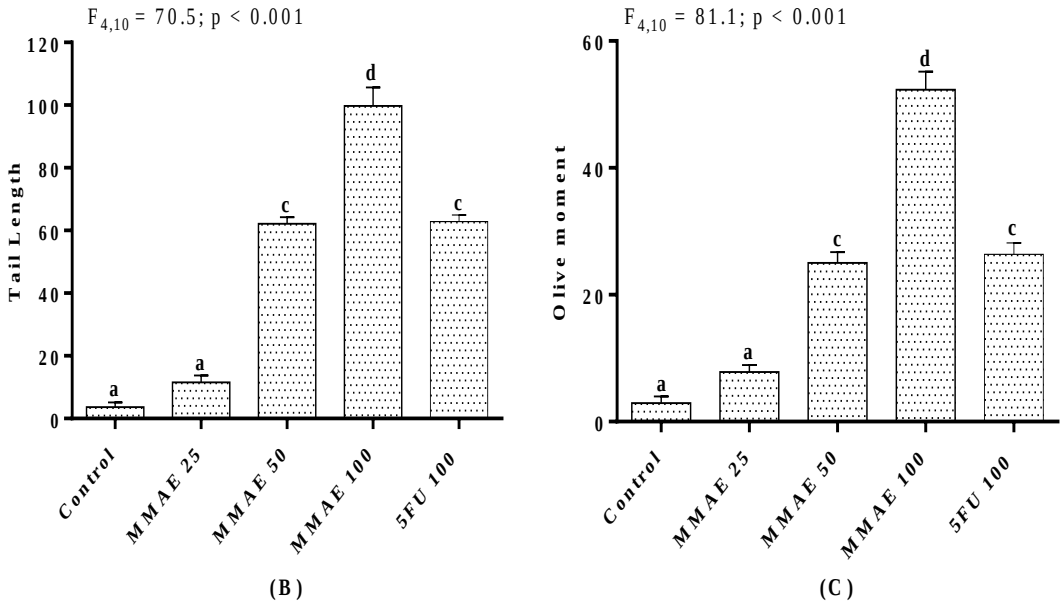


Figure 5.4. (A) Fluorescence images of Comets observed in control and HCT 116 cells treated with different concentration of MMAE; (B & C) The extent of DNA damage expressed in terms of Tail length and Olive moment. Control: HCT 116 cells without treatment; MMAE 25, MMAE 50, MMAE 100 and 5FU 100: HCT 116 cells treated with 25, 50, 100 µg/mL of aqueous extract of *M. macrophylla* and 100 µg/mL of 5FU, respectively. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.5. Antioxidants/Oxidant Status

Treatment of HCT 116 cells with MMAE significantly increased glutathione (GSH) concentration and the activities of glutathione-s-transferase (GST) and superoxide dismutase (SOD) when compared to the untreated control (Figure 5.5A-C). In an effort to investigate whether MMAE treatment affect intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was also assessed. Consistent to the increased antioxidant enzyme activities, the oxidative stress level was significantly reduced in HCT 116 cells after treatment with MMAE (Figure 5.5D).

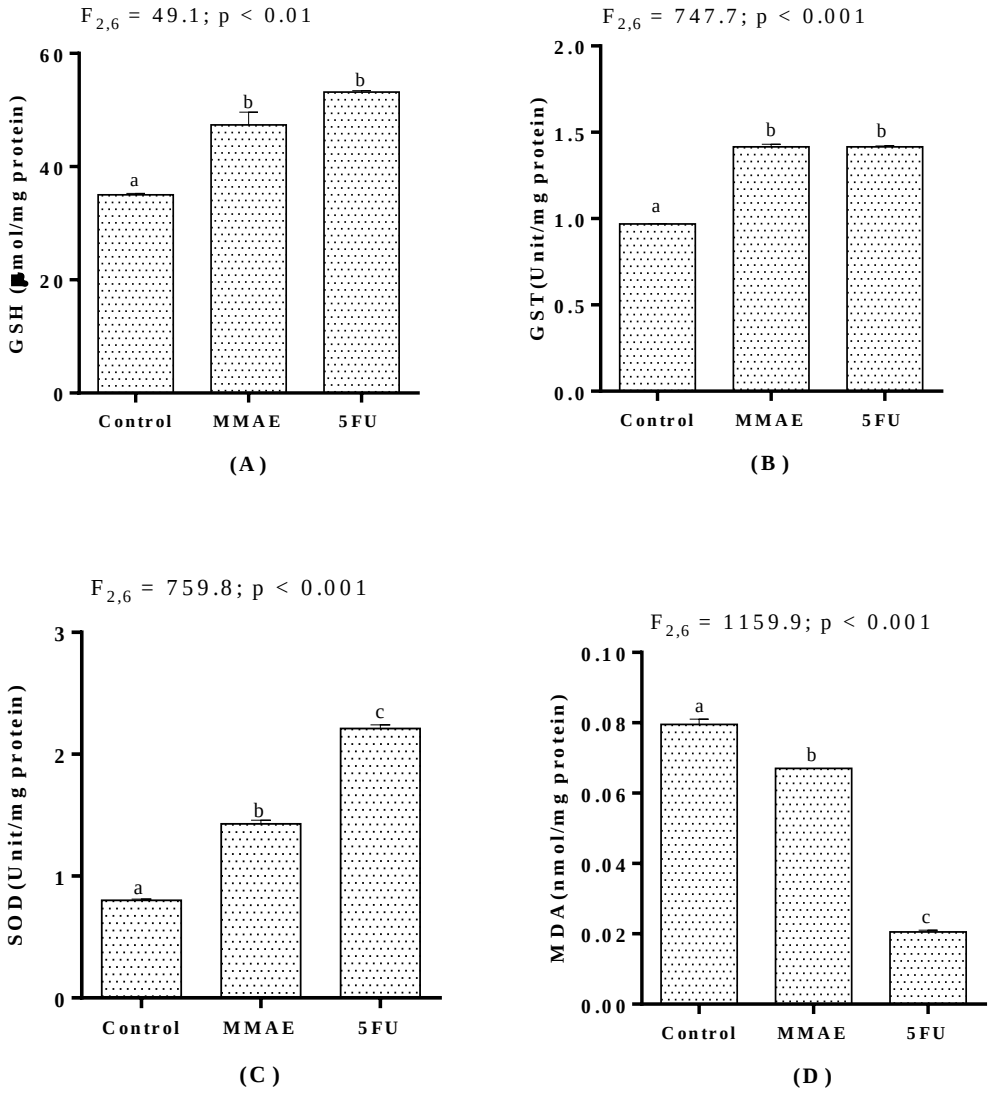
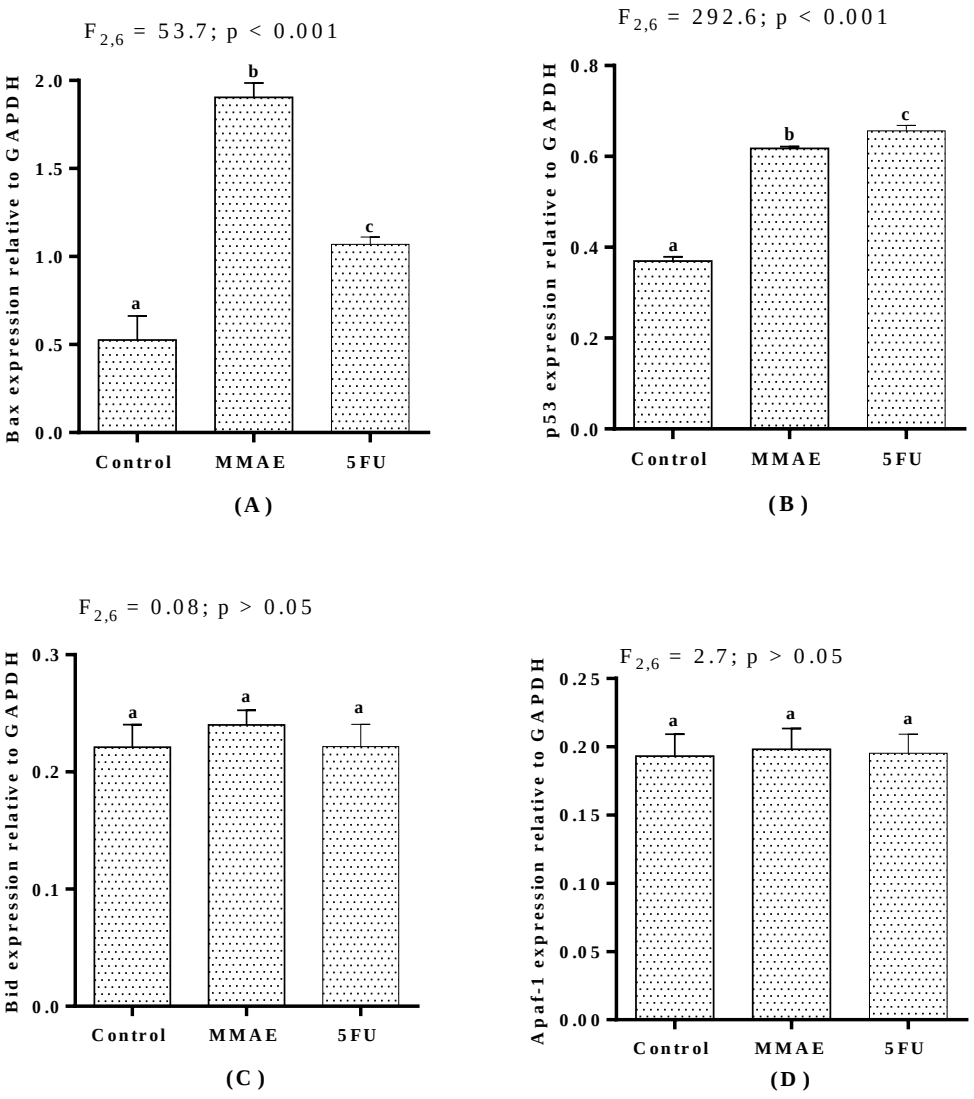


Figure 5.5. Effects of the aqueous extract of *M. macrophylla* on (A) glutathione level (GSH); (B) glutathione-s-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; and (D) lipid peroxidation (LPO) expressed in malondialdehyde (nmol/mg protein) in HCT 116 cells after 48 h treatment. Control: HCT 116 cells without treatment; MMAE: HCT 116 cells treated with 50 µg/mL of aqueous extract of *M. macrophylla*. 5FU: HCT 116 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.6. Effect of MMAE on the relative expression of pro-apoptotic and anti-apoptotic genes

The mRNA expression levels of both pro-apoptotic and anti-apoptotic genes were also investigated in HCT 116 cells using qPCR techniques. We found that treatment of HCT 116 cells with 50 µg/mL MMAE for 48 h induced up-regulation of the pro-apoptotic genes Bax and p53 by 3.62, and 1.67 folds respectively and down-regulation of anti- apoptotic genes Bcl-X_L and PARP by 1.94 and 6.67 folds respectively, when compared to the untreated control. However, no significant change was observed in the level of mRNA expression of pro-apoptotic genes (Bid and Apaf-1) between the control and MMAE treated groups (Figure 5.6A-F).



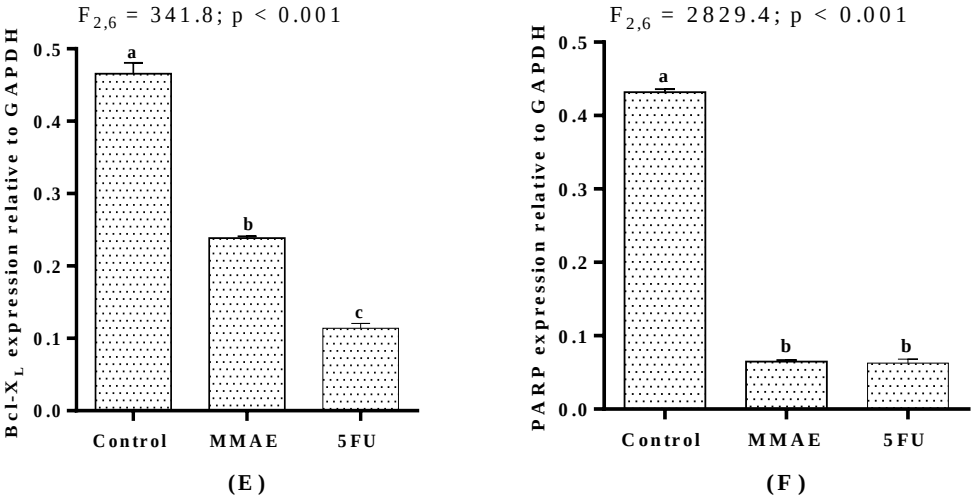


Figure 5.6. Effects of the aqueous extract of *M. macrophylla* on mRNA expression levels (A) Bax; (B) p53; (C) Bid; (D) Apaf-1; (E) Bcl- X_L and (F) PARP in HCT 116 cells after 48 h treatment. Control: HCT 116 cells without treatment; MMAE: HCT 116 cells treated with 50 µg/mL of aqueous extract of *M. macrophylla*. 5FU: HCT 116 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

3.7. Activation of caspase-3/6 by MMAE on HCT 116 cells

Caspase-3/6 plays an important role in execution of apoptosis in cancer cells. Effect of MMAE on HCT 116 cell apoptosis was assessed by caspase-3/6 activity. Treatment of HCT 116 cells with 50 µg/mL of MMAE for 48 h resulted in significant increase in the activities of caspase-3 and caspase-6 by 1.61 and 1.42 folds respectively, compared to the untreated control (Figure 5.7).

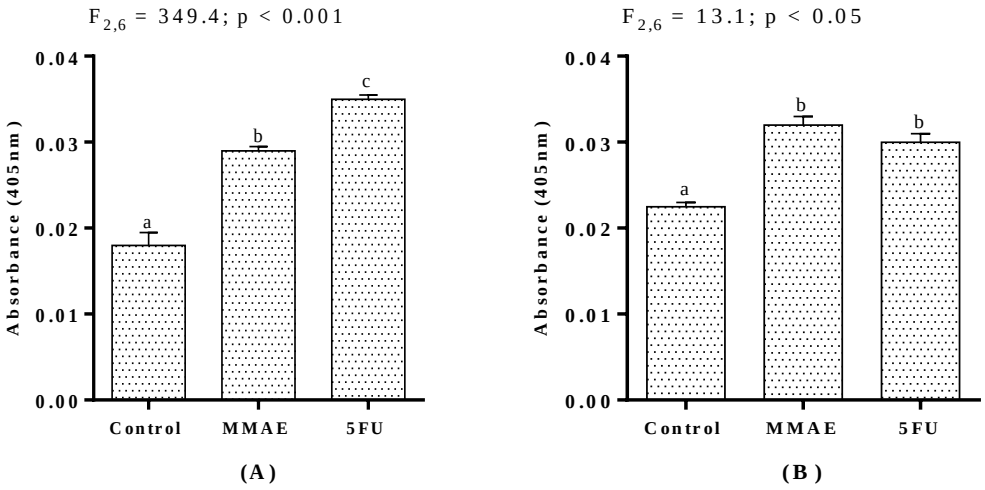


Figure 5.7. Effects of the aqueous extract of *M. macrophylla* on activities of (A) Caspase-3 and (B) Caspase-6 in HCT 116 cells after 48 h treatment. Control: HCT 116 cells without treatment; MMAE: HCT 116 cells treated with 50 µg/mL of aqueous extract of *M. macrophylla*. 5FU: HCT 116 cells treated with 100 µg/mL of 5FU. Values are expressed as Mean ± SEM. Different letters indicate significant variation.

4. DISCUSSION

In this chapter, we report the cytotoxic effects of *M. macrophylla* in human colon carcinoma cells (HCT 116) and its role in inducing apoptotic activity. *M. macrophylla* has mostly been studied for its medicinal properties such as anti-microbial, antioxidant, anti-diabetic and thrombolytic activities with no report on its anticancer activity. The different plants of the genus *Mussaenda* has been known for exhibiting anticancer activities. The sepals of *M. phillipica* were reported to exhibit antitumor effects by triggering the antioxidant defense system in HCT116 and NCI-H460 cell lines (Renilda & Fleming, 2016). *M. dona aurora* (Vidyalakshmi and Rajamanickam, 2009), *M. roxburghii* (Chowdury et al., 2015), *M. luteola* (Shylaja and Sathiavelu, 2017) and *M. glabrata* (Lipin and Darsan, 2017) have been shown to exhibit anticancer properties through various molecular mechanisms. Therefore, in this study, we employed techniques that focus on the mechanism of anticancer activities of *M. macrophylla* extract in HCT 116 cell line. Interestingly, *M. macrophylla*, like other *Mussaenda* species, shows anti-cancer properties, as evidenced from our current research.

The chloroform, methanol and aqueous extracts of *M. macrophylla* were screened for their cytotoxicity by MTT assay at different concentrations and time point to determine the IC_{50} value. The MTT (3- [4, 5-dimethylthiazol2-yl]-2, 5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations, the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the *in vitro* cytotoxic effects of drugs on cell lines or primary patient cells. Treatment of HCT 116 cells with different concentrations of *M. macrophylla* extracts resulted in a dose dependent increase in cytotoxicity as measured using MTT assay. Among the various extracts of *M. macrophylla*, aqueous extract of *M. macrophylla* (MMAE) was found to possess the highest cytotoxic effect at 48 h treatment duration with an IC_{50} of 29.03 ± 2.46 µg/mL (Figure 5.1D). The ability of cancer cells to form colonies enables them to communicate and build into solid tumor. Thus, a significant parameter to assess the effectiveness of treatment on cancer cells is to determine their reproductive integrity and colony-forming capability. MMAE treatment was found to reduce the

reproductive integrity of HCT 116 cells in a concentration dependent manner which correlated well with the cytotoxicity test, and the surviving fraction of HCT 116 cells after treatment with 100 µg/mL MMAE was only 1.21% (Figure 5.2B) indicating the effectiveness of the plant extract in inhibiting colony formation in the cancer cell.

The use of natural phytochemicals for inhibiting cancer cell proliferation and inducing apoptosis contributes to promoting cancer cell death (Wang et al., 2012; Guon and Chung, 2017). Many clinical anticancer drugs are known to exert their activities by inducing apoptosis (Debatin, 2000). Apoptosis is a gene-regulated response and, from the morphological point of view, is distinguished by the specific structural changes in cells, such as plasma membrane bleb formation, cell and nuclear shrinkage, oligonucleosomal DNA fragmentation, and chromatin condensation (Bai et al., 2018). To investigate whether MMAE inhibited HCT 116 cell proliferation via apoptosis, acridine orange/ethidium bromide (AO/EtBr) dual staining was used to identify and quantify the apoptotic morphology. HCT 116 cells treated with different concentrations of MMAE exhibited characteristic apoptotic morphology with condensed and brightly orange red nuclei (intact or fragmented) compared to the untreated control cells which showed round and intact green nucleus representing the live cells (Figure 5.3A) A progressive rise in apoptotic index (%) was observed in a concentration-dependent manner in the treated cells when compared to the control (Figure 5.3B). Many studies have revealed that various anticancer drugs work by inducing apoptosis in cancer cells, and several compounds isolated from medicinal plants have been proven to exhibit anticancer activity through induction of apoptosis (Wang and Fang, 2009).

Rise in intracellular ROS either due to increased productions or weakened antioxidant system has been invariably associated with pathophysiology of various diseases including cancer (Dickinson and Chang, 2011; Weidinger and Kozlov, 2015; Reczek and Chandel, 2017). The antioxidant system consists of both enzymatic (such as SOD, CAT, GST, and GPx) and non-enzymatic (such as GSH, ascorbic acid and lipoic acid) (Hanschmann et al., 2013). In most cancer cells, high levels of ROS have been observed, that are responsible for cancer cell dissemination and promoting disease progression via alteration of several signalling pathways (Tochhawng et al., 2012). The key to disease progression or improvement of many

cancer types is the balance between oxidants and antioxidants in the cell. This is accounted to the known role of accumulation of intracellular ROS and its association with cancer progression (Tafani et al., 2016). Therefore, to counterbalance the increased ROS levels in the cancer cell, the cells antioxidant mechanisms have to be efficient. Unfortunately, in many cancer types the activities of the antioxidant system are overwhelmed by the high ROS levels. Therefore, many cancer treatments require external agent to assist the cellular antioxidants. Since high ROS ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$) levels have close association with tumor initiation, angiogenesis, cell invasion, metastasis and chemoresistance in different cancer models (Galadari et al., 2017), use of antioxidants or agents that enhance antioxidant system may provide an opportunity to reduce intracellular ROS-mediated tumorigenesis and cancer progression. *Prunus spinosa* (Condello and Mischini, 2021) and Visfatin/eNampt (Buldak et al., 2015) have been reported to possess anticancer activities in HCT 116 cells via elevation of antioxidant defence system and reduction of lipid peroxidation. Consistently in the present study, increase in the level of GSH and activities of GST and SOD, and decreased lipid peroxidation as evidenced by the significant decrease in MDA levels after MMAE treatment clearly demonstrates its antioxidant nature which may be responsible for its anticancer activity in HCT 116 cells.

To assess whether MMAE treatment causes DNA strand breaks, single-cell gel electrophoresis assay (Comet assay) was performed. Anticancer therapy relies heavily on DNA damage and the induction of apoptosis in response to anticancer drugs. In proliferating cells, DNA damage activates a pathway that halts cell division and allows for either DNA repair or apoptosis-induced cell death. Necrotic or apoptotic cells can form comets with small or non-existent heads and large diffuse tails (known as a "hedgehog" comet) in alkaline conditions, as can be seen on the HCT 116 cells after treatment with MMAE. Our results showed that MMAE possessed DNA-damaging effect in a dose-dependent manner which was evident from the increased comet tail length and olive moment in HCT 116 cells treated with MMAE (Figure 5.4). The Bcl-2 (B-cell lymphoma/leukemia-2) family of pro- and anti-apoptotic proteins, play a key role in the mitochondrial-mediated intrinsic apoptosis through their interactions, via regulation of mitochondrial membrane

permeabilization (Xiong et al. 2014). Currently, there are around twenty members of the Bcl-2 family, six of which have anti-apoptotic activity and 14 of which have pro-apoptotic activity. Therefore, the fate of a cell is highly dependent on the balance between these two groups of proteins (Jourdan et al., 2009). An excess in pro-apoptotic Bcl-2 proteins at the mitochondrial surface results in membrane-permeabilization and release of pro-apoptotic proteins such as cytochrome c, Smac/Diablo, and apoptosis inducing factor (AIF) and finally downstream activation of caspase-3 and caspase-6 (Arnoult et al., 2003). Therefore, alterations in the expression of these genes contribute to the pathogenesis and progression of cancers thus making them potential targets for the development of anticancer drugs. The relative mRNA expression of pro-apoptotic genes (Bax, p53, Bid and Apaf-1) and anti-apoptotic genes (Bcl-X_L and PARP) was assessed using qPCR techniques in order to determine whether Bcl-2 family proteins are involved in MMAE induced apoptosis. Our findings suggest that MMAE- induced apoptosis in HCT 116 cells could be regulated, at least in part, via the mitochondrial pathway as indicated by the up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes.

Caspase-3 and caspase-6 cleave various critical proteins for the execution of both the intrinsic (the mitochondrial mediated) and extrinsic (the death receptor mediated) pathways of apoptosis, including inhibitor of caspase-activated DNase (ICAD), poly (ADP ribose) polymerase (PARP), and intra-nuclear proteins (Porter et al., 1999). This cleavage mediates disassembly of the cell into the apoptotic morphological changes including cell shrinkage, chromatin condensation and nuclear fragmentation (Elmore, 2007). Thus, activation of caspase-3 and caspase-6 are a strong biomarker for cells undergoing apoptosis. Treatment of HCT 116 cells with MMAE led to increased caspase-3 and caspase-6 activities strongly indicating that MMAE-induced apoptosis was executed through a caspase-dependent pathway. In conclusion, the mechanisms of MMAE anticancer activities involve induction of mitochondria-mediated apoptosis via increased activities of caspase-3 and caspase- 6, and upregulation of pro-apoptotic genes including Bax and p53 and down-regulation of anti-apoptotic genes including Bcl-X_L and PARP suggesting that MMAE could be regarded as a new candidate for cancer treatment.

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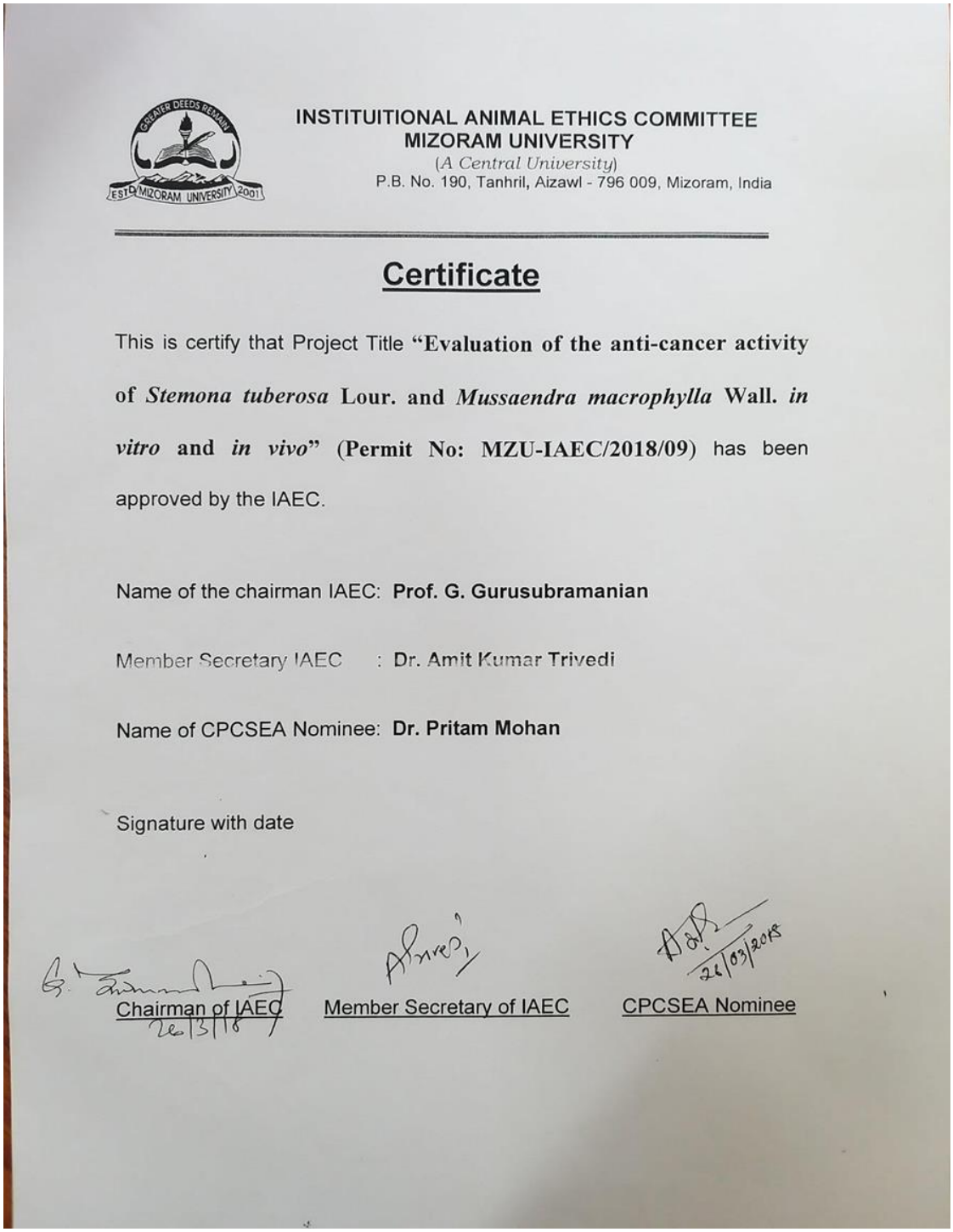
ABBREVIATIONS

MMCE	*Mussaenda macrophylla* chloroform extract
MMME	*Mussaenda macrophylla* methanol extract
MMAE	*Mussaenda macrophylla* aqueous extract
mL	Millilitre
µg	Microgram
kg	Kilogram
g	Gram
h	Hour
min	Minute
sec	Second
BCG	Bacillus Calmette-Guerin
UV	Ultra-violet
IC_{50}	Half maximal inhibitory concentration
LC-MS	Liquid Chromatography-Mass Spectrometry
DOX	Doxorubicin
5FU	5-Fluorouracil
µmol	Micromole
nmol	Nanomole
mM	Millimole
M	Moles
nm	Nanometer
cm	Centimeter
mm^3	Cubic millimeter
OD	Optical Density
ANOVA	One-way analysis of variance
SEM	Standard error of mean
PBS	Phosphate buffered saline
DLA	Dalton's Lymphoma Ascites
OECD	Organisation for Economic Co-operation and Development

i.p	Intraperitoneal
b. wt.	Body weight
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
mRNA	Messenger RNA
RQ1DNase	RNA Qualified DNase
CO_2	Carbon dioxide
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time-PCR
RT	Real time
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
p53	Tumor Protein p53
Bcl-2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma extra large
Bax	Bcl-2 Associated X, Apoptosis Regulator
Apaf-1	Apoptotic protease activating factor 1
PARP	Poly [ADP-ribose] polymerase
Bid	BH3 interacting-domain death agonist,
DR-5	Death Receptor 5
MDA	Malondialdehyde

ANIMAL ETHICS APPROVAL CERTIFICATE



INSTITUITIONAL ANIMAL ETHICS COMMITTEE
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Certificate

This is certify that Project Title "**Evaluation of the anti-cancer activity of *Stemona tuberosa* Lour. and *Mussaendra macrophylla* Wall. *in vitro* and *in vivo*" (Permit No: MZU-IAEC/2018/09)** has been approved by the IAEC.

Name of the chairman IAEC: **Prof. G. Gurusubramanian**

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1. International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET)
2. International Conferences of Recent Advances in Animal Sciences (ICRAAS)

National:

1. National Seminar on Animal Handling, Maintenance & Care (Department of Zoology, Mizoram University)
2. National symposium on Avian Biology & Comparative Physiology (Department of Zoology, Mizoram University)
3. Workshop on Statistical and Computing Methods for Life-Science Data Analysis (BAU, ISI Kolkata, Department of Botany, Mizoram University
4. Hands-on Training Workshop on 'Medical Imaging: Preclinical Imaging in Drug Discovery' (DBT-NER Biotechnology/Bioinformatics Training Centre, ACTREC), Kharghar, Navi Mumbai, India on 7-11th January, 2019.

State:

1. State level seminar on 'Climate change adaptation and Disaster risk reduction (MAS, Department of Environment, Forest & Climate change, MISTIC and the Department of Disaster management and rehabilitation, Gov't of Mizoram)
2. Workshop on Techniques in Animal and Microbial Cell culture (Department of Biotechnology, Mizoram University)
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1. Free radical scavenging and antioxidative potential of various solvent extracts of *Mussaenda macrophylla* Wall. *In vitro* and *ex vivo* study, on The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) and International Conference on Biodiversity, Environment and human health: Innovations and Emerging Trends (BEHIET 2018), Mizoram University, Aizawl on 12-14th November, 2018.

National:

1. Assessment of free radicals scavenging and antioxidant activities of various extracts of *Ganoderma applanatum* (Family: Ganodermataceae), in the Mizoram Science Congress 2018, a National Conference, held at Pachhunga University College during 4-5th October, 2018.

2. Anti-tumor activities of aqueous extract of *Mussaenda macrophylla* Wall. Against Dalton's Lymphoma Ascites (DLA) bearing mice, on the International Conferences of Recent Advances in Animal Sciences (ICRAAS) organised by Department of Zoology, Pachhunga University College, 6-8th November, 2019.

PUBLICATIONS:

1. **Marina Lalremruati,** Lalmuansangi C, Zothan Siama: Free radical scavenging activity and antioxidative potential of various solvent extracts of *Mussaenda macrophylla* Wall: An *in vitro* and *ex vivo* study. *Journal of Applied Pharmaceutical Science*, 9(12), 094-102, 2019. http://www.japsonline.com
2. **Marina Lalremruati**, C. Lalmuansangi, Mary Zosangzuali, Lalchhandami Tochhawng, Amit Kumar Trivedi, Nachimuthu Senthil Kumar and Zothan Siama: *Mussaenda macrophylla* Wall. exhibit anticancer activity against Dalton's lymphoma ascites (DLA) bearing mice via alterations of redox-homeostasis and apoptotic genes expression. *The Journal of Basic and Applied Zoology*, 83(6), 2022. https://doi.org/10.1186/s41936-022-00268-9
3. C. Lalmuansangi, **Marina Lalremruati** and Zothansiama: Assessment of free radical scavenging activities antioxidative potential of the tuber extracts of Stemona tuberosa Lour. *Current Trends in Biotechnology and Pharmacy*, 13 (3), 347-358, 2020. 10.5530/ctbp.2020.3.36
4. Mary Zosangzuali, **Marina Lalremruati**, C. Lalmuansangi, F. Nghakliana, Lalrinthara Pachuau, Priyanka Bandara & Zothan Siama: Effects of radiofrequency electromagnetic radiation emitted from a mobile phone base station on the redox homeostasis in different organs of Swiss albino mice. *Electromagnetic biology and medicine*, 1-15, 2021. https://doi.org/10.1080/15368378.2021.1895207
5. C. Lalmuansangi, Mary Zosangzuali, **Marina Lalremruati**, Lalchhandami Tochhawng & Zothan Siama: Evaluation of the protective effects of *Ganoderma applanatum* against doxorubicin-induced toxicity in Dalton's Lymphoma Ascites (DLA) bearing mice. *Drug and Chemical Toxicology* (2020). https://doi.org/10.1080/01480545.2020.1812630
6. F. Nghakliana, C. Lalmuansangi**,** Mary Zosangzuali, **Marina Lalremruati** and Zothansiama. Anti-oxidative potential and anti-cancer activity of *Elaeagnus caudata* (Schltdl) against Type-II human lung adenocarcinoma, A549 cells. *Indian Journal of Biochemistry and Biophysics*, 58, 543-556, 2021. IJBB 58 (6) 543-556 Dec 2021.pdf

BOOK CHAPTERS:

1. **Marina Lalremruati**, C. Lalmuansangi, Mary Zosangzuali, F. Nghakliana and Zothansiama. *Mussaenda macrophylla* Wall.: Chemical Composition and Pharmacological Applications. Bioactives and Pharmacology of Medicinal Plants. Apple academic Press, USA (distributed by CRC Press of Taylor & Francis).
2. C. Lalmuansangi, **Marina Lalremruati**, Mary Zosangzuali, F. Nghakliana and Zothansiama. Medicinal properties and bioactive compounds of *Stemona tuberosa* Lour. (Family: Stemonaceae). Bioactives and Pharmacology of Medicinal Plants. Apple academic Press, USA (distributed by CRC Press of Taylor & Francis).
3. Mary Zosangzuali, **Marina Lalremruati**, C. Lalmuansangi, F. Nghakliana and Zothansiama. Pharmacological importance and chemical composition of *Mallotus roxburghianus* Muell.Arg. Bioactives and Pharmacology of Medicinal Plants. Apple academic Press, USA (distributed by CRC Press of Taylor & Francis).
4. F. Nghakliana, Mary Zosangzuali, **Marina Lalremruati**, C. Lalmuansangi, and Zothansiama. Ethnopharmacological applications and phytochemical compounds of the genus *Callicarpa* L. Bioactives and Pharmacology of Medicinal Plants. Apple academic Press, USA (distributed by CRC Press of Taylor & Francis).

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INVESTIGATION OF ANTI-CARCINOGENIC ACTIVITIES OF *MUSSAENDA MACROPHYLLA* WALL. (FAMILY: RUBIACEAE)

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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APRIL, 2022

ABSTRACT

INVESTIGATION OF ANTI-CARCINOGENIC ACTIVITIES OF *MUSSAENDA MACROPHYLLA* WALL. (FAMILY: RUBIACEAE)

BY

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Submitted

In partial fulfillment of the requirements of the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

CONSOLIDATED ABSTRACT

Cancer is a leading cause of death and an important barrier to increasing life expectancy throughout the world. Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020. The global cancer burden is expected to be 28.4 million cases in 2040, a 47% rise from 2020, with a larger increase in transitioning versus transitioned countries due to demographic changes. In India, the projected number of patients with cancer is around 1.3 million for the year 2020. Furthermore, Aizawl is currently renowned as India's cancer capital due to its high cancer incidence rate. As a result, cancer is a major public health problem around the world, and the treatment and cure processes are still a work in progress. Conventional treatment approaches to cancer treatment include chemotherapy, surgery, radiation therapy, hormonal therapy, targeted therapy, and immunotherapy. Depending on the type of cancer and its stage, several techniques to cancer treatment have been employed. Despite significant improvements in the currently available treatments for cancer patients and the positive impact of these treatments on survival, these treatments can cause a variety of traumatic side effects and hence, the development of more effective and less toxic anticancer drugs has become an urgent approach in treatment of cancer. Of the hundreds of chemicals that have been and are being evaluated for their anticancer activities, natural products derived from medicinal plants seem to be the most promising due to their safety, efficacy and lesser side effects when compared with synthetic drugs. The beneficial effects of plants in cancer treatment have been extensively researched and have yielded promising results. We have selected *Mussaenda macrophylla* for this study because it is one of the most commonly used traditional medicinal plants by the local people of Mizoram. Previous studies with *M. macrophylla* have revealed its anti-bacterial, anti-coagulant, anti-inflammatory and hepatoprotective activities. However, the molecular mechanisms underlying the anticancer effect of *M. macrophylla* have not yet been explored. This thesis is broadly divided into six chapters. **Chapter 1** is the

general introduction highlighting the global incidence of cancer, treatment modalities, characteristics and causes of cancer. **Chapter 2** describes the extraction of *M. macrophylla* and the analysis of its phytochemical constituents in depth. **Chapter 3** describes the *in vitro* and *ex vivo* free radical scavenging activities and anti-oxidative potential of *M. macrophylla* extracts. **Chapter 4** describes the anticancer activities of the aqueous extract of *M. macrophylla* (MMAE) in Dalton's Lymphoma Ascites (DLA) bearing Swiss albino mice. **Chapter 5** describes the anticancer potential of the aqueous extract of *M. macrophylla* (MMAE) in Type-II human lung adenocarcinoma (A549) cells. **Chapter 6** describes the anticancer properties of MMAE in human colon cancer (HCT116) cells.

Chapter I: General introduction

Chapter II: Preparation of extracts, estimation of phytochemical contents, and identification of bioactive compounds of different extracts of *Mussaenda macrophylla* Wall.

The leaves of *Mussaenda macrophylla* were collected, dried in shade and powdered. The powdered leaves were subjected to sequential Soxhlet extraction with increasing polarity of different solvents such as petroleum ether, chloroform, methanol and distilled water. The liquid extracts were filtered and concentrated using a rotary evaporator and finally freeze dried. We also investigate the phytochemical composition using qualitative and quantitative analysis and LC-MS profiling. Preliminary phytochemical screening revealed the presence of alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids from various extracts of *M. macrophylla*. Among the various extracts of *M. macrophylla*, aqueous extract has the highest total phenolic (387.61 ± 14.10 mg gallic acid equivalent/g) and flavonoid (5,761.65 ± 38.5 mg quercetin equivalent/g) contents. LC-MS analysis also revealed the presence of 73 major bioactive compounds in the aqueous extract of *M. macrophylla*. Some of the compounds such as Chaetocin, Daphnoretin and Brefeldin identified from the LC-MS analysis have been previously reported to exhibit anticancer activities.

Chapter III: Free radical scavenging activities and antioxidative potential of various extracts of *Mussaenda macrophylla* Wall. *in vitro* and *ex vivo* systems

In this chapter, free radical scavenging activities and antioxidative potential of various extracts of *Mussaenda macrophylla* was investigated. The antioxidative potential of *M. macrophylla* extracts was measured by their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions ($O_2^{\bullet-}$), and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in a cell free system. The aqueous extract of *M. macrophylla* possessed the highest scavenging activities for DPPH, $O_2^{\bullet-}$, and ABTS with IC_{50} of 25.92 ± 0.33 μg/mL, 4.12 ± 0.94 μg/mL, and 17.20 ± 0.50 μg/mL, respectively. Furthermore, the scavenging activities of the aqueous extract of *M. macrophylla* against ABTS and $O_2^{\bullet-}$ were found to be more effective than ascorbic acid which was used as standard. The total reducing power of *M. macrophylla* extracts was also determined by measuring the transformation of Fe^{3+} into Fe^{2+} and the methanolic extract was found to exhibit the highest reducing power. The extracts were also analyzed for their anti-hemolytic and inhibitory effect on lipid peroxidation in an *ex vivo* condition using mice erythrocyte and liver, respectively. The aqueous extract of *M. macrophylla* showed the highest inhibitory activities against mice erythrocyte hemolysis and lipid peroxidation in the liver homogenate with an inhibition rate of 80.53% and 65.33%, respectively.

Chapter IV: Investigation of the anticancer activity of *Mussaenda macrophylla* Wall. in Dalton's lymphoma ascites (DLA) bearing Swiss albino mice

Mussaenda macrophylla is a shrub widely used in Mizo traditional practice for treatment of cancer, fever, cough, ulcer and dysentery. In this study, we explore the anticancer activity of the aqueous extract of *M. macrophylla* (MMAE) using Dalton's Lymphoma Ascites (DLA) bearing mice as our model. Effects of MMAE on survival, weight change and antioxidants/oxidant status were determined in DLA mice by administering different doses of MMAE for 9 consecutive days. Doxorubicin (DOX) was used as a standard drug. Effects of MMAE on cytotoxicity, serum enzymes activities and hematological parameters were also determined. Comet assay and qPCR were employed to study DNA damage and differential apoptotic gene expression induced by MMAE respectively. MMAE significantly

inhibited tumor growth and increased survival time of the tumor bearing DLA mice. MMAE significantly increased the glutathione levels; and glutathione-s-transferase and superoxide dismutase activities. Consistently, MMAE decreased lipid peroxidation levels in DLA mice. Reduced RBC and hemoglobin levels were significantly reversed by MMAE treatment. MMAE also lowers the activities of aspartate aminotransferase, alanine aminotransferase, and creatinine levels that were otherwise elevated in the DLA control animals. Induction of DNA damage, up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes in DLA bearing mice following MMAE treatment provide an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our findings demonstrate the role of the aqueous extract of *M. macrophylla* as a potential anticancer agent possibly targeting the apoptotic pathway.

Chapter V: *In vitro* anticancer activity of *Mussaenda macrophylla* Wall. extracts on human lung adenocarcinoma cell line (A549 cells)

The incidence of lung cancer has increased globally along with the increased in the mortality rate due to lung cancer. In this chapter, we explore the anticancer activity of *Mussaenda macrophylla* extracts against Type-II human lung adenocarcinoma cells (A549). MTT assay was used to investigate the cytotoxicity of chloroform (MMCE), methanol (MMME), and aqueous (MMAE) extracts of *M. macrophylla*, and MMAE was shown to be the most effective extract, with an IC_{50} of 49.6 ± 1.12 µg/mL. As a result, MMAE was used for the subsequent tests. Effects of MMAE on reproductive integrity and colony-forming ability of A549 cells was assessed using clonogenic assay and MMAE was found to significantly inhibit cancer cell proliferation in a dose-dependent manner. Effects of MMAE on antioxidants/oxidant status were also determined and MMAE decreased the glutathione (GSH) levels and glutathione-s-transferase (GST) and superoxide dismutase (SOD) activities while consistently increasing the level of lipid peroxidation (LPO) in A549 cells. Fluorescent dual staining, comet assay, qPCR and caspase-3/6 assay were employed for the analysis of apoptotic cell morphology, DNA damage, differential apoptotic gene expression and execution of apoptotic pathway induced by MMAE, respectively. Induction of cell death, DNA damage, up-regulation of pro-apoptotic

genes (Bax and p53) and elevation of caspase 3/6 activities in A549 cells following MMAE treatment provide an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our findings demonstrate the role of the aqueous extract of *M. macrophylla* as a potential anticancer agent targeting the apoptotic pathway.

Chapter VI: Anti-carcinogenic activities of *Mussaenda macrophylla* Wall. leaf extracts on human colon cancer cells, HCT 116 cells

Mussaenda macrophylla is a traditionally widely used shrub for treatment of various ailments including cancer, ulcer, dysentery, fever and cough. In this chapter, we determine the anticancer effects of *M. macrophylla* using colon cancer cell line (HCT 116). The cytotoxicity of various extracts of *M. macrophylla* such as chloroform (MMCE), methanol (MMME) and aqueous (MMAE) extracts were examined using MTT assay and the inhibitory concentration (IC_{50}) was measured wherein MMAE was found to be most effective extract with an IC_{50} of 29.03 ± 2.46 µg/mL. Thus, only MMAE was used for the subsequent tests. The extract was screened for their inhibitory effects on HCT 116 cells using clonogenic assay. Additionally, acridine orange/ethidium bromide (AO/EtBr) staining verified apoptotic features in HCT 116 cells following MMAE treatment through fluorescent microscopic analysis. Antioxidants/oxidant assay, comet assay, qRT-PCR analysis of pro-apoptotic and anti-apoptotic gene expression and caspase-3/6 activity assay were performed to investigate the anticancer effects and possible mechanism of actions leading to apoptosis in HCT 116. Our results showed that MMAE inhibited the cell proliferation of HCT 116 cells and significantly increased the level and activities of antioxidants including glutathione (GSH), glutathione-s-transferase (GST) and superoxide dismutase (SOD), while decreasing the lipid peroxidation (LPO) level in HCT 116 cells. Treatment of HCT 116 cells with MMAE also resulted in DNA damage, up-regulation of pro-apoptotic genes (Bax and p53), down-regulation of anti-apoptotic genes (Bcl-X_L and PARP) and elevation of caspase-3 and caspase-6 activities providing an insight into apoptosis-based anticancer activities of *M. macrophylla*. Our study showed that MMAE could be a promising agent for colon cancer therapy targeting the mitochondrial apoptotic pathway.