SYNERGISTIC EFFECTS OF *Chrysanthemum cinerariaefolium SESQUITERPENE* LACTONES WITH SESAME LIGNANS ON INTERLEUKIN-7(IL-7) EXPRESSION AND CANCER IMMUNOTHERAPY

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Declaration

"I, **Murungi Japhael Mbabu** do hereby attest that I am the sole author of this thesis and that its contents are only the result of the readings and research I have done''

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May GOD Bless you

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Abstract

Ethnopharmacological relevance: Pyrethrum *(Chrysanthemum cinerariefolium)* has been widely used as a source of botanical insecticide for over a hundred years. A part from pyrethrine used as a natural biocide, the flowers of Pyrethrum contain potential defense compounds known as sesquiterpene lactones (STLs). Sesquiterpene lactones have been reported as being anti-cancer as well as anti-inflammatory agents and have been isolated and reported to exhibit potent cytotoxic effects towards ovarian cancer cells. Sesame lignans have been associated with stimulation of osteoblast differentiation in adipose stem cells and have been reported to have anticancer properties against human lymphoid leukemia cells. This study sought to find out whether there is synergistic effect of the pyrethrum STLs and Sesame lignans as an immunotherapeutic remedy for cancer.

Methodology: The sesquiterpene compounds present in ***Chrysanthemum cinerariaefolium*** were characterized with Gas Chromatography–Mass Spectrometry (GC-MS). Sixteen sesquiterpenes were identified, mainly hydrocarbons but also derived oxygenated compounds. Sesquiterpenes were acyclic, monocyclic, byciclic and tryciclic. All the sesquiterpenes were identified by mass spectral data, linear retention indices (LRI), literature data and injection of standards where available. GC-MS analysis indicated various secondary phytochemicals; Alkaloids, terpenoids, tannins, flavonoids, glycosides, phenols and saponins as present in the plant's extracts. The presence of these phytochemical could be implicated in the medicinal value of the two plants. In comparison, phytocompounds such as flavonoids, alkaloids, saponins and terpenoids have been shown to be potent anticancer agents, giving rise to numerous convectional drugs, which are used in mainstream medicine and in the primary therapeutic strategies for numerous conditions including cancer

In this study, Intestinal Epithelial Cells (IEC) were used to produce IL-7 cytokine which works on the immune system and on the postulation that pyrethrum STLs and Sesame lignans extracts work by up regulating IL-7. The cell counting kit-8 (CCK-8) proliferation assay procedure was

used to asses cell proliferation. STLs and lignans extracts concentration ranging from 0.00001 to 100µg/ml was used in the cell proliferation assay. IEC-6 CELL grown on media supplemented with 100µg/ml of pyrethrum STLs and lignans at a ratio of 1:1 was used in *in vivo* and RT-PCR technique employed to evaluate up- regulation of IL-7. The extract with the best IL-7 expression was selected for farther evaluation *in vivo* at 30mg/mice/day in mice infected with CT-26 colon tumor cells. Cyclophosphamide at 20gm/mice/day was taken as the positive control drug. Flow cytometry was employed to evaluate the peripheral blood $CD4^+$and $CD8^+$ lymphocytes. The extracts were further treated *in vitro* on CT-26 cells at 100 and 200µg/ml to explore direct inhibition.

Results:

The extracts showed proliferative activities towards EIC-6. The plant extracts synergistically inhibited the growth of cancer cells CT-26. 1µg/ml was found to give optimum IEC-6 cell growth however; 100µg/ml was determined as the ED_{50} and used as the minimum evaluation concentration for IL-7 expression. The combined pyrethrum and sesame extracts were found to up-regulate IL-7 best. The cells exposed to extract for longer time gave optimal IL-7 expression of over 1.2 times at 100µg/ml .The STLs and lignans extract was found to increase the CD4+ and CD8+ lymphocytes *in vivo* in mice a reflection on IL-7 up regulation in the intestinal mucosa and further to inhibit CT-26 cell growth in a significant way at $P \leq 0.05$. IEC-6 cells can be used to produce increased IL-7 Cytokine responsible for resultant adaptive immunity and cancer immuno surveillance.

1.0 INTRODUCTION

1.1 Background

1.1.1 Cancer

Cancer is highly complex and miscellaneous series of genetic and molecular factors, both intrinsic and extrinsic, create a class of fatal disease called Cancer. Cancer is among the leading causes of death worldwide with approximately 14 million new cases every year and this is expected to rise to 22 million within the next 2 decades (WHO, 2014). Above all the problems associated with the treatment of cancer, metastasis, the spread of cancer cells from the primary tumor to seed (colonize) other distant tumors, is one of the greatest challenges that we are facing in cancer treatment today (Schroeder et al., 2012). Although the conventional anti-cancer therapies are improving on effectively managing the primary tumors, more systemic, specific and targeted cancer treatments are needed to control the metastatic cancer cells (Steeg P.S, 2006).The complexity of cancer has been recently simplified by defining the hallmarks of most, if not all, types of cancer (Hanahan, D. and Weinberg, R.A, 2011); Self-sufficiency in growth signals, resistance to growth suppressors, avoiding apoptosis, limitless proliferation, inducing angiogenesis, invasion and metastasis, reprogramming energy metabolism and lastly, evading the host's immune system. Further, Hanahan and Weinberg emphasis that these acquired functional properties of cancer cells can be made possible by two characteristics; development of genomic instability which generates random mutations, and tumor-promoting inflammation in which, the infiltrating immune cells (mostly innate immune cells) have paradoxical effect by providing growth factors, survival factors, proangiogenic factors, extracellular matrix-modifying enzymes and inductive signals in to the tumor microenvironment that cancer cells may benefit. There is no doubt that defining these hallmarks of cancer is already opening a gate for the development of new, more specific and effective anti-cancer agents which targets a particular key hallmark to act on. Cancer immunotherapy is one of these new inventions that are a result of these new developments.

1.1.2 Conventional therapies against Cancer

Surgery, chemotherapy and radiation therapy have long been considered as the traditional treatments of cancer. Even though there have been major improvements in these treatments for the past decade, radiation and chemotherapy still can only kill a fraction of tumor cells with an adverse effect of a high level of cytotoxicity against the healthy cells (Urruticoechea et al.,2010). Despite the effectiveness of surgery to cure cancer, this treatment is highly restricted to benign and confined metastases which are only 10-15% of the cancer cases (Ohlsson et al., 1998). Combination of these treatments, such as Chemoradiotherapy, has also been commonly used to treat many tumor kinds such as breast and central nervous system (Urruticoechea et al., 2010). Bone Marrow Transplantation and Peripheral Blood Stem Cell Transplantation is mostly used for the patients that receive these high doses of chemotherapy and/or radiation therapy in order to restore the ability of the body to produce blood cell (National Cancer Institute, 2010). The drawbacks of these traditional anti-cancer treatments are their severe adverse effects due to the lack of selectivity and their tendency to cause drug-resistance cancer cells (Guillemard, V. and Saragovi, H.U, 2004).

1.1.3 Immunotherapy

Cancer immunotherapy, also called biological therapy of cancer, means the modulating and using of the patient's own immune system to target the cancer cells rather than using an extrinsic means of therapy. In that manner, cancer immunotherapy focuses on developing agents that activates or enhances the immune system's recognition and killing of the cancer cells (Sharma et al., 2011). Cancer immunotherapy, as an alternative modality of treatment, has significantly developed during the last decades and completes the therapeutic arsenal. In contrast to the other therapeutic concepts, immunotherapy primarily aims to prevent the metastatic spread of the disease and to improve quality of life of the affected individuals. Approaches that are applied in immunotherapy are based on complementation or stimulation of the immune system via a plethora of compounds, such as lymphokines, vaccines, in vitro-stimulated effector cells of the immune system or antibodies. Immune surveillance is the concept that envisages prevention of the development of tumors by the early destruction of abnormal cells by the immune system of the host. Consequently, a lack of (or escape from) immune surveillance plays an essential role in cancer development, and

may be associated with an evasion of tumor cells from the surveillance of the immune system, which in particular seems to be an escape from specific T cell-mediated immunity (Ochsenbein et al.,2001).

1.1.4 The immune system

The immune system is a network of proteins, cells, tissues and organs that work together to defend the body against infections. It identifies and destroys pathogens and tumor cells. It also 1 clears the body of foreign substances and dead cells. The key to a healthy immune system distinguishing between the body's own molecules and foreign molecules, recognized as self and non-self. This ability is necessary to protect the organism from invading pathogens and to eliminate modified or altered cells (e.g. malignant cells). In addition, the immune system is capable of generating immunological memory against an infectious agent and prevents repeated infections (Murphy et al., 2008). The immune system is divided into innate and adaptive immunity and both these systems are necessary to maintain protective immunity of the individual. The main cells of the adaptive immune system are two types of lymphocytes, the B and the T cells. They recognize foreign antigens through specific receptors. B cells mature in bone marrow but T cells finish their development in thymus. The adaptive immune system can be divided into the humoral and the cellular immune system. B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response. B cells most often require help from T cells to become activated and the activated B cells then differentiate into antibody-secreting plasma cells and memory B cells. Activation of T cells results in several types of effector T cells and memory T cells (Murphy et al., 2008).While T cells undergo development in the thymus, the immature cells that recognize self-major histocompatibility complex (MHC) receive survival signals (positive selection) and those that interact strongly with self-antigens are removed from the repertoire (negative selection). The few T cells that survive selection mature and leave the thymus as naïve but mature T cells. They then circulate between the bloodstream and lymphoid tissues, where they may encounter APCs. APCs present antigens to the naïve T cells in connection with the MHC molecules. There are two types of MHC molecules, class I and class II. When naïve T cells recognize specific peptides expressed on MHC molecules they become activated. Activation leads to clonal expansion and differentiation and gives rise to a clone of effector T cells of identical antigen specificity. These effector T cells are attracted to the sites of infection (Murphy et al.,

2008).T cells fall into two major classes, cells that express the CD4 and cells that express the CD8 co- receptors. CD8+ T cells recognize MHC class I molecules and activated CD8+ T cells, cytotoxic T cells, are specialized to kill tumour and virus-infected cells.CD4+T cells recognize MHC class II molecules and activated CD4+T cells differentiate into a number of different effector T cells with a variety of functions. The main functional subsets of CD4+ effector T cells are Th1, Th2, Th17 and regulatory T cells (Murphy et al., 2008; Wan et al., 2009; Zhu et al., 2008). Th1 cells play a role in activation of infected macrophages, destruction of pathogens that persist in macrophage vesicles, providing help to B cells for production of antibodies directed at extracellular pathogens and induction of class switching. Th2 cells stimulate production of antibodies by B cells, especially IgE antibodies. Th17 cells play a role early in the adaptive immune response. They activate multiple cell types, e.g. fibroblasts and epithelial cells, to secrete soluble mediators such as chemokines that can recruit neutrophils to sites of infectionwhere they play an important role in protection against extracellular pathogens and establishment of chronic inflammation (Murphy et al., 2008; Pulendran et al., 2010). Regulatory T cells tend to suppress the adaptive immune responses from becoming uncontrolled and prevent autoimmunity. addition to providing effector T cells, the primary T cell response also generates memory T cells, which are long-lived cells that give an accelerated response when they encounter the same antigen that activated them initially.

1. 1.5 Immunostimulants

The use of immunostimulants for cancer therapy is one of the earliest approaches in immunotherapy. It is a non-specific approach that aims to enhance, in general, the activity of the lymphocytes that are already attacking the tumor cells but are insufficient to produce a full-powered immune response. In this manner, this strategy uses the patient's own immune system as the effecting factor. In the late 1990s and early 2000s, the most important cytokines for cancer therapy, Interleukin-2 (IL-2), Interleukin-7(IL-7) and Alpha-Interferon (IFN-α), demonstrated their anti-cancer properties and were approved by the U.S. Food and Drug 7 Administration (FDA) for the treatment of diverse types of cancers including metastatic melanoma and renal cell carcinoma (Kirkwood et al., 2008). Alpha-Interferons are proteins belonging to the type-I IFN family which was discovered decades ago for its anti-viral properties (Belardelli, F, 1995). The human IFN-α family consist of at least 13 functional subtypes which share the same receptor

system and very similar biological functions (Mogensen et al., 1999). These diverse biological functions include the activation and regulation of both innate and adaptive immune system by enhancing the effects of macrophage and natural killer (NK) cells, increasing the expression of MHC class I antigens, and regulating the proliferation and survival of both helper and cytotoxic T-cells (Belardelli et al., 2002). IFN-α has also direct effects on cancer cells by its apoptotic, antiangiogenic and antiproliferative properties (Belardelli et al.,2002). In today's immunotherapy regimes, IFN-α is the most used cytokine for the treatment of more than a dozen types of cancer, such as hairy cell leukemia, chronic myeloid leukemia, B and T cell lymphomas, melanoma, renal carcinoma and Kaposi's sarcoma (Pfeffer et al., 1998 and Belardelli et al., 2002). Interleukin-2 is a glycoprotein which is a strong T-cell and Natural Killing (NK) cell growth factor that plays a key role in immune regulation and lymphocyte proliferation (Smith, K.A., 1988). Unlike IFN-α, IL-2 has only indirect anti-cancer effects through the activation of the effector lymphocytes which are also called lymphokine-activated killer cells (Fang et al., 2008). Clinical trials with systemic administration of high-dose IL-2 demonstrated that this regime provides consistent, however low, overall response rate of ~13–17% (Atkins et al., 1999). The drawbacks of this immunotherapy are its high cost and its severe but reversible adverse effects. However, to this date, IL-2 remains to be an indispensible immunotherapeutic agent for the treatment of metastatic melanoma (Fang et al., 2008). Molecular modification of IL-2 is also being developed at the moment which is called BAY 50-4798. This agent has two modified amino acids that aim to have the same response as IL-2 but without the adverse cytotoxicity to healthy cells (Margolin et al., 2007). Apart from Interleukin-2 and Alpha-Interferon, Bacillus Calmette-Guerin (BCG) (Morales et al.,1976), Levamisole (Renoux, G, 1980), Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Waller, E.K, 2007) have also being used as immunostimulants over the years for immunotherapy but mostly in combinations with other immunotherapies or other strategies for anti-cancer therapeutics such as chemotherapy, or with other immunotherapies such as cancer vaccines and adoptive cell transfer therapy (Dimberu, P.M. and Leonhardt, R.M, 2011).

1.1.6 Interleukin 7(IL-7)

IL-7 is mainly produced by non-hematopoietic cells including keratinocytes in the skin, fibroblastic stromal cells in the bone marrow, epithelial cells in the thymus, prostatic epithelium and the intestine (Mazzucchelli et al., 2009). Immune cells, such as dendritic cells (DCs) can also

produce IL-7 (Guimond et al., 2009). The immune system protecting the organism from cancer relies on the size of its T lymphocyte pool, especially the CD8+ T cell pool. T lymphocytes are mainly generated in the thymus where IL-7 is necessary for their development. This pool is maintained in a dynamic balance. Antigen-specific effector T cells fulfill their mission and subsequently die. Then expansion of new T cells supplements this pool. IL-7 retains this balance in three ways: thymopoiesis, homeostasis proliferation and life-support.

1.1.7 IL-7 for Immune Reconstitution in Cancer Patients

Cancer patients often suffer from immunosuppression with low T cell counts. This situation deteriorates when the patients receive chemotherapy as a side-effect of drugs via myelosuppression. IL-7 administration can enhance their immune reconstitution, mainly from the homeostatic expansion of peripheral T cell populations. IL-7 therapy not only increases both cell counts (Luh et al.,2008) and the diversity of T cells recognizing different antigens , but it also promotes the homing of T cells to lymphoid tissues where antigen recognition occurs . It has also been reported that IL-7 can increase thymopoiesis to fill-up the pool of naïve T cells. RhIL-7 has been applied in a phase I study with a significant increase in peripheral CD4+ and CD8+ T lymphocytes in patients with refractory malignancy (Sportes et al., 2010).The presence of tumor infiltrating lymphocytes (TILs) within the tumor microenvironment is considered to be an indication of the host immune response to tumor antigens (Eliichi et al., 2005), and is thought to reflect the dynamic process of "cancer immune editing" Intestinal epithelial cells (IEC) have been implicated in IL-7 synthesis (Madrigal-Estebas, 1997; Mwitari et al., 2014).IL-7 plays an important role in immune processes in our bodies. Several studies have indicated that IEC may play an important role in mucosal immune responses by helping to regulate intestinal intraepithelial lymphocytes (IEL) (Hua yang et al, 2005; Mwitari et al., 2014). Interleukin-7 has been implicated and proposed as an important link to finding the right treatment for cancer ailments. IL-7 has a potential for adoptive immunotherapy (Mwitari et al., 2014). Importance and usefulness of Cytokine IL- 7 as a tool in cancer treatment research has been demonstrated before (Cytheris, 2012; Mwitari et al., 2014).

1.1.8 Plants extract Immunomodulation.

It is known that plant extracts are able to affect a great number of physiological and pharmacological aspects in the human body and therefore screening for biologic activities should be diversified to increase the chances of detecting a variety of useful and meaningful biological activities which would otherwise go undetected. Common plants such as Carica papaya, the pawpaw (Clagget et al., 1974; Balandrin et al., 1985) and Allium sativum, the garlic, have been reported to possess broad therapeutic properties ranging from anticancer, antibacterial, and antidiabetic to anticoagulant (Venugopal and Venugopal, 1995). Extracts of Melia azederach has been demonstrated to possess antiviral activity against a number of viruses (Wachsman et al, 1983). Andrei et al., (1988) further demonstrated that the antiviral effect was due to the induction of refractory state to viral infection in mammalian cells by a mechanism independent of interferon. In addition to antimicrobial effects of plant extracts, a number of them have been demonstrated to possess immunomodulatory activities. Alternanthera tenella aqueos extracts were shown to enhance production of antibodies (Guerra et al., 2003).

1.1.9 Plants investigated

Botanical description of pyrethrum plant

Chrysanthemum cinerariaefolium is a perennial herbaceous plant belonging to the Asteraceae family with leaves having surface which is green and shining. It grows up to 60 cm high. The stems are unbranched with few short scattered hairs below the flower. The leaves are long petioled, finely cut and silky beneath with distinct segments. The plant bears numerous flower heads which measure 6-9 mm when closed and 9-12 mm, when open. The involucre consists of two to three mm of yellowish or greenish yellow, lanceolate hair bracts (Beentje, 1994).

1.1.10 Synergy and phytotherapeutics

It is known that plants contain a wide diversity of specialized micromolecules (secondary metabolites), and this diversity presupposes a high likelihood of interactions. The therapeutic indications of medicinal plant-based extracts are, in most cases, empirical, and practitioners of phytotherapy intuitively believe that a total extract acts better than an equivalent dose of an isolated substance. Through synergy studies, it is now possible to prove the validity of this sentence, with

some recent works confirming the presence of interactions and amplified effect in different plant extracts, and also between components of the same extract (Nelson et al, 1999). Synergy is defined as the combined or cumulative effects produced by interactions between various forces, particles, elements, substances, parts or individuals in a given context. In pharmacology, since the last century, the medical clinic has been aware of the benefits of combining drugs. It is observed that isolated medical substances with action in just one molecular target are, in general, less effective for treating a disease than a therapy with combined medical substances, which act on various targets simultaneously (multi-target), potentializing the therapeutic effect. Over the last 10 years, classical medicine has gradually abandoned therapies with a single substance (monotherapy), in favor of treatments with a combination of drugs (multitherapy), like the now well-established treatments for AIDS, cancer, malaria, hypertension and infections (Csermely et al, 2005). Multi-drug therapy (multi-target, multitherapy) is more effective and less vulnerable to adaptive resistance because the biological systems are less able to compensate the action of two or more substances simultaneously. As a result, mono-target drugs are incapable of effectively combating complex pathological conditions like cancer and infectious diseases (Keith and Zimmermann, 2004). The understanding that acting on a single target is in most cases, insufficient to restore the balance in a biological system, has led to the search for new drugs that act on multiple targets, to maximize (or potentialize) the therapeutic effect. Thus, the principle of synergy becomes a major contemporary challenge in the discovery of new therapeutic agents (Lehar et al., 2007).

1.1,11 Sesquiterpenes lactones

Sesquiterpenoids are extensively dispersed in plant kingdom. They are natural products with 15 carbons (Cane *et al* 1997(99), Davis *et al* 200). Numerous of them show cyclic chemical structures (Chen *et al*., 2003, Degenhardt *et al* 2003) These compounds are synthesized by plants in several organs such as leaves, fruits or roots (Aharon *et al* .,2003, Martin *et al* .,2004, Pichersky *et al*.,2001, Rodriguez *et al* .,2001).The flower heads of pyrethrum consist of a collection of small flowers: ray and disc florets that are set on a receptacle. Microscopic examination of the disc florets shows that the surface of the achenes is densely covered by glandular trichomes filled with a mixture of compounds dominated by sesquiterpene lactones (STLs) (Ramirez et al., 2012).

Sesquiterpene lactones (STLs) are a sub-class of sesquiterpenoids and are typical secondary compounds of the Asteraceae plant family (Picman et al., 1986. Seaman et al., 1982). In the last

several years, the potent bioactivities of STLs, such as anti-malarial activity of artemisinin (Klayman et al., 1985), drew significant interests in the biochemistry of STLs (Gupfert *et al*., 2009). They show anti-microbial activities, serve as antifeedants Knight et al ., 1995, Mori et al ., 1993,Mullin *et al*., 1995), as an antimigraine (Pfaffenrath *et al*., 2002,Tassorrelli *et al*.,2005], anti-inflammatory, for treatment of stomach-ache, skin infection (Heinrich *et al*.,1998), antitumor, antiulcer, neuro-cytotoxic, cardiotonic activities (Chao et al 2000) and antineoplastic activity and hence make lead structures for the development of therapeutic agents.

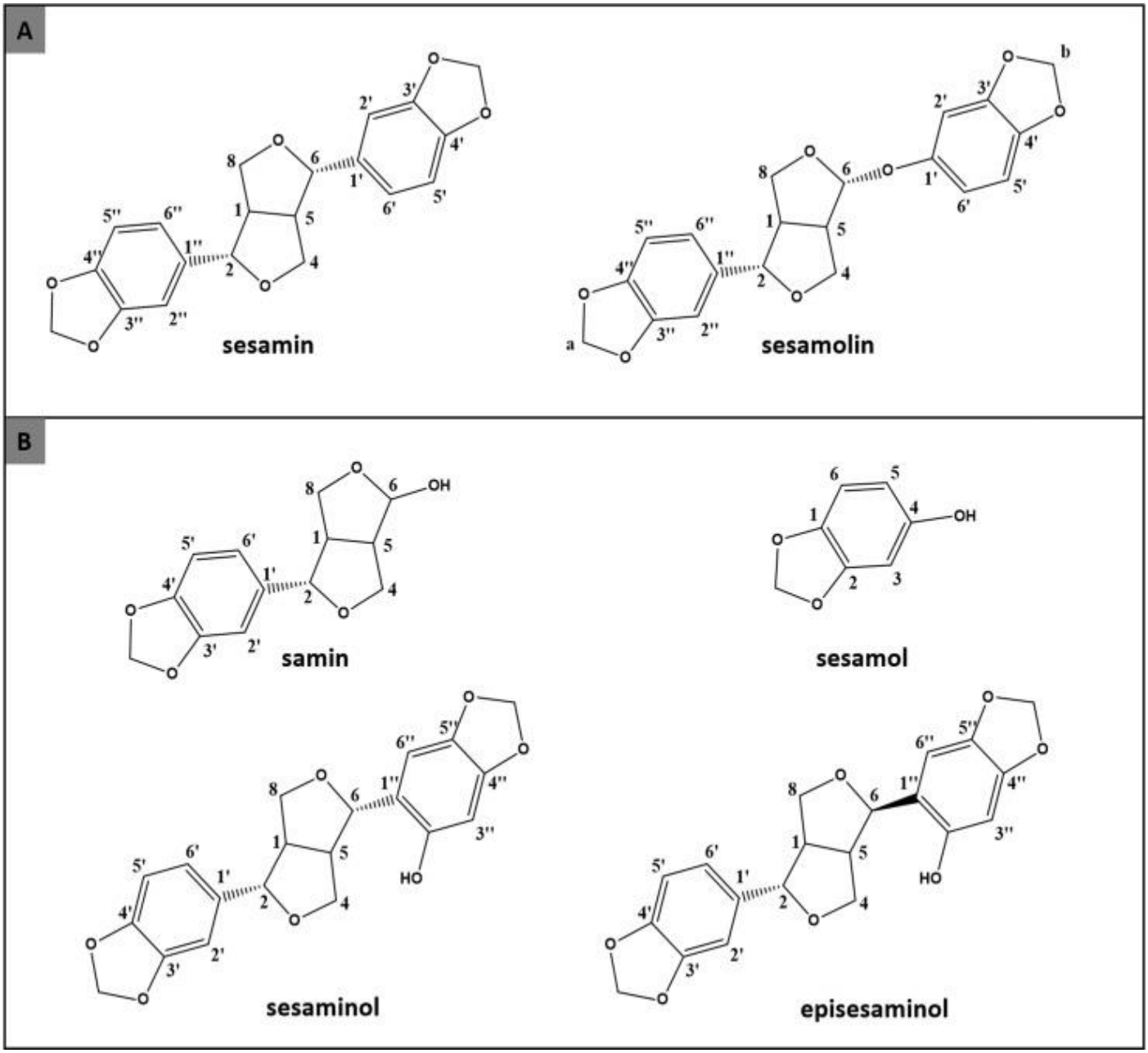
According to scientific evidence, the lactone fraction obtained from *Artemisia annua* L have greater potential for pain relief revealed by chemical-induced nociception assays in mice (Fellin et al., 2014). Similarly several experiments have shown the antinociceptive potential of terpenes in different painful conditions. They were able to reduce significantly the nociceptive response in various models of nociception with possible involvement of muscarinic, opioid, dopaminergic, adenosinergic and glutamatergic systems and the involvement of ATP-sensitive K^+ channels (Batista et al., 2008). Citronellal, a monoterpene possess antinociceptive potentials with the involvement of opioid system (Quintans et al., 2010, Qiuntans-Junior., 2011), similarly carvacrol, p-cymene, hydroxydihydrocarvone (monoterpene) possess antinociceptive potentials in several models of animals (Guimaraes *et al*., 2012, Brito *et al*., 2013, Goncalves *et al*., 2008). Through structural analysis and the mechanism proposed by scientific studies, the monoterpenes like myrcene, linalool, citronellol and citronellal usually have antinociceptive potentials via acting on the opioid system (Salminen *et al* ., 2008).

1.1.12 Sesame lignans

Lignans are products of secondary metabolism produced by several plants to serve as molecules of defense against the predators. Chemically are phenyl propane dimers that additionally exhibit varied biological activities. Sesamum indicum is a major source of sesame lignans. Several different fat soluble aglycon lignans and six different insoluble lignan glycosides have been reported from sesame oil and oil-free sesame seed meal. Kamal-Eldin *et al* (2011). Two major lignans namely sesamin and sesamolin have been extensively studied by virtue of their antioxidant property that makes them resist rancidity and thereby conferring a long shelf life to sesame oil. Sesamin has been shown to exhibit anti- cholesterolemic and antihypertensive properties (Nishant et al., 2008) and its associated with stimulation of oesteoblast differentiation in adipose stem cells

(Wanachewin *et al.,* 2012). Sesamolin has anticancer properties against human lymphoid leukaemia cells (Miyahara *et al.*, 2001).Whereas the minor lignans of Sesame such as sesamol and Pinoresinol inhibits membrane lipid peroxidation and DNA damage (Parihar *et al.*, 2006).

Figure 1: Chemical structures of major (**A**: sesamin and sesamolin) and minor (**B**: samin, sesamol, sesaminol, and episesaminol) lignans isolated from sesame oil.



Due to the high pharmacological interest of sesamin and sesamolin, there are several works regarding the isolation and purification of these lignans from *Sesamum indicum* materials like sesame seeds, sesame meal, and SO (Lee and Choe, 2006; Wang et al., 2009; Reshma et al., 2010; Dar et al., 2015; Hammann et al., 2015; Jeon et al., 2016). In the literature, silica gel column

is referred as separation technique of SO extract, which is mostly applied for laboratory purposes (Lee and Choe, 2006). Moreover, crystallization method is carried out, providing mixture of lignans (Reshma et al., 2010))

In view of these, this study focused on the synergistic effects of STLs isolated from pyrethrum and Sesame lignans on interluken 7 for Immune Reconstitution in Cancer Patients with following Specific objctives:

- To isolate and purify Sesquiterpene lactones (STLs) from C.cinerariefolium and sesame lignans from sesame oil.

- To assess the synergistic effects of C, cinerariefolium STLs extract with sesame lignans on proliferation of IEC cells in vitro using the cell counting kit -8 (CCK-8).

- To determine the level of IL-7 mRNA expression in IEC-6 cells grown in media supplemented with C,cinerariefolium STLs extract and sesame lignans using the RT534 PCR technique

- To evaluate synergistic inhibitory effects of C. cinerariefolium STLs extract with sesame lignans in mice infected with CT-26 colon cancer tumor cells.

- To determine the synergistic effect of the C.cinerariefolium STLs extract with sesame lignans on lymphocytes CD4+ and CD8+ 538 associated with immunomodulation and cancer cells immunosurveillance *in vivo* in mice using the flow cytometry technique.

2. METHODS

2.1 Plant materials

Powdered flowers of *Chrysanthemum cinerariaefolium* was provided by the director Center for traditional medicine and drug research (KEMRI-CTMDR).

2.1.1 Extraction fractionation and isolation of sesquiterpene lactone

Powdered flowers (10 kg) Chrysanthemum *cinerariaefolium* was soaked in commercial grade hexane with occasional stirring. Filtered the whole suspension after 22 days and repeated this process three times. Then combined all the filtrates and concentrated through rotary evaporator under reduced pressure at 40 °C. 1 kg of greenish-black extract was obtained. The crude extract was added to 5 L distilled water followed by the addition of an equal volume of hexane and shaken gently in a separating funnel. Collected the hexane layer from the separating funnel and continued this process till there appeared no color in the hexane when added further. Then combined the entire hexane portion and subjected to rotary evaporator at 45 °C, 120 g hexane fraction (12%) was collected. The process was repeated for ethyl acetate (95 %) to obtain its respective fraction. The remaining aqueous portion left was approximately 210 g (21%).

2.1.2 Chemical characterization of crude extract and isolation of sesquiterpene lactone

The hexane and ethyl acetate extracts were tested for the presence of various phytochemicals (Kamar *et al*., 2015). The fraction showed positive result for terpenoids, flavonoids tarnnins and glucosides. were subjected to column chromatography for the isolation and purification of the compounds by elution with 5% ethyl acetate: *n*-hexane. It gave the compounds 1-8. TLC was used to examine and separate the compounds from the extracts, eight compounds were isolated which were then analyzed and bioactive components identified using GC-MS.

2.1.3 GC MS analysis.

Gas chromatography and mass spectrometry (GC - MS) analysis is a common confirmation test. It is best used to make an effective chemical analysis. This analysis provided a representative spectral output of all the compounds that got separated from the samples. The first step of GC MS was done by injecting the samples to the injection port of the Gas chromatography (GC) device. The GC instrument vaporized the sample and then separated and analyzed the various components. Each component ideally produced a specific spectral peak that was recorded on a paper chart electronically. The time elapsed between elution and injection called the "retention time". Differentiated between the compounds. The peak is measured from the base to the tip of the peak. GC MS analysis was done at JKUAT food and technology center. Identification of bioactive constituents by GC -MS. GC- MS analysis was performed using a THERMO Gas Chromatography- TRACE ULTRA VER: 5.0 [20]. The oven temperature was maintained at 220ºC at a rate of 6ºC/min; the carrier gas with a flow rate of 1 ml/min. The split sampling technique was used to inject the sample in the ratio of 1:10. Retention indices (RI) of the compounds were determined by comparing the retention times of a series and identification of each component was confirmed by comparison of its retention index with data in

2.1.4 Identification of bioactive constituents by GC- MS

Interpretation of Mass-Spectrum was carried out by using the database of National institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components which was stored in the NIST library. The molecular weight, name, chemical structure and molecular formula of the components of the test materials were ascertained. The peak in GC- MS of hexanel extract of flowers of *Chrysanthemum cinerariaefolium* showed the presence of the secondary phytochemical compounds like phenolics, fatty acids and its esters.

2.2 Cell proliferation assay

IEC-6 cells were seeded at 50,000 cells per well in corning 96 well flat bottomed micro titer plates and incubated overnight at a volume of 100 µl. 10µl of pyrethrum STLs, sesame lignans s and a combination of both at a ratio of 1: 1 at eight different concentrations each serially diluted were added and cell incubated for a further 72hrs.10µl of CCK-8 (Dojindo Molecular Technologies,

Beeijing,China) was added and incubated in a high humid environment at 37^0 C and 5% CO_2 for 3 hours and optical density (OD) read at 460nm in a 96 well micro titer plate Tecan i-control infinite 200 OD reader. The tests were done in triplicates. Data was analyzed using unpaired Student's T-test .Values are expressed as mean ± S.D.

A second assay was carried out to determine the reliability and cell numbers by plating 100µl of a known number of cells in a clear 96 well plate (Cell numbers ranging from 2x104-2x106). 10 µl CCK-8 solution was then added and incubated for 3 hours under identical conditions to first assay and absorbance determined.

2.3 C T-26 Cell cytotoxicity assay

CT-26 colon cancer cells (ATCC) were seeded at 50,000 cells per well in corning 96 well flat bottomed micro titer plates and incubated overnight at a volume of 100µl. Pyrethrum STLs and sesame lignans extracts was tested at two concentrations of 100 & 200µg/ml by adding 10µl of each and cells incubated at 37ºC, 5% CO_2 in high humidity for a further 72hours. Two controls were similarly set up; a negative control without drug and a positive control where Cytoxin at 100µg/ml was added. 10µl of CCK-8 (Dojindo Molecular Technologies, Beijing, China) was then added and incubated in a high humidity environment at 37^0C and 5% CO_2 for 3 hours and optical difference (OD) read at 460 nm in a 96-well microtiter plate Tecan i-control infinite 200 OD reader. The test was done in triplicate. The data was analyzed using unpaired Student's T-test. Results are presented as mean ±S.D and in form of a bar chart.

2.4 RT-PCR assay technique

In this study, STLs and lignin bearing extracts from pyrethrum and sesame respectively were investigated on IEC-6 cells in vitro. The levels of ex- pression of IL-7 mRNA were determined by RT-PCR methods as a measure of their immune modulating enhancement effect. Ap- propriate controls were set up without the extracts.

RNA extraction, Amplification and gel electrophoresis

Extraction and amplification was done according RNeasy® MiniKit (Qiagen).

The IEC-6 cells were cultured in 6 well plates in media supple- mented with various extracts at concentrations of 100µg/ml for 72, 6 or 3 hours. Briefly the media was decanted and cells washed

in PBS (D-hanks solution) and 1ml Trizol (Invitrogen) added. 0.2ml of Chloroform at 4^{o}C was added per 1ml shaken vigorously using a vortex for 30 seconds and incubated at room temperature (about 20o C) for a period of 4 minutes. This was followed by cen- trifuging for 15 minutes at 12000 revolutions per minute (rpm). A colorless aqueous layer formed at the top of which 500µl was pipetted out carefully. A similar volume (500µl) of isopropyl alco- hol at minus 20o C was added to the RNA fraction and vortexed properly and incubated at room temperature (about 20o C) for a period of 25 minutes. The mixture was centrifuged for 10 minutes at 12000 revolutions per minute (rpm) at 4o C. The supernatant was discarded and RNA pellet washed with 1ml of 75% ethanol and vortexed properly and, centrifuged for 10 minutes at 8000 revolutions per minute (rpm) at 4o. A refrigerated centrifuge was used each time. The supernatant was discarded and RNA pellet air dried before re-dissolving in 20 µl RNAse free water. The Optical difference (OD) was measured at 260/280nm to determine the quality of RNA using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and con- centration (ng/µl) obtained. The concentration of RNA (ng/µl) was used to calculate the volumes of RNA and water (H2O) for use in reverse transcription by first dividing 500 by ng/µl of RNA obtained to give volume of RNA in µl and then this was substracted from 6.5 to give volume of H2O to be used. Reverse tran- scription and cDNA amplification was done according to RNe- asy® Mini Kit (Qiagen), and the following genes were targeted for amplification; GAPDH with primers sequence 5' to 3' sense ACC ACA gTC CAT gCC ATC AC and antisense TCC ACC ACC CTg TTg. CTg TA and, IL-7 sense gAg TTT CAg ACg gCA CAC AA and antisense gAA ACT TCT ggg Agg gTT CC (from Sangon co.ltd) at reaction conditions (94o C for 3 minutes, 94o C for 30 seconds, 60.5o C for 30 seconds, 72o C for 30 seconds, 4o C indefi nitely) and at 22 cycles for GAPDH and 38 cycles for IL-7. All amplified cDNA were analyzed by 1.5% agarose gel electropho resis and stained with ethidium-bromide for visualization under UV illumination (GeneGenius) and photographed. The size of amplified DNA was identified by comparison with DNA marker (100 bp DNA ladder, TaKaRa Biotechnology (Dalian) co., Ltd) and volumes of the DNA calculated for each band.

2.5. Experimental apparatus and extraction procedure of sesame lignans

Hundred grams of Sesame oil (SO) were mixed with methanol in different ratios (1:1, 1:2 and 1:0.5 w/v), and was then placed in an extraction vessel consisting of a three necked flask equipped with a motor-driven stirrer, reflux condenser, thermometer assemblage and heating mantle. The mixture of Sesame oil and methanol, in different ratios, as described, was then subjected to continuous stirring at different extraction temperatures (50, 60 and 70 C) for 10 min, after which the mixture temperature was lowered to 50 C. The mixture was then transferred into a separating funnel and, after 15 min of settling time, the methanol extract and residual oil were separated. The separated residual oil from the first extraction was stripped of solvent and subjected to a second extraction with a fresh batch of methanol, as has been described and, likewise, 10 sequential extractions were performed. The methanolic extracts separated from the 10 sequential extractions were pooled and stripped of solvent using a flash evaporator to obtain the methanolic extract concentrate. The resultant residual oil from the 10 sequential extractions was also concentrated and yields were determined by gravimetry. The sequential extraction process was repeated with three batches of fresh SO at an oil to solvent ratio of 1:1(w/v) and temperature of 70 C. 2.3. Crystallization of lignans from methanolic extract concentrate The methanolic extract concentrate, as obtained above, was dispersed in petroleum ether (1:0.5 w/v) and the mixture was subjected to cryoscopic temperature conditons of 4 to 10 C for a time duration of 24–48 h in order to facilitate crystallization of the lignans. The lignan crystals were separated from the mixture by vacuum-filtration, washed with chilled petroleum ether until oil-free, dried in a vacuum oven at a temperature below 60 C for 1 h and weighed.

2.5.1. Separation of lignans from methanolic extract concentrate by saponification

Saponification of the crystal-removed methanolic extract was carried out by adding (1:1 w/v) of potassium hydroxide (KOH) in water (60:40 w/v) and ethanol (1:6 v/v) to the extract and then refluxing it in a boiling water bath for 1 h. After completion of saponification, water (1:4 v/v) was added to the mixture which was then extracted with petroleum ether (1:10 v/v) six times, each time separating the petroleum ether phase. The separated petroleum ether fractions from the six extractions were pooled and then washed with 10% ethanol until alkali-free. The combined alkali free petroleum ether extract was flash-evaporated under reduced pressure and then dried in a vacuum oven at a temperature below 60 C for 30 min to 1 h to get the unsaponifiable matter (USM).

2.5.2. Purification of USM

Purification of the unsaponifiable matter was carried out by washing it with petroleum ether (1:0.7 w/v) 10 times, followed by vacuum-filtration to remove the impurities and it was then dried in a vacuum oven below 60 C for 1 h to obtain the purified USM

2.6 In vivo studies of the pyrethrum STLs and sesame lignans

Experimental animals

45 female Balb/C mice 6 weeks old weighing on average 18.2g and infected with CT-26 Colon cancer tumor cells were employed to assess the immune boosting action of the pyrethrum STLs and sesame lignans extracts. They were housed in an animal facility well lighted by natural light and well ventilated. They were kept in groups of five per cage. They received normal mice feed and water ad li- bitum. The experimental period was 17 days with two days to acclimatize and one day after 14 days of treatment. The Animal experiment was carried out in accordance with Kenya Medical Institute (KEMRI) guidelines on laboratory animal's use and care.

2.6.1 In-vivo assay

The mice were divided into 3 groups of 15 mice each in a completely randomized design consisting of a negative control which received 0.2ml normal saline orally, a positive control group which received 20mg/ml of an anti-cancer drug Cyclophosphamide (Cytoxin) through IP route and, a test group which received 30mg of the pyrethrum STLs and sesame extracts orally.

The treatments were administered on a daily basis for two weeks except Cytoxin which was administered for 10 days. Weights of the animals were taken at 0 days, 10 days, and 15th day before they were sacrificed and blood, tumor, liver, spleen, thymus and lungs excised and properly labeled. The blood was collected in 0.4ml of 6mg/ml EDTA to prevent coagulation and stored at 4 degrees centigrade. The organs were weighed and either stored at minus 20 or minus 80 degrees centigrade and histopathology evaluation done. Lysed Whole blood immunofluorescence staining was per formed.

2.7 Flow Cytometry- Immunofluorescence staining of mice Cells by Lysed Whole Blood Method

Whole blood (peripheral) was drawn from the tail into 1.5 ml centrifuge tubes. The tubes were pre-coated with 0.4ml of 6mg/ ml EDTA as an anticoagulant. The tubes were well rinsed with the 0.4ml of EDTA by corking and swilling round to cover all sides of the tube before opening to dry the sides and collecting the blood. All tubes were properly labeled. Briefly, 100µl of well- mixed anti-coagulated whole blood was added to the bottom of a well labeled tube. The appropriate primary antibody was then added to each tube as described in the following manner, to 100ul (1x106) Cells was added 0.25µg of FITC Hamster Anti-Mouse CD3e, 0.25µg of APC Rat Anti-Mouse CD4 and 1.0µg of PE Rat Anti-MouseCD8a. The tube was capped and vortexed gently to mix and, incubated for 25 minutes in the dark at room tempera- ture (20° to 25°C). The tube was removed from dark chamber and mixed well. 1.5ml of lysing solution was added to and vortexed. After which it was incubated at room temperature in the dark for 10-15 minutes, until cells become clear and centrifuged for 5 minutes at 1000 rpm (200 x g). The supernatant was removed by aspiration, vortexed and 2 ml washing solution added and, centrifuged for 5 minutes at 1000 rpm (200 x g). The supernatant was again removed by aspiration. The cells were fixed by re-suspending in 2% paraformaldehyde buffer for 30 minutes at room temperature, and washed with wash buffer. The cells were re-suspended in 500µl wash buffer and stored in the refrigerator at 4°C for 24 hours in the dark before reading in a Merck- Millipore flow cytometer. This exercise was repeated for all samples. A negative control was set up in one tube by titrating 50ul of cells and adding 100ul PBS and taken through the same procedure.

2.7 Data analysis

Statistical analysis was done using excel data sheets and SPSS version 14.0. The expression of IL-7 mRNA relative to GAPDH mRNA was calculated and bar charts drawn. The differences between the control and the treatments in these experiments were tested for statistical significance by unpaired Student's T-test, one-way ANOVA and Dunnet's multiple comparisons were performed A probability level less than5%(value of $P<0.05$)was considered to indicate statistical significance. Values are expressed as mean ± standard deviation

3.0 Results

3.1 Extraction and compounds identification

The phytochemical components of the crude C.*cinereriafolium* extract were analyzed qualitatively by the common methods using chemicals. Flavonoids, glycosides, tannins, terpenoids, steroids, alkaloids and carbohydrates were found in the flower extract of this plant (Table 1).

Table 1: Phytochemical components in the flower of *Chrysanthemum cinerariaefolium*

S/NO.	Phytochemical component	Hexaneextractof *Chrysanthemum cinerariaefolium*	ethyl acetate extract
1	Flavanoids	+	+
2	alkaloids	+	+
3	Terpenoids	+	+
4	Tannins	+	+
5	Glucosides	+	+

GC-MS is the best techniques to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids, esters, etc. Peak area, retention time and molecular formula were used for the confirmation of phytochemical compounds. The active principles with their

Retention time (RT), Molecular formula, Molecular weight (MW) and peak area in percentage were recorded. GC-MS analysis of hexane extract of pyrethrum flowers revealed the existence of Hexadecanoic acid, methyl ester, Pentadecanoic acid, 14-methyl-methyl ester (36.23, Ethyl 9, 12, 15-octadecatrienoate (33.12 and4-(4-Chlorobenzoyl)-1-cyclohexyl-5tosylamino-1 H-1, 2,3-triazole among other phytochemical constituents. From the GC-MS analysis of pyrethrum flowers the presence of the phytochemical constituents revealed indicate the medicinal quality of the plant (Table 2, 3and 4)

3.2 GC-MS indicated the following Phytochemical constituents of C.*cinerariefolium* flower hexane extract

Lists of compounds

Table 2

1	cis-5,8,11,14,17-Eicosapentaenoic acid	74.6	34275	1.116	70367	
2	cis-5,8,11,14,17-Eicosapentaenoic acid	75.78	48669	1.167	78144	
3	2H-Pyran, 2-(7-dodecynyloxy)tetrahydro-	70.35	114293	1.304	195134	
4	Ethylbenzene	80.86	237069	1.774	265774	
5	Benzene, 1,3-dimethyl-	96.24	1058935	1.831	12266…	
6	p-Xylene	87.8	327724	2.02	345869	
7	2,5-Octadecadiynoic acid, methyl ester	75.05	15871	2.048	18188	
8	cis-5,8,11,14,17-Eicosapentaenoic acid	72.77	8545	2.5	13435	
9	cis-5,8,11,14,17-Eicosapentaenoic acid	72.41	35870	2.821	38841	
10	2,5-Octadecadiynoic acid, methyl ester	75.18	27571	3.324	26353	
11	2,5-Octadecadiynoic acid, methyl ester	74.29	44919	3.576	43147	
12	2,5-Octadecadiynoic acid, methyl ester	69.84	50041	4.074	38256	
13	Octadecane, 1-chloro-	67.29	107191	4.12	80714	
14	Methyl 5,7-hexadecadiynoate	72.6	41014	4.314	27857	
15	cis-5,8,11,14,17-Eicosapentaenoic acid	74.8	25337	4.44	33209	
16	2,5-Octadecadiynoic acid, methyl ester	75.27	19794	4.629	20636	
17	Limonen-6-ol, pivalate	69.41	90273	4.675	79473	
18	2,5-Octadecadiynoic acid, methyl ester	75.23	16387	4.846	14994	
19	Methyl 4,7,10,13-hexadecatetraenoate	76.11	56469	5.275	79118	
20	Limonen-6-ol, pivalate	67.97	77367	5.333	77555	
21	Methyl 4,7,10,13-hexadecatetraenoate	77.63	23816	5.842	51185	
22	Methyl 4,7,10,13-hexadecatetraenoate	72.75	29208	6.248	47627	
23	cis-5,8,11,14,17-Eicosapentaenoic acid	73.86	11584	6.42	22069	
24	Methyl 4,7,10,13-hexadecatetraenoate	77.14	12227	6.637	25310	
25	cis-5,8,11,14,17-Eicosapentaenoic acid	75.43	25826	7.101	61134	
26	cis-5,8,11,14,17-Eicosapentaenoic acid	77.96	50822	7.404	149889	
27	cis-5,8,11,14,17-Eicosapentaenoic acid	74.35	71678	7.49	136365	
28	Methyl 4,7,10,13-hexadecatetraenoate	77.48	15578	7.57	36255	
29	cis-5,8,11,14,17-Eicosapentaenoic acid	76.79	8970	8.022	16896	
30	cis-5,8,11,14,17-Eicosapentaenoic acid	76.27	9250	8.114	14887	
31	cis-5,8,11,14,17-Eicosapentaenoic acid	76.83	16892	8.171	30411	
32	cis-5,8,11,14,17-Eicosapentaenoic acid	74.61	17096	8.4	28215	
33	tert-Hexadecanethiol	71.98	48148	8.508	56326	
34	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	75	33645	8.566	49709	
35	cis-5,8,11,14,17-Eicosapentaenoic acid	75.9	9308	8.617	14079	
36	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	72.63	61359	8.737	113479	
37	3-Dodecene, 1-(benzyloxy)-4-methyl	71.15	17999	8.8	19411	
38	cis-5,8,11,14,17-Eicosapentaenoic acid	76.43	16330	9.115	17982	
39	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	75.25	33220	9.235	45252	
40	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	76.51	72662	9.287	111311	
41	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	74.28	95442	9.332	129476	
42	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	75.22	35508	9.407	72183	
43	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	75.99	15375	9.544	18804	
44	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	77.94	11300	9.721	54360	
45	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	76.78	22061	10.151	28277	
46	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	78.36	13050	10.408	22832	
47	Estra-1,3,5(10)-trien-17.beta.-ol	75.3	74888	10.74	163429	
48	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	77.43	29562	10.814	76402	
49	Hexadecanoic acid, ethyl ester	76.88	95349	11.116	227424	
50	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1…	74.55	35960	11.172	77199	
51		75.64	13685		21218	

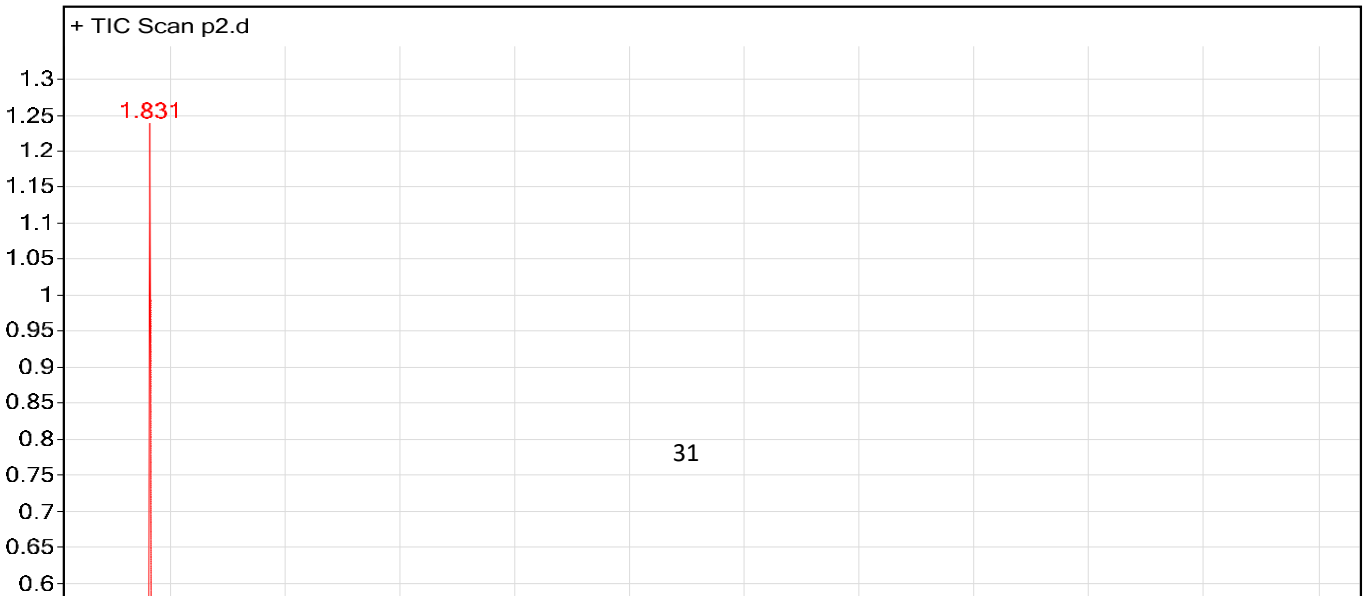
Table 3

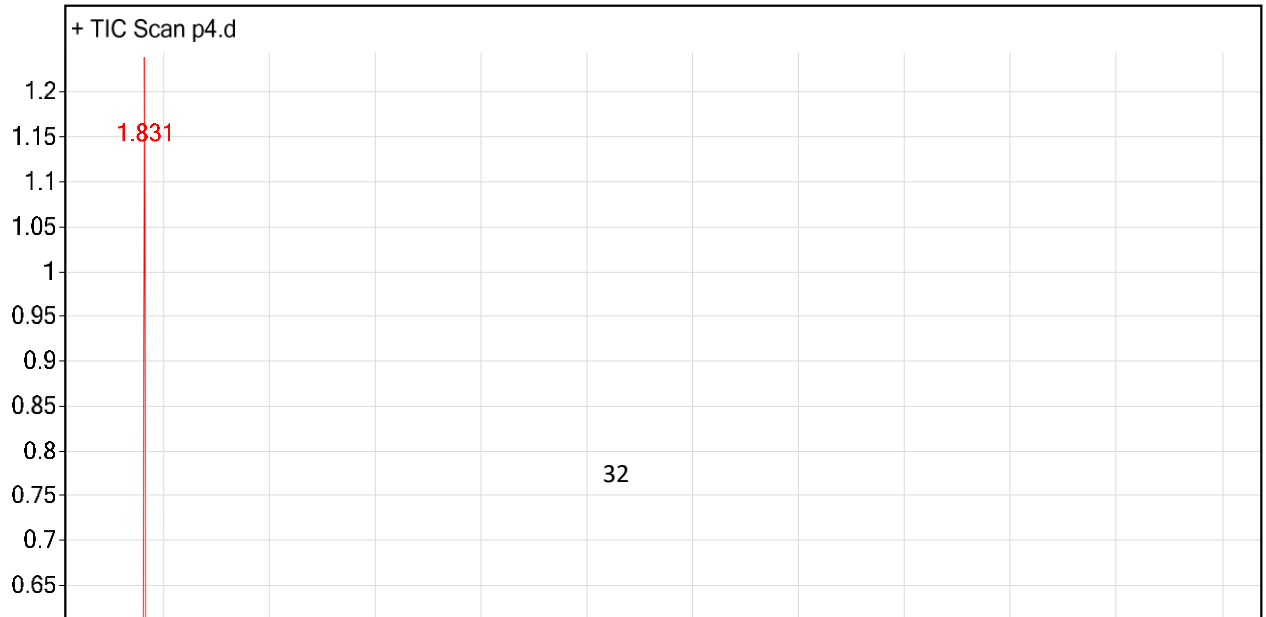
1	cis-5,8,11,14,17-Eicosapentaenoic acid	76.91	31963	1.121	71212	10	NIST11.L
2	cis-5,8,11,14,17-Eicosapentaenoic acid	76.84	45417	1.161	90438	10	NIST14.L
3	2H-Pyran, 2-(7-dodecynyloxy)tetrahydro-	71.43	123115	1.316	249382	10	NIST14.L
4	10-Chlorotricyclo[4.2.2.0(1,5)]dec-7-ene	74.79	161449	1.774	214737	10	NIST14.L
5	p-Xylene	93.81	759354	1.836	978172	10	NIST14.L
6	p-Xylene	82.69	241539	2.02	303584	10	NIST14.L
7	Doconexent	74.1	16600	2.792	13752	10	NIST14.L
8	cis-5,8,11,14,17-Eicosapentaenoic acid	73.38	28368	2.821	27418	10	NIST14.L
9	cis-5,8,11,14,17-Eicosapentaenoic acid	74.25	17333	2.935	12568	10	NIST14.L
10	cis-5,8,11,14,17-Eicosapentaenoic acid	73.82	15275	3.513	24059	10	NIST14.L
11	cis-5,8,11,14,17-Eicosapentaenoic acid	73.33	37240	4.074	30368	10	NIST14.L
12	cis-5,8,11,14,17-Eicosapentaenoic acid	71.86	24657	4.12	18532	10	NIST14.L
13	cis-5,8,11,14,17-Eicosapentaenoic acid	75.85	78521	4.451	135806	10	NIST14.L
14	2,5-Octadecadiynoic acid, methyl ester	75.1	24259	4.629	26580	10	NIST14.L
15	cis-5,8,11,14,17-Eicosapentaenoic acid	75.44	22171	4.675	38527	10	NIST14.L
16	cis-5,8,11,14,17-Eicosapentaenoic acid	74	10194	4.852	9846	10	NIST14.L
17	cis-5,8,11,14,17-Eicosapentaenoic acid	74.2	14887	5.069	15121	10	NIST14.L
18	cis-5,8,11,14,17-Eicosapentaenoic acid	73.88	40073	5.275	45091	10	NIST14.L
19	cis-5,8,11,14,17-Eicosapentaenoic acid	74.23	45323	5.333	53896	10	NIST14.L
20	cis-5,8,11,14,17-Eicosapentaenoic acid	74.12	16590	6.242	26576	10	NIST14.L
21	cis-5,8,11,14,17-Eicosapentaenoic acid	74.85	10395	6.408	21395	10	NIST14.L
22	2,5-Octadecadiynoic acid, methyl ester	76.8	35128	7.404	90204	10	NIST14.L
23	cis-5,8,11,14,17-Eicosapentaenoic acid	74.22	32445	7.49	55295	10	NIST14.L

Table 4

1	cis-5,8,11,14,17-Eicosapentaenoic acid	75.91	31963	1.12	71212	10	NIST11.L
2	cis-5,8,11,14,17-Eicosapentaenoic acid	76.84	45417	1.161	90438	10	NIST14.L
3	2H-Pyran, 2-(7-dodecynyloxy)tetrahydro-	71.43	123115	1.316	249382	10	NIST14.L
4	10-Chlorotricyclo[4.2.2.0(1,5)]dec-7-ene	74.79	161449	1.774	214737	10	NIST14.L
5	p-Xylene	93.81	759354	1.836	978172	10	NIST14.L
6	p-Xylene	82.69	241539	2.02	303584	10	NIST14.L
7	Doconexent	74.1	16600	2.792	13752	10	NIST14.L
8	cis-5,8,11,14,17-Eicosapentaenoic acid	73.38	28368	2.821	27418	10	NIST14.L
9	cis-5,8,11,14,17-Eicosapentaenoic acid	74.25	17333	2.935	12568	10	NIST14.L
10	cis-5,8,11,14,17-Eicosapentaenoic acid	73.82	15275	3.513	24059	10	NIST14.L
11	cis-5,8,11,14,17-Eicosapentaenoic acid	73.33	37240	4.074	30368	10	NIST14.L
12	cis-5,8,11,14,17-Eicosapentaenoic acid	71.86	24657	4.12	18532	10	NIST14.L
13	cis-5,8,11,14,17-Eicosapentaenoic acid	75.85	78521	4.451	135806	10	NIST14.L
14	2,5-Octadecadiynoic acid, methyl ester	75.1	24259	4.629	26580	10	NIST14.L
15	cis-5,8,11,14,17-Eicosapentaenoic acid	75.44	22171	4.675	38527	10	NIST14.L
16	cis-5,8,11,14,17-Eicosapentaenoic acid	74	10194	4.852	9846	10	NIST14.L
17	cis-5,8,11,14,17-Eicosapentaenoic acid	74.2	14887	5.069	15121	10	NIST14.L
18	cis-5,8,11,14,17-Eicosapentaenoic acid	73.88	40073	5.275	45091	10	NIST14.L
19	cis-5,8,11,14,17-Eicosapentaenoic acid	74.23	45323	5.333	53896	10	NIST14.L
20	cis-5,8,11,14,17-Eicosapentaenoic acid	74.12	16590	6.242	26576	10	NIST14.L
21	cis-5,8,11,14,17-Eicosapentaenoic acid	74.85	10395	6.408	21395	10	NIST14.L
22	2,5-Octadecadiynoic acid, methyl ester	76.8	35128	7.404	90204	10	NIST14.L
23	cis-5,8,11,14,17-Eicosapentaenoic acid	74.22	32445	7.49	55295	10	NIST14.L
24	2,5-Octadecadiynoic acid, methyl ester	76.69	8433	7.907	20822	10	NIST14.L
25	cis-5,8,11,14,17-Eicosapentaenoic acid	77.9	16087	8.474	16127	10	NIST14.L
26	cis-5,8,11,14,17-Eicosapentaenoic acid	77.08	19401	8.508	33762	10	NIST14.L
27	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	74.06	54377	8.731	114450	10	NIST14.L
28	cis-5,8,11,14,17-Eicosapentaenoic acid	74.99	47805	9.286	66893	10	NIST14.L
29	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	74.77	41008	9.338	55777	10	NIST14.L
30	cis-5,8,11,14,17-Eicosapentaenoic acid	75.47	12081	9.401	26939	10	NIST14.L
31	cis-5,8,11,14,17-Eicosapentaenoic acid	75.27	11953	9.544	12879	10	NIST14.L
32	cis-5,8,11,14,17-Eicosapentaenoic acid	76.45	12479	9.716	17365	10	NIST14.L
33	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	78.31	18568	9.973	45183	10	NIST14.L
34	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	76.14	13953	10.15	14578	10	NIST14.L

3.3 Chromatograms of the coresponding hexane extract of *C.cinerariefolium*.

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1.831
32
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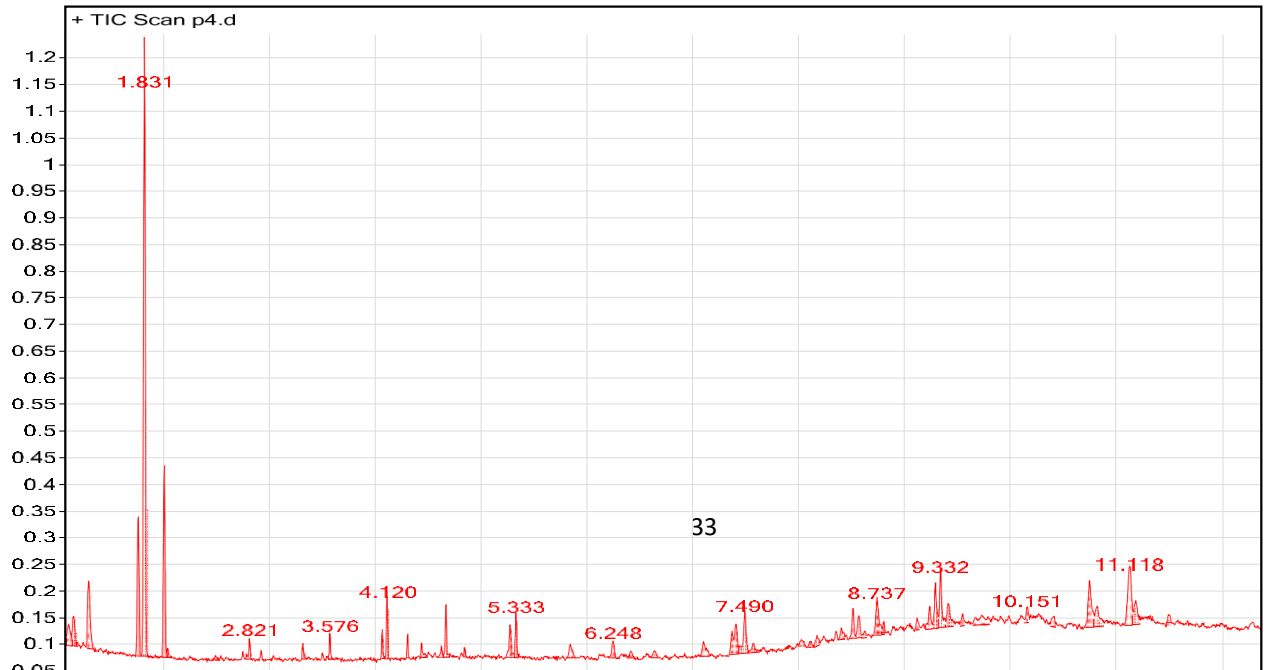
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65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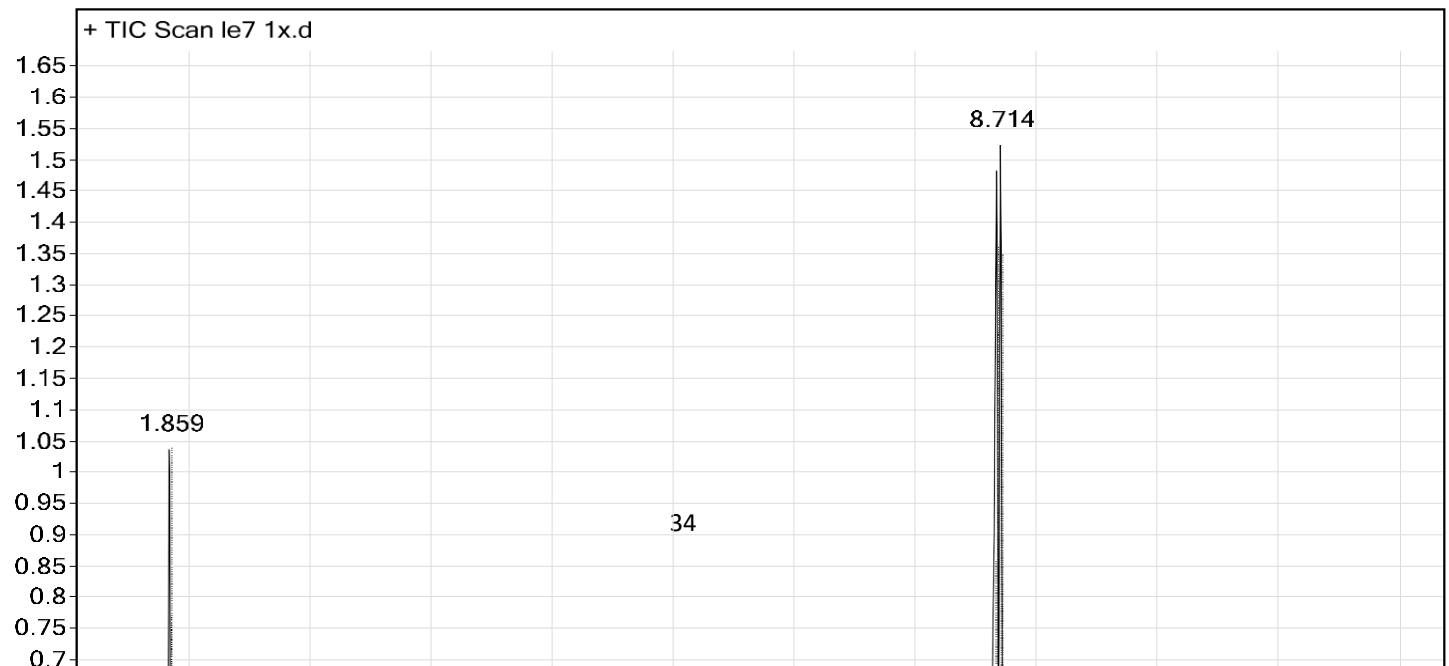
9.332
10.151
11.118
33


+ TIC Scan le7 1x.d
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1.6
1.55
1.5
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
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0.9
0.85
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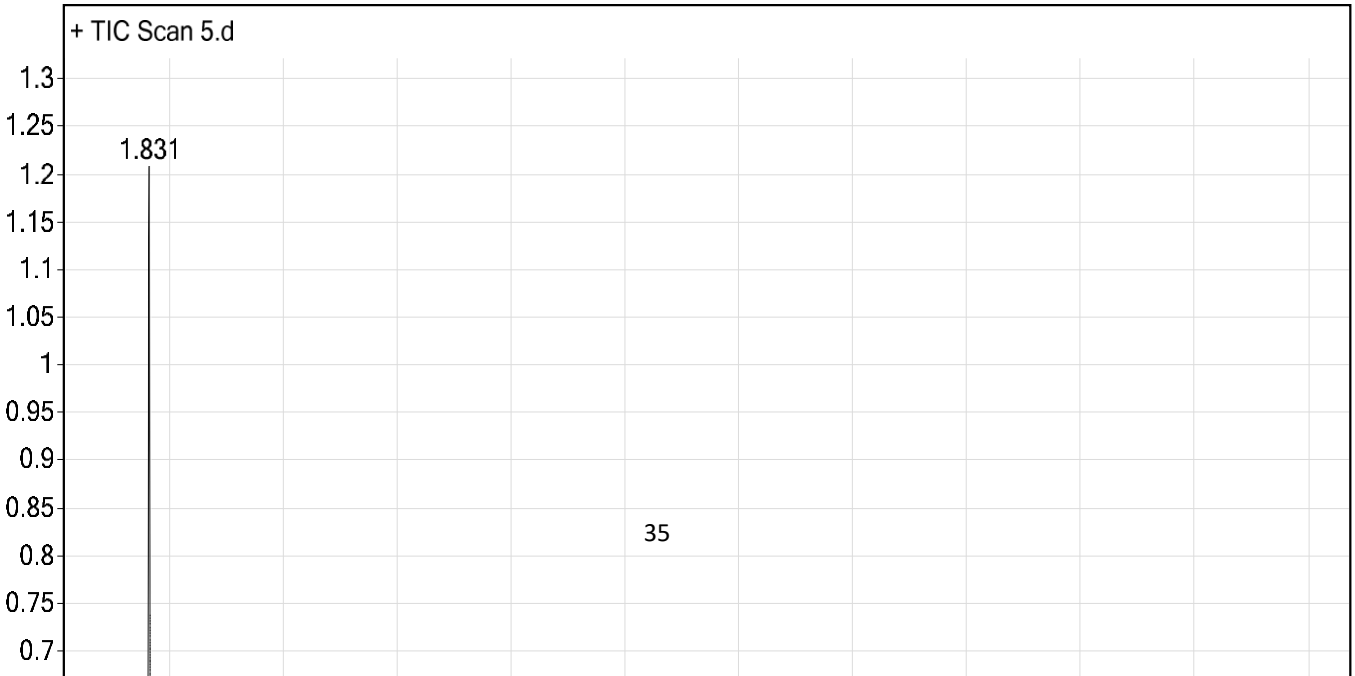
0.75
0.7
1.859
8.714
34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1.3
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1.2
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1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
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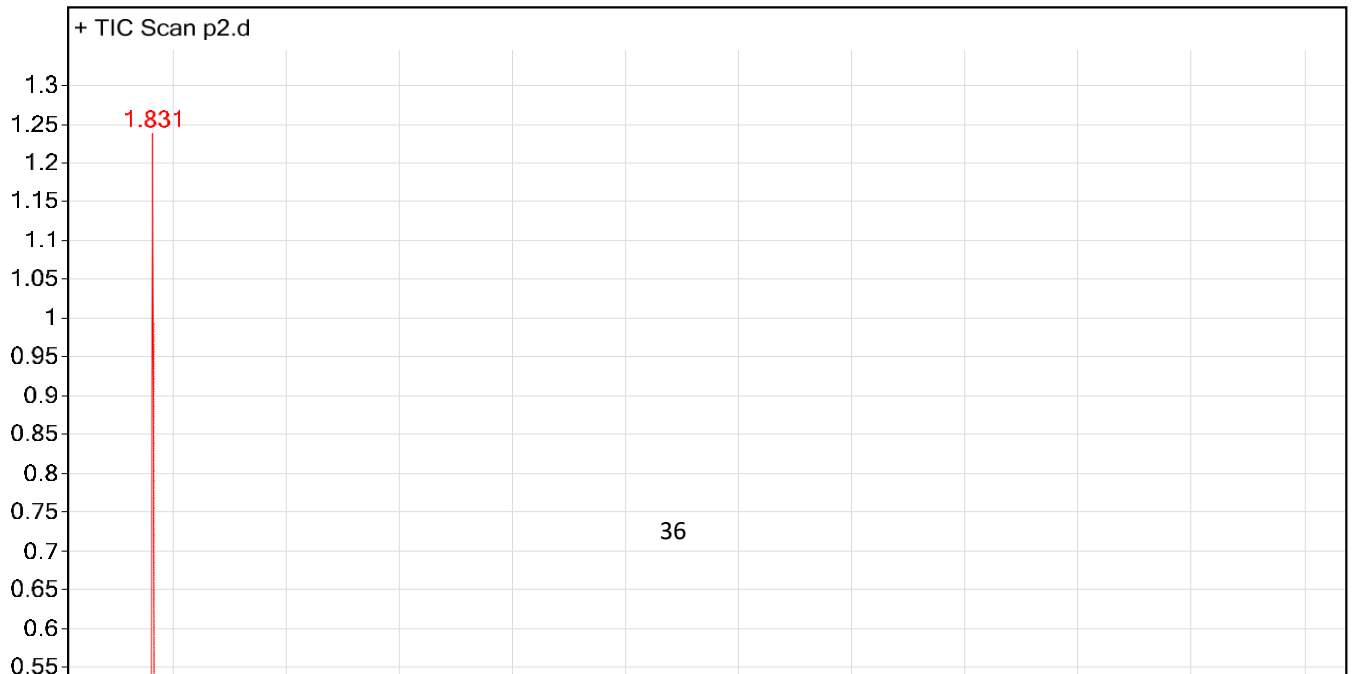
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1.25
1.2
1.15
1.1
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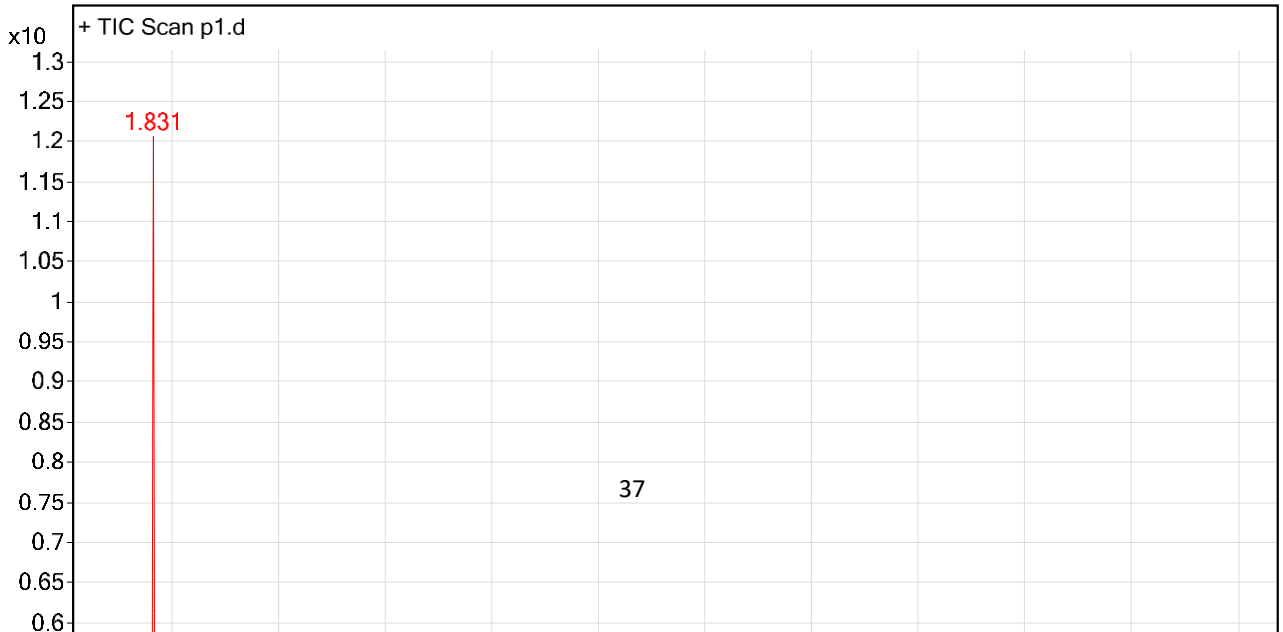
0.85
0.8
0.75
0.7
0.65
0.6
0.55
36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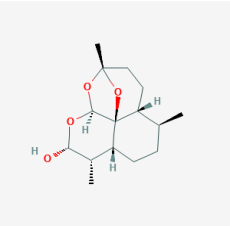
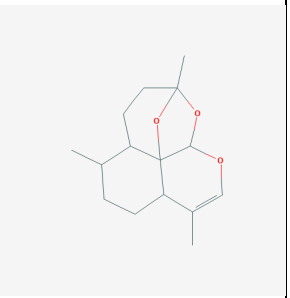
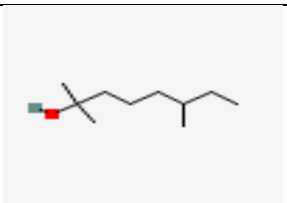
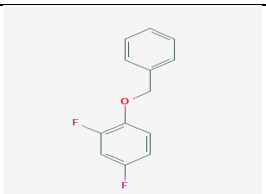
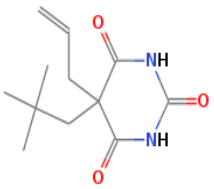
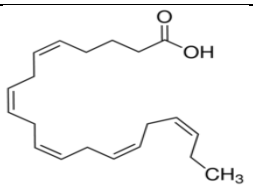
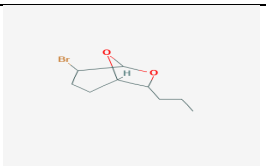
x10
1.3
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
1.831
37

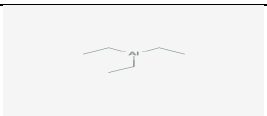
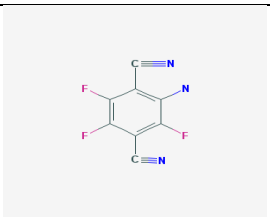
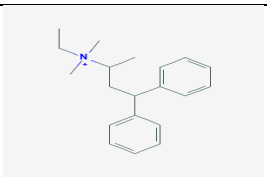
3.4 Biological properties of the identified constituents from GC -MS analysis

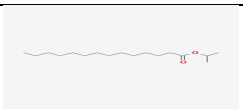
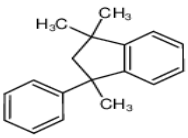
Table 1

S/NO	RT	Peak area %	Name of the compound	Molecular formula	Molecular waight (g/mol)	Chemical structure	Biological properties

	9.412	10980	Dihydroartemisinin,3-desoxy	$C_{17}H_{24}O_4$	268.353		• Antimalarial resistant, me falciparum W2
	171		1,5,9-Trimethyl-11,14,15-trioxatetracyclo[10.2.1.04,13.08,13]pentadec-9-ene	$C_{15}H_{22}O_3$	250.33 g/mol		•
2	1.11	18101	2-0ctanol, 2-6 dimethul	$C_{10}H_{22}O$	158.285		• Drug for genita • Drug for derma • Anti-acne agen • Ophthalmic ag • Antiinfective(a • Used as fragra

2		58.543	2,4Difluorobenzene,1 benzyloxy	$C_{13}H_{10}F_2O$	220.219		• Drug for derma • Drug for disord • Non central inflammatory a steroidal anti-i • Antineoplastic • Immunomodul
3	1.247	-	Barbituric acid, 5-allyl-5-neopentyl	$C_{12}H_{18}N_2O_3$	238.2829		• Drug for disord • Centrally actin • Antimigraine a
	1.121	77876	cis-5,8,11,14,17-Eicosapentaenoic acid	$CH_3(CH_2CH=CH)_5(CH_2)_3CO_2H$	302.45		• Essential for he • It's a li thromboxane A • Has potential cardiovascular • Decreases the very low densi
	1.247	11127	4-bromo-7-propyl-6,8-dioxabicyclo[3.2.1]octane	$C_9H_{15}BrO_2$	235.121		• Preservation o parts • Biocide,pests r

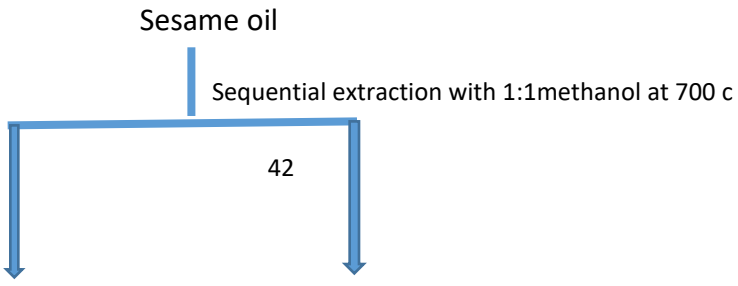
							• Used as herbic
	1.304	205663	Triethylaluminum	C6H15AL	114.168		• General fuels, fuel • Catalyst inter olefins,expecia production of alcohols; gas p • Intermediates i
	8.354	19691	2-Amino-3,5,6-trifluoroterephthalonitrile	$C_8H_2F_3N_3$	197.12		• Pesticic chemic
			1-Fluorododecane	$C_{12}H_{25}F$	188.33		• Preparations fo • Preparations f cavity or of toothpastes; M • Anti-perspirant preparations,w
			dasycarpidan-8(16h)-ethanol,318-didehydro-1-(hydroxymethyl)-,(2.,xi.,4.xi)-	$C_{20}H_{28}N+$	282.451		• A muscarinic treatment of uri absorbed from not cross the blood-brain barrier

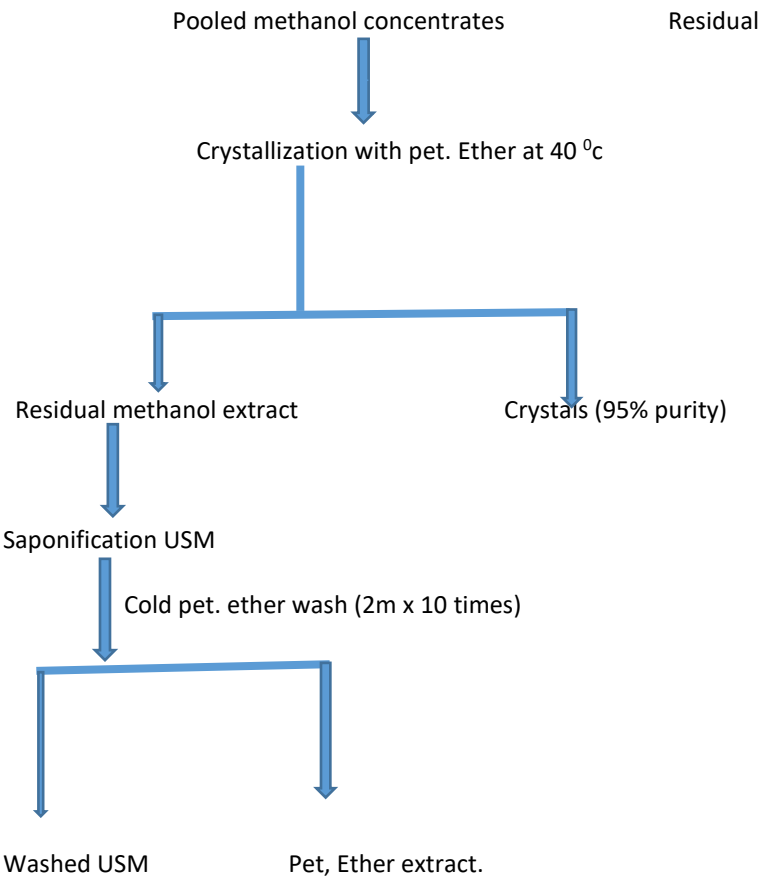
							Drugs used in the treat diseases such as PROSTATIC HYPE DYSFUNCTION.
			Isopropyl myristate	$C_{17}H_{34}O_2$	270.457		• effective for th • Flavouring A Additives: CA
	8.737	70246	**1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-**	$C_{18}H_{20}$	236.3514		• General adhes variety of uses • Fillers for pai products to pro •

3.5 Extraction of Saseme lignans

Sesame oil had a residual lignan content of 0.2 ± 0.02%, indicating that the optimised conditions favoured maximum extraction of lignans. Saponification of the crystal-removed methanolic concentrate (9.4 ± 0.19 g) for different time durations revealed that 1 h of saponification was sufficient to complete the reaction. Washing of the USM (6.04 ± 0.5 g) with chilled petroleum ether was found to increase the lignan content in the USM due to the removal of pigments from the USM. By this purification step, the total lignan content in USM was increased from 58% to 64%, along with a 9% loss in USM weight (2.5 ± 0.02 g).

Flow diagram for recovery of lignanas from the Sesame oil

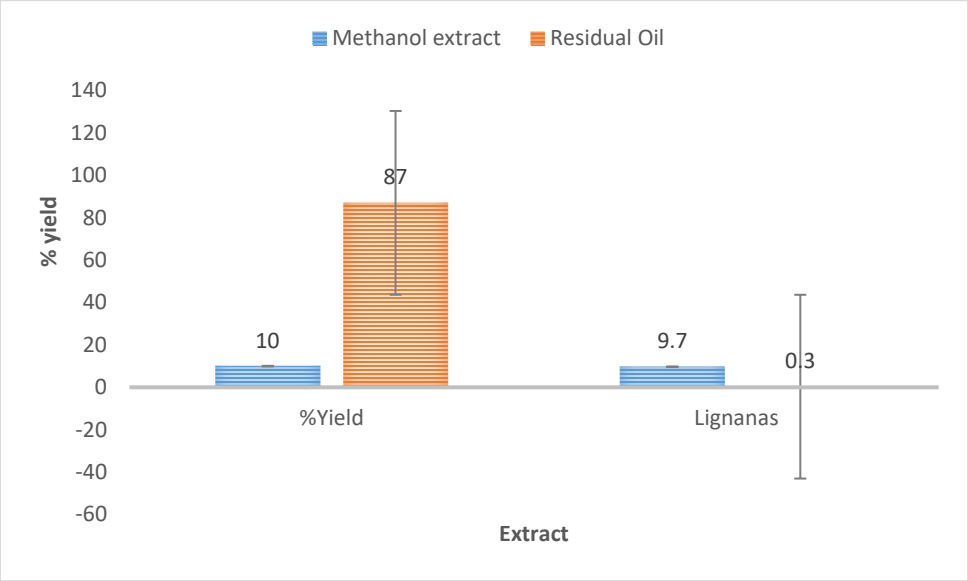




Lignan content of Sesame oil, Methanol extract and the residual 0il

OIL/EXTRACT	% YIELD	LIGNANAS
Sesame oil	100	1.27 ±0.04
Methanol extract	10.03 ± 0.12	9.9±0.19
Residual oil	87.07 ±0.02	0.3 ±0.02

Total lignans content from methanol extract and residual oil



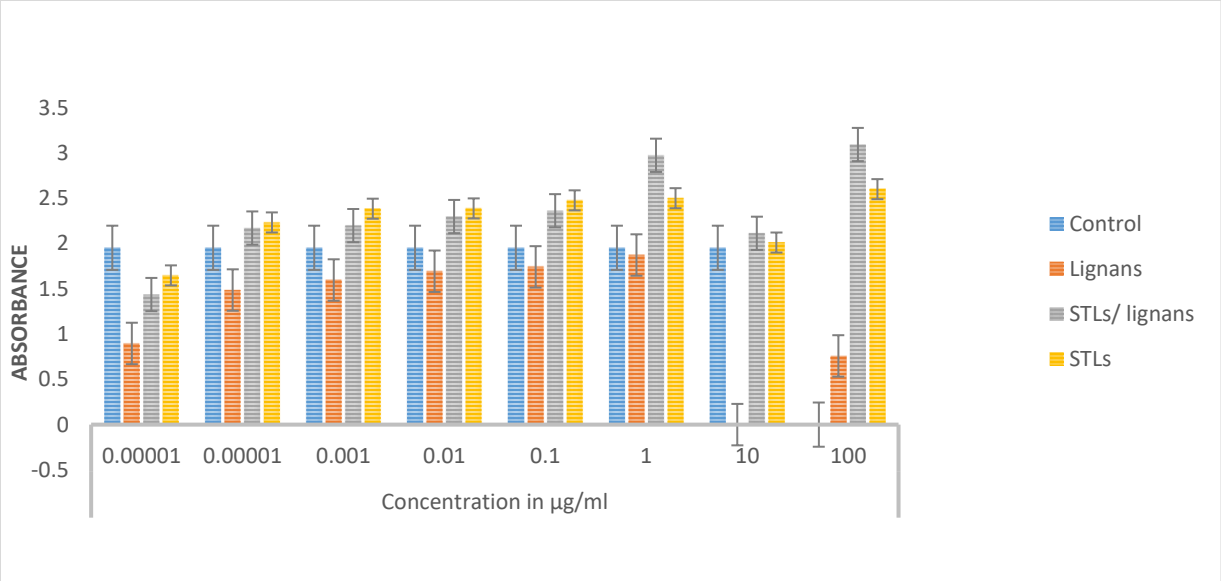
Cell Proliferation assay

A calibration chart was drawn to ascertain reliability and help enable cell number determination from absorbance values. The cell numbers were observed to have a linear relationship with the absorbance values. A high number of cells gave a corresponding high absorbance. A concentration of 1µg/ml gave the best proliferative effect on IEC-6 cells for the all tested extracts (Table1). Pyrethrum and sesame extracts investigated had no visible cytotoxic effects and promoted cell growth normally even at concentrations above 100µg/ml in 6 well plates. The ED_{50} was determined to be approximately 100 µg/ml.

Table 1. IEC-6 cell proliferation assay of pyrethrum and sesame extracts evaluated at different concentrations using the CCK-8 assay protocol.

Treatment	Concentration in µg/ml							
	0.00001	0.00001	0.001	0.01	0.1	1	10	100
Control	1.954	1.954	1.954	1.954	1.954	1.954	1.954	1.954±
Lignans	0.897	1.487	1.598	1.695	1.743	1.874	1'682	0.759
STLs/ lignans	1.437	2.172	2.199	2.299	2.363	2.976	2.114	3.095

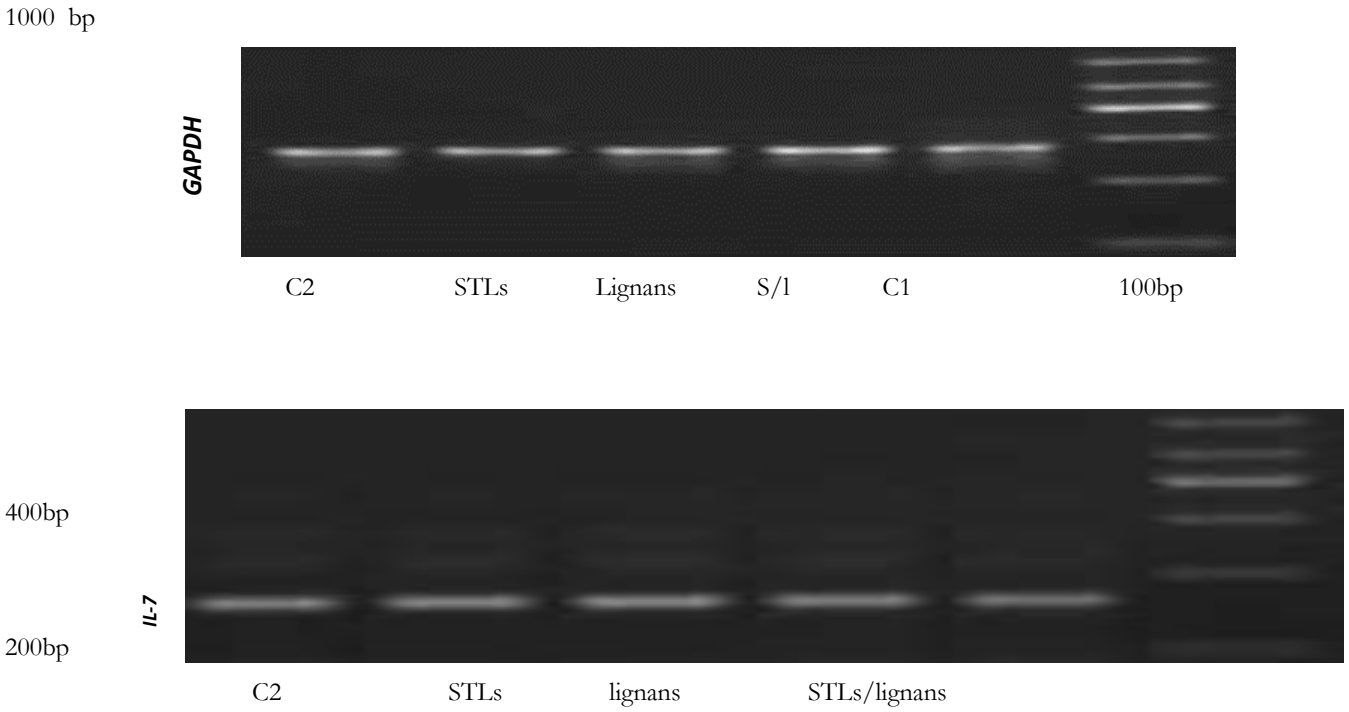
STLs	1.649	2.234	2.386	2.389	2.478	2.502	2.012	2.602



Mouse IEC-6 cells subjected to STLs and lignans extracts tested for their effect on the expression of GAPDH and IL-7 mRNA at 3.33mg/ml exposed for 72 hours

Pyrethrum STLs and lignans Extracts were selected as best in up regulating IL-7.) Compared to the control and the STLs combined with lignans

Figure 1 Expression of GAPDH and IL-7 by Mouse IEC-6 cells subjected to various extracts at a concentration of 100µg/ml

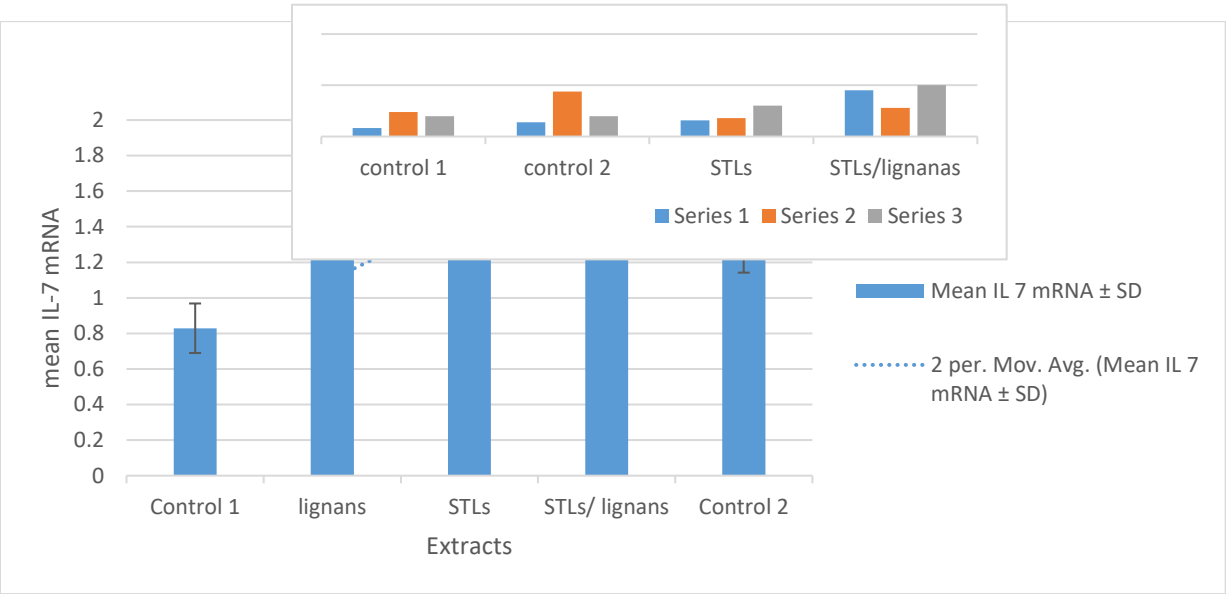


IEC-6 cells were shown to express IL-7 Cytokine in a constant manner. This cytokine is associated with immunomodulation and cancer cells Immunosurveillance. IEC- 6 cells subjected to STLs, Sesame lignans and combination of the two extracts at a ratio of 1:1 mRNA expression effects

Table 2. Mean relative IL-7 mRNA expression.

	Mean IL 7 mRNA ± SD
Control 1	0..829
lignans	1.381
STLs	1.582
STLs/ lignans	1.592

Control 2	1.281



Invivo assay

Table 3: Effect of treatment on the tumor weight of Balb/C mice infected with Colon cancer CT-26 tumor cells

Group	Tumor index ± SD

Normal	0.0000
Negative	0.422
Positive	0.301
Test	0.989

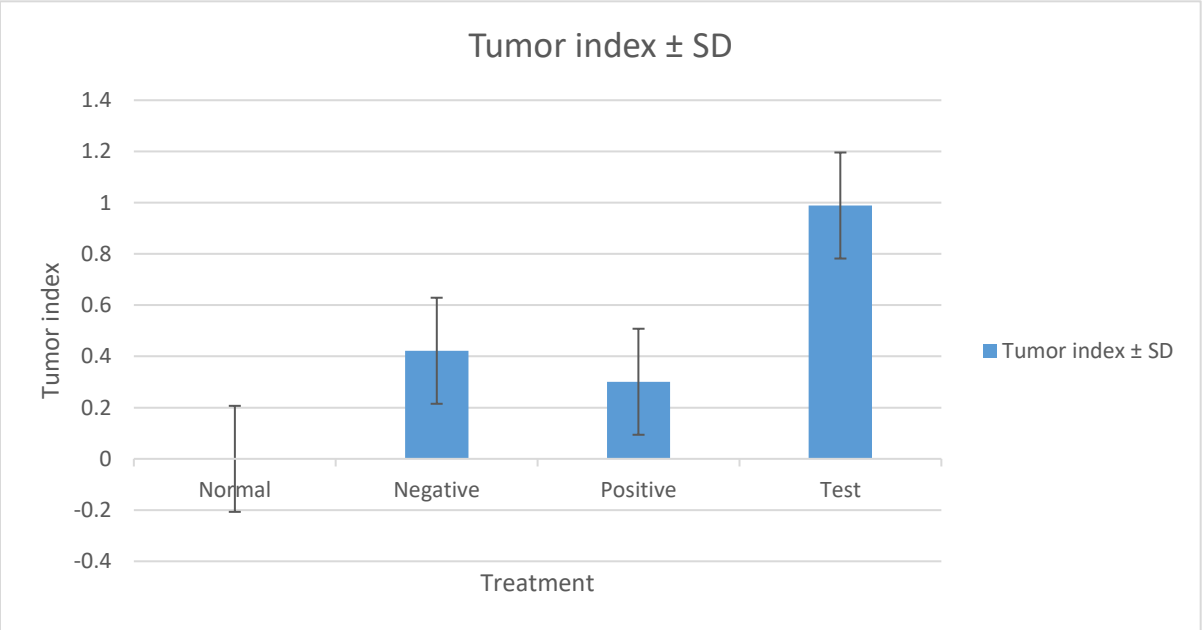
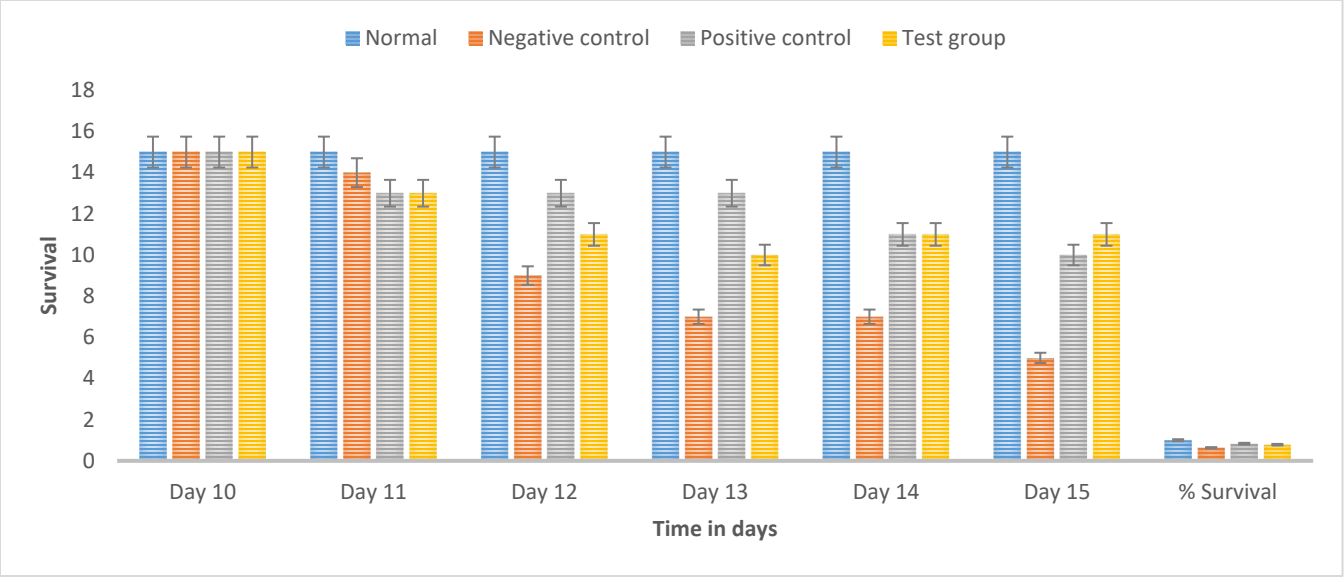


Figure 3: The mean tumor index in the normal group and three experimental groups of Balb/C mice infected with Colon Cancer tumor cells. Test group has a significant difference compared to the untreated negative control group.

Bulb /C mice infected with CT- 26 tumour cells. Tumour resulting from the infection

Table 4. Mortality rate of mice infected with colon cancer tumor cell

Treatment	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	% Survival
Normal	15	15	15	15	15	15	100%
Negative control	15	14	9	7	7	5	63.33%
Positive control	15	13	13	13	11	10	83.33%
Test group	15	13	11	10	11	11	78.33%



The % survival rate on day 15 in the infected mice treated with either Cyclophosphamide or STLs and sesame lignan extracts was higher at 78.33% compared to 63.33% for the negative control

Figure 5: The % survival at the end of *in vivo* assay for the Normal healthy group and three infected groups. The mice that received no treatment

Table 6: Effect of treatment on the weight of immunity organs in Balb/C mice infected with CT-26 Colon cancer tumor cells

Index	Normal healthy mice	Negative control	Positive control	Test group
The spleen index	4.159	7.102	7.32	10.430
The thymus index	1.979	0.681	0.593	0.413

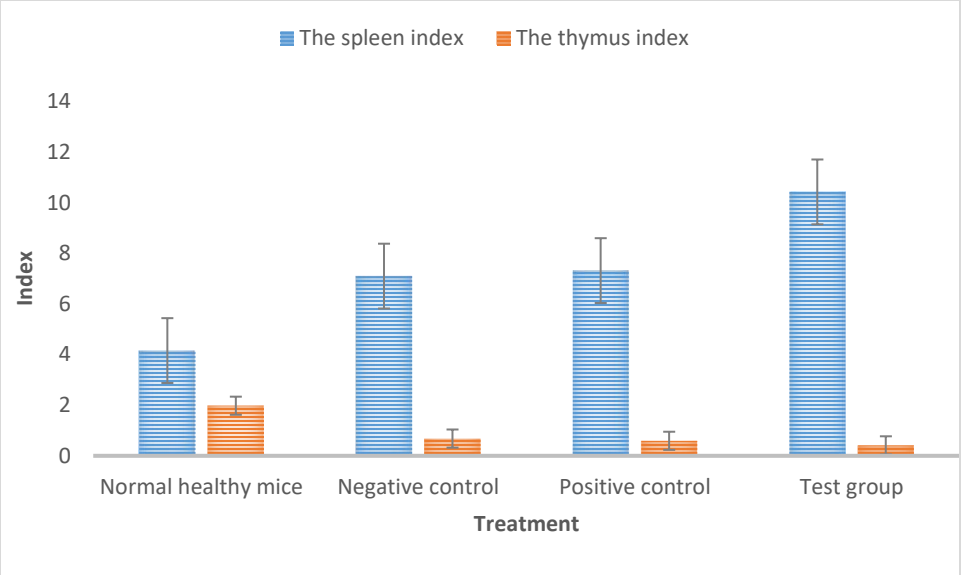


Figure 7**: The mean thymus index in the normal group and three experimental groups of Balb/C mice infected with Colon Cancer tumor cells. No significant difference noted in mean thymus index among the infected groups but all infected groups indexes are significantly different from the healthy group (p≤0.05).**

Mortality rate of mice infected with colon cancer tumor cell

Treatment	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	% Survival
Normal	15	15	15	15	15	15	100%
Negative control	15	14	9	7	7	5	63.33%
Positive control	15	13	13	13	11	10	83.33%
Test group	15	13	11	10	11	11	78.33%

Mean Weight Indexes of Mice Tumor and Immune Organs

There was a slight decrease in weight for all groups at the commencent of the treatment but the tread reversed and started to increase gradually over the treatment period. The mean tumor weight indices in the extracts fed test group infected with Ct-26 colon cancer cells did show a significant difference at p≤0.05. Conversely the mortality in the negative control (no treatment) group was higher and only about half of the mice 53.3% survived while in the positive control and test groups, the survival rate was over 73% . The mean weights indices of immunity organs, the Thymus and the Spleen were significantly different from those of the normal uninfected mice. The Thymus weight indexes were significant in a negative way by having lower values and the Spleen weight indexes were significant (p≤0.05) in a positive way by having higher values. A larger spleen size may suggest a heightened

baseline activation state or pre-expansion of a responding cell population

Flow -Cytometry- Immunofluorescence staining of mice Cells by Lysed Whole Blood Method.

Flow Cytometry analysis of whole mouse peripheral blood was done to determine the effect of different treatments on the lymphocytes associated with immunomodulation and cancer cell Immunosurveillance. Total $CD3^+$, $CD4^+$ and $CD8^+$ cell numbers were assessed. The $CD4^+$ and $CD8^+$ cells are represented as a % of the total $CD3^+$ cells. The $CD4^+$ and $CD8^+$ cells in the mice which received the STLs and lignans extracts were found to be significantly different ($p \leq 0.05$) from the untreated control mice (Table 6, Figures 8, 9, 10).

Table 7. Effect of treatment on the Lymphopoiesis in Balb/C mice infected with colon cancer tumor.

Treatment	CD4+ compared to CD3 cells as a %	CD8+ compared to CD3 cells as a %	Both CD4+ and CD8+ compared to CD3 cells as a %
Normal	79.424	21.369	0.972
Negative control	73.728	23.100	0.983
Positive Control	78.566	21.654	0.988
Test group	83.502	17.604	2.403

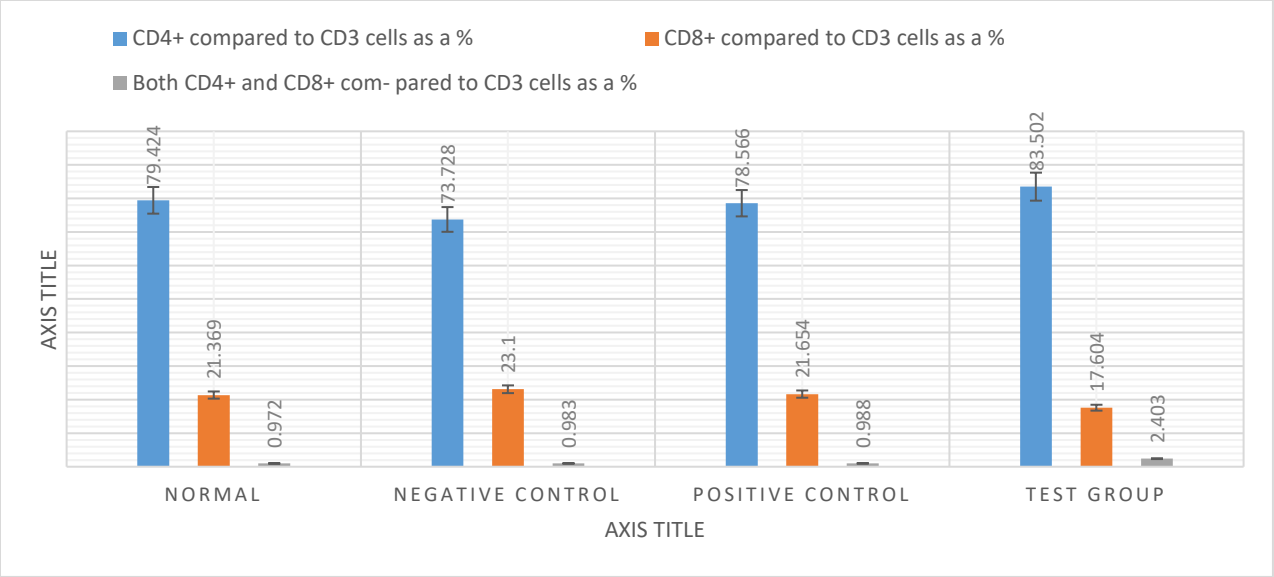


Figure 8: The mean number of $CD4^+$ cells as a % of the total $CD3^+$ positive cells in the three experimental groups

The mean number of CD4+ cells is significantly higher in the STLs Test group than the untreated negative control group ($P \leq 0.05$). The mean number of $CD8^+$ cells as a % of the total $CD3^+$ cells in the three experimental groups. The mean number of $CD8^+$ cells is significantly lower in the lignansTest group compared to the Negative control group ($P \leq 0.05$).

The mean number of both $CD4^+$ and $CD8^+$ cells are significantly higher in the S/L test group compared to the untreated negative control group ($P \leq 0.05$).

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4.0 Discussion

Several anticancer activity studies have focused on the ability of single pure compounds; however, there have been reports that the total contents of a whole herb show a significantly better effect than a single isolated active ingredient (i.e., synergistic action); that is, the potential anticancer effects of herbal combinations are more effective than using any single constituent alone (Anand, et al, 2002; Ma XH, et al, 2009).

Synergistic combination of two or more agents can overcome the toxicities or other side effects of a single pure compound (Ma XH et al, 2009). For instance, the glycyrrhizin and saponin fractions of ginsenosides were individually reported to be ineffective but collectively useful for reducing ulcerative colitis in male Wistar/ST rats caused by 2,4,6-trinitrobenzene sulfonic acid, (Kawashima et al,2007).

Sesquiterpene lactones are of considerable interest due to their potent bioactivities, including cancer cell cytotoxicity and antineoplastic efficacy in in vivo studies. Among these compounds, artesunate, dimethylaminoparthenolide, and L12ADT peptide prodrug, a derivative of thapsigargin, are being evaluated in the current cancer clinical or preclinical trials. Based on the structures of several antitumor sesquiterpene lactones, a number of analogues showing greater potency have been either isolated as natural products or partially synthesized, and some potential anticancer agents that have emerged from this group of lead compounds have been investigated extensively. There are various scientific reports indicating that STLs are pharmacologically active molecules and have been shown to have various potentials like antitumor, anti-inflammatory, antibacterial, antifungal, antiviral, antiprotozoal, antihelminthic, antiulcer, molluscicidal, hepatoprotective, hepatocurative, and antidepressant effects. (Chaturvedi et al, 2011).

World Health Organization (WHO), as recommended the first-line therapy against malaria caused by Plasmodium falciparum which is a combination therapy of a STL (artemisinin) and its derivatives with other antimalarials, such as mefloquine and amodiaquine, (Aquino et al, 2011). Semisynthetic derivatives with improved pharmacokinetic profiles include the active principle dihydroartemisinin, artemether, artelinic acid and artesunate, Aquino et al, (2011).

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The cytotoxic and apoptotic effects of STLs have been investigated in vitro against several cancer cell lines (Cotugno et al, 2012: Choi et al,2009). In result of all the chemical and pharmacological research on STLs, substances such as parthenolide and its synthetic analog, dimethylaminoparthenolide (DMAPT), thapsigargin, the artemisinin derivatives artemether and artesunate are presently in cancer clinical trials(Foster et al, 2011). Similarly thapsigargin pro-drug (G-202, thapsigargin coupled with a masking peptide which is cleaved at the tumor site, releasing

47

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IEC-6 cells exposed to C.cinereriaefolium and sesame lignans extracts at two concentrations of 100µg/ml and 3.33mg/ml positively ex-pressed IL-7 cytokine. Intestinal epithelial cells (IEC) have been implicated in IL-7 synthesis before (Madrigal- Estebas et al, 1997). IL-7 has been associated with potential for adoptive immunotherapy (Itisam Sarangi et al,2006). IL-7 cytokine produced can be associated with the immunomodulation medicinal value present in C.cinereriaeflolium and sesame lignans. Chrysanthemun cinereriaefolium have been previously reported as having protective mechanism against cancer is their capacity to stimulate the immune system response where IL-7 is presumed to activate certain immune cells and proteins that attack cancer, in-cluding macrophages, T-cells, natural killer cells, and other interleukins. The cells exposed to 6 hours and 3 hours express IL-7 mRNA in a time dependent way. The expression at 3 hours was not signi cantly different from the control but a significant difference was noticed on exposure to 6 hours. At 100µg/ml the expression of IL-7 is better than at 3.33mg/ml. The difference might be explained away as a result of shock due to sudden exposure to a high dose despite lack of noticeable toxicity therefore possibly needs more time as shown by results due to exposure for 72hours. Cells might thus require a longer adjustment time to the new micro-environment. In the lower dose cells continue with normal metabolic processes catalyzed by the extracts hence the better expression at the lower dose. The lower dose would possibly be more acceptable in drug dosage design.

The thymus weight indexes in the tumor cells infected test groups of mice are signicantly lower than in the untreated healthy mice. This is possibly indicative of the role played by thymus gland in immune responses to tumor cells. The production of lymphocytes in great numbers possibly

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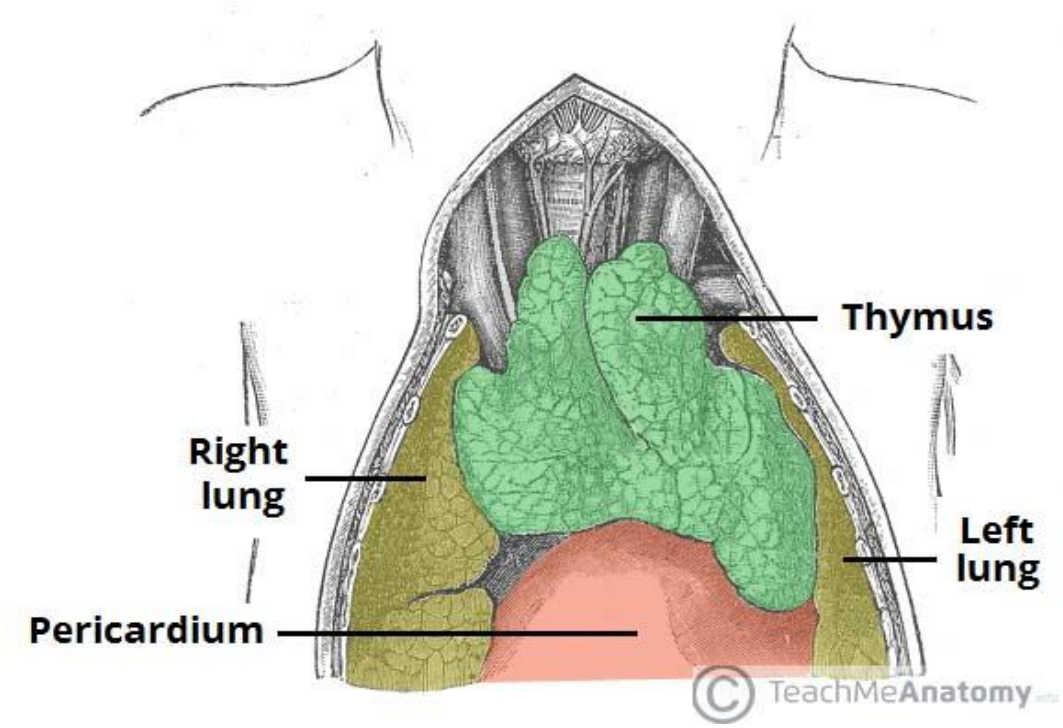


The function of thymus gland in immune responses is demonstrated in this research. The converse is true for the spleen weight indices. The spleen indices are significantly higher in mice infected with tumor cells and more so higher in mice treated with STLs and lignans.

This may be indicative of formation of more splenocytes as a response to the positive challenge by the tumor cells. This too may be indicative of better treatment outcome in mice subjected to

the STLs and lignans. This may be a further demonstration of the immunological response including expansion of the lymphocytes pool initiated in the spleen due to the presence of the foreign cancer cells. T-cells stored in the spleen are activated and mobilized to perform their immune functions.

The thymus gland is very active from before birth until puberty, and it functions as both a lymphatic organ and an endocrine organ (an organ of the endocrine system that produces hormones). In order to understand the role the thymus gland plays in immunity, it's helpful to first distinguish between T lymphocytes and B lymphocytes.T cells (also known as Tlymphocytes or thymus derived lymphocytes) mature in the thymus gland and play a central role in cell-mediated immunity, meaning that the cells themselves are active in fighting off foreign invaders such as bacteria, viruses, cancer cells, and more.In contrast, B lymphocytes are part of the humoral immune system and produce antibodies directed at specific invaders.As part of the adaptive immune system, the thymus can be thought of as the training ground for T lymphocytes. During childhood, immature T cells (called progenitor cells) that originate in the bone marrow travel via the bloodstream to the thymus gland where they mature and differentiate into specialized T cells.T cells in the thymus differentiate into three primary types.

- **Cytotoxic T cells.** The word cytotoxic means "to kill." These cells are responsible for directly killing infected cells.
- **Helper T cells.** These cells are responsible for both causing production of antibodies by B cells and activating other types of T cells to address a foreign invader.
- **Regulatory T cells.** These cells function as "police." They suppress both B cells and other T cells

The immature T cells that leave the bone marrow enter the thymus in the cortex (known as the classroom of the thymus). During "training," these cells are taught to recognize antigens associated

with foreign cells and matter in a process called positive selection. Cells are positively selected for usefulness.

Once the T cells have learned to recognize specific pathogens, they travel to the medulla to undergo "negative selection." In the medulla, the mature T cells are introduced to the body's own antigens. Since T cells that would react with the body's antigens could attack a person's own cells, these cells are eliminated. T cells are negatively selected for autoimmunity, and these self-attacking cells either die or are turned into regulatory cells.The thymus gland produces several hormones including:

- thymopoietin and thymulin, which are hormones that assist in the process where T cells differentiate into different types
- thymosin, which accentuates the immune response as well as stimulating pituitary hormones such as growth hormone
- thymic humoral factor, which acts similarly to thymosin, but increases the immune response to viruses in particular

The thymus gland may produce small amounts of some hormones produced in other areas of the body, such as melatonin and insulin. Cells in the thymus gland (such as epithelial cells) also have receptors through which other hormones can regulate its function..T cells are part of the adaptive immune system, in which each T cell has been trained to recognize a particular antigen. When exposed to a foreign cell, cytotoxic T cells lock onto the cell and kill it with assistance from helper and regulatory T cells.This is also referred to as cell-mediated immunity, as it involves the use of immune cells to fight infections.

In general, T cells are barricaded in the cortex of the thymus so they do not become sensitized to the body's own cells. However, the process of negative selection in the medulla is used to get rid of cells that accidentally have become sensitized to "self."

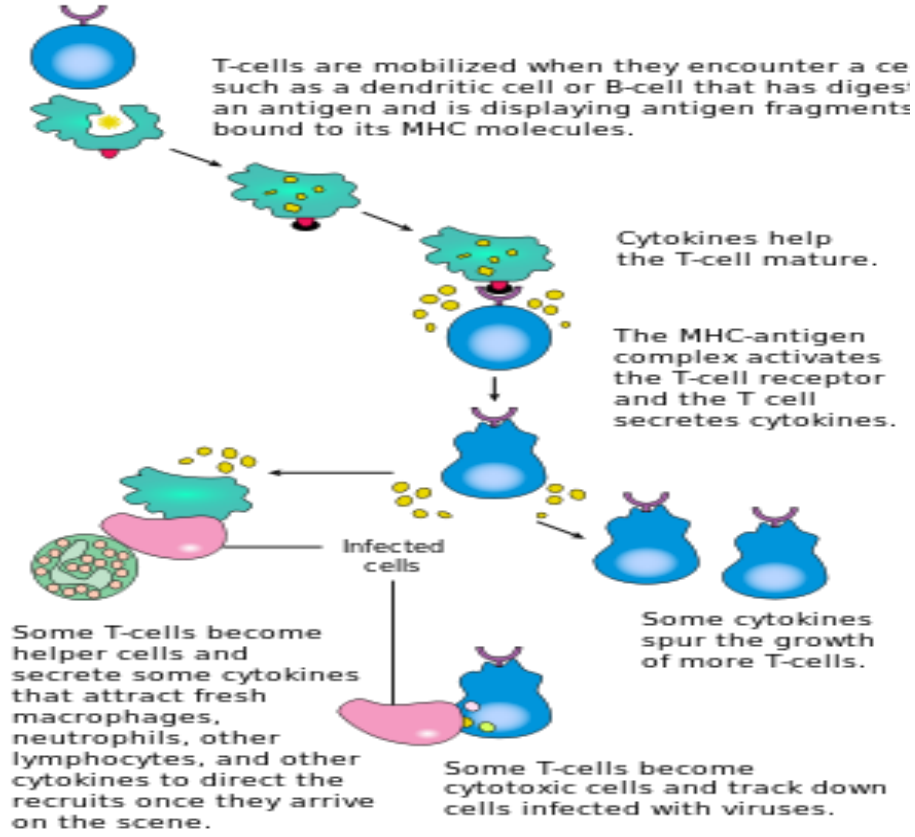
This function helps prevent the development of autoimmune disorders, which are medical conditions in which the body attacks its own tissues rather than foreign invaders. If the thymus gland is removed early in life, a person has an increased risk of developing one of these disorders.

T helper cell

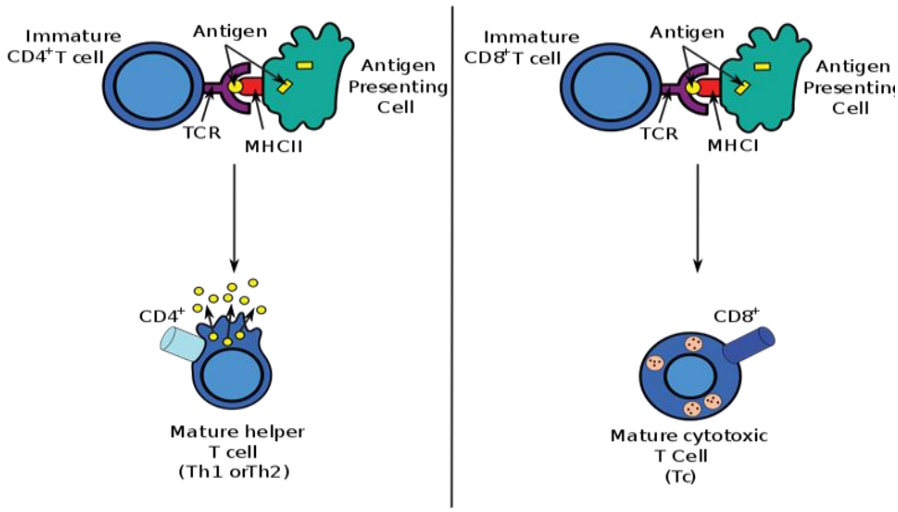
The T helper cells (T_h cells), also known as $CD4^+$ cells, are a type of T cell that play an important role in the immune system, particularly in the adaptive immune system. They help the activity of other immune cells by releasing T cell cytokines. T cell provides help to other cells in the immune response by recognizing foreign antigens and secreting cytokines that activate T and B cells. T-helper cells fall into two main classes: those that activate other T cells to achieve cellular inflammatory responses; and those that drive B cells to produce antibodies in the humoral immune response. These two classes of response are generally incompatible with one another and require coordination by substances called cytokines to promote one response while dampening the other.

CD4 is, by contrast, a type of protein found on certain immune cells like T-cells, macrophages, and monocytes. CD4 T-cells are considered "helper" cells because they do not neutralize infections but rather trigger the body's response to infections. In response, CD8 T-cells — classified as such because of the type of protein on their surface — play the part of "killer" cells by producing substances (antibodies) that help fight off viruses and other foreign invaders.

The T lymphocyte activation pathway: T cells contribute to immune defenses in two major ways; some direct and regulate immune responses; others directly attack infected or cancerous cells.



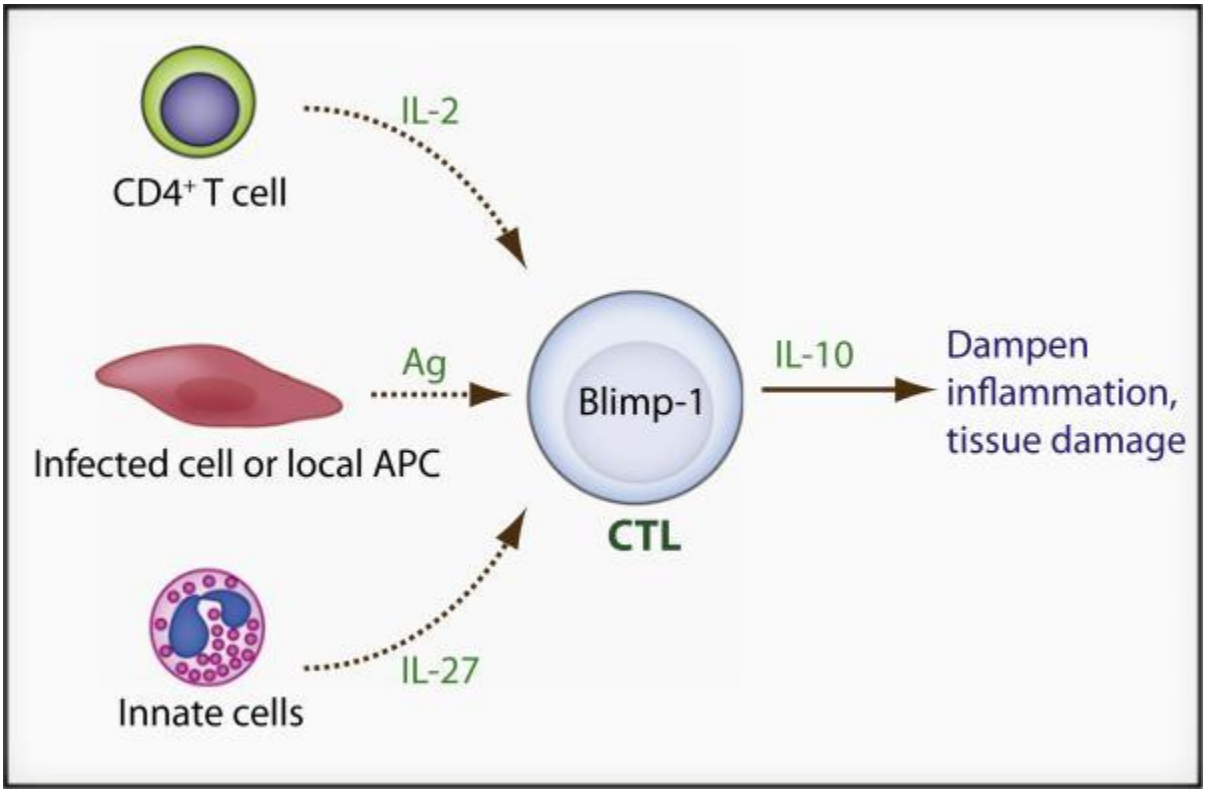
Activation of naive helper



Antigen presentation stimulates T cells to activate "cytotoxic" CD8+ cells or "helper" CD4+ cells. Cytotoxic cells directly attack other cells carrying certain foreign or abnormal molecules on their

surfaces. Helper T cells, or Th cells, coordinate immune responses by communicating with other cells. In most cases, T cells only recognize an antigen if it is carried on the surface of a cell by one of the body's own MHC, or major histocompatibility complex, molecules.

CD8$^+$ T Cells: Foot Soldiers of the Immune System



Self-Control by Effector CD8$^+$ T Cells

In infected peripheral tissues, some effector CD8$^+$ (CTL) T cells receive additional local signals including antigen, CD4$^+$ T cell-derived IL-2, and innate cell-derived IL-27, and transiently acquire the ability to secrete IL-10 in a Blimp-1-dependent manner. CTL-derived IL-10 is critical to control local inflammation and tissue damage.

CD8 (cluster of differentiation 8) is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Double-positive cells (CD4$^+$/CD8$^+$) that interact well with MHC class II molecules will eventually become CD4$^+$ cells, whereas thymocytes that interact well with MHC class I molecules mature into CD8$^+$ cells. A T cell becomes a CD4$^+$ cell by down-regulating

expression of its CD8 cell surface receptors. Resting naive $CD8^+$ T cells have an astounding capacity to react to pathogens by massive expansion and differentiation into cytotoxic effector cells that migrate to all corners of the body to clear the infection. The initial interaction with antigen-presenting cells in the central lymphoid organs drives an orchestrated program of differentiation aimed at producing sufficient numbers of effectors to get the job done without resulting in clonal exhaustion. Interactions with antigen-presenting cells and other immune cells continue at the site of infection to regulate further on-site expansion and differentiation, all with the goal of protecting the host with minimal bystander tissue damage.

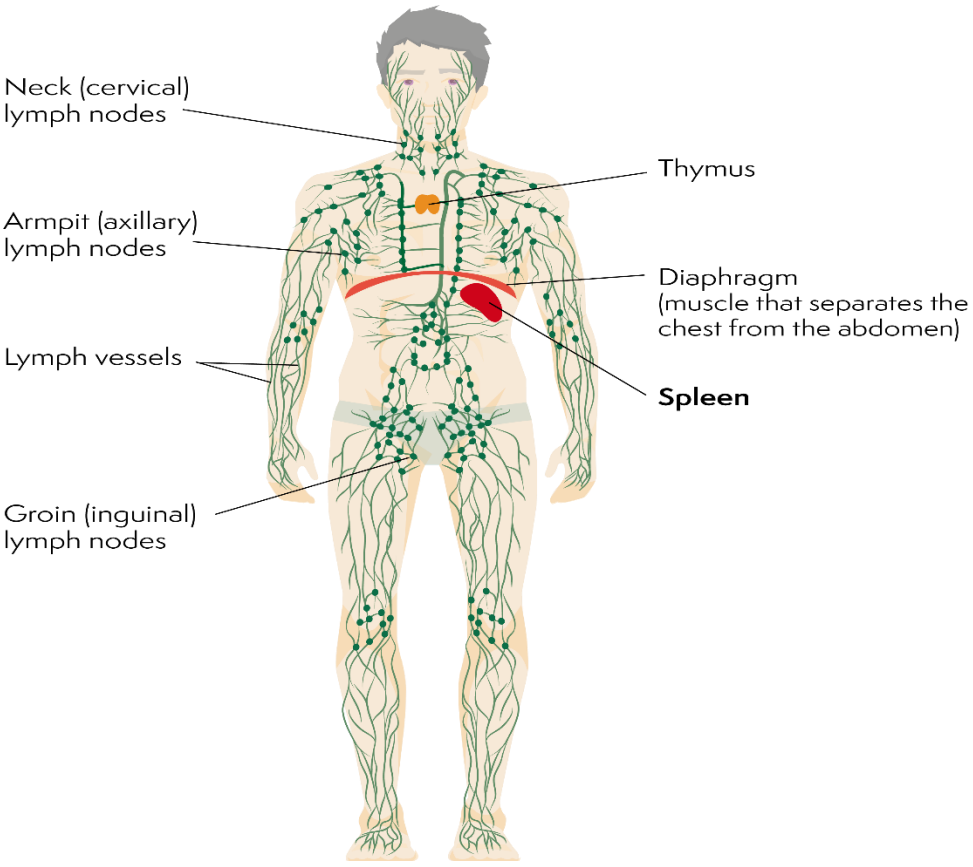
In vivo administration of IL-7 has been demonstrated to enhance peripheral T-cell functional capacity and expand the peripheral T-cell population (Hua young et al, 2005). Geiselhart *et al*, 2001, have reported that IL-7 administration alters the peripheral T-cell CD4:CD8 ratio, and results in increases in peripheral T-cell numbers and altered function. The percentage of CD8+ T cells is higher in the spleen, leading to an inverse CD4/CD8 ratio. Thus, the distribution of the different lymphocyte subsets is markedly different between spleen and peripheral blood, inferring an important and distinct role for the spleen in CD4+ and CD8+ T cell activation (Terry, *et al*,2001). There is a possibility that other organs besides the thymus and spleen are involved in generation and or maturation of T-lymphocytes. The Intestinal mucosa could possibly be a source of the T lymphocytes. The higher proportion of CD4+ and CD8+ cells in the STLs and lignans treatment group comparable to conventional treatment is thought to be indicative of better prognosis in disease outcome. CD4+ T cells, also known as T-helper (Th) cells, play an important role in orchestrating adaptive immune responses to various infectious agents (Rutishauser *et al* 2010). They are also involved in the induction of autoimmune and allergic diseases. Upon T-cell receptor (TCR)-mediated cell activation, naive CD4+ T cells can differentiate into at least four major lineages, Th1, Th2, Th17, and iTreg cells that participate in different types of immune responses (Rutishauser *et al* 2010). This study has confirmed the postulation that IL-7 is thought to be produced by the intestinal epithelial cells (IEC) and, IL-7 has been indicated as having potential for adoptive immunotherapy and is capable *in vivo* of causing CD4+ T cell dependent destruction of tumor cells (Itisam Sarangi, et al 2006). CD4+ T cells play a critical role in the development of effective anti-tumor immunity (Feng *et al* ,2008). IL-7 is thought to function in the immune system

by providing the right cells in sufficient num-bers to support and improve specific immune responses against infectious agents and malignant cells (Cytheris et al 2012).

STLs and saseme lignans would seem to rightly as so speculate to be acting as the stimulants in IEC IL -7 synthesis which leads to the cascade events leading to higher CD4+ cells observed. The mechanism of action of STLs and lignans could therefore be through stimulation of Intestinal Epithelial Cells which causes up regulation of IL -7 which then stimulates immune organs including the intestinal mucosa to produce more CD4+ and CD8+ lymphocytesas well as ensures their long survival. This is supported by the up regulation of IL 7 mRNA in the small intestines in the test group. Positive effect of STLs and saseme lignans is also confirmed by the lower mortality rate in the test group at the end of assay and lower tumor weight indices. STLs and lignanas have been shown to produce reduction in tumor size and prolong the survival time of tumor bearing mice.Elevated levels of IL-7 in serum could be associated with T-cell lymphopenia and, could be reflecting a homeostatic response, recovery of CD4+ counts should therefore lead to a decline in IL-7 levels. The levels of IL-7 observed in the circulation are likely to reflect more significant alterations of available IL-7 within the tissue microenvironment in the thymus and would be compatible with IL-7 as a primary regulator of T-cell homeostasis. Similarly, circulating IL-7 levels might only rise after increases have already occurred in the thymus tissue to a level sufficient to allow “spillover” into the circulation. Elevation of IL-7 within the lymphoid microenvironment in mice with CD4+ depletion contributes to the increased peripheral expansion, accumulation of activated cells, and thymic rebound observed CD4- depleted hosts.The inducible IL-7 production and enhanced IL-7 activity after lymphopenia are implicated in the activation of CD4+ and CD8+ T cells in autoimmune diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis and type I diabetes Infiltration of the tumors by the CD4+ and CD8+ cells signifies better prognosis in cancer cases (Klebanoff, *et al* 2011).

The spleen

The function of the spleen in general is related to the immune system or blood supply



The spleen has several roles.

- Removing bacteria and viruses from the bloodstream
- Making antibodies and storing several types of white blood cells. The spleen also:
- filters out old and misshapen red blood cells (that carry oxygen around the body) and platelets (cells that reduce bruising and bleeding) from the bloodstream
- Stores a small supply of red blood cells and platelets for the body to use in an emergency
- Works as a back-up site for making new blood cells if bone marrow (which usually makes blood cells) is not working as it should

Conclusion

Sesquiterpenes lactones (STLs) have been isolated from numerous genera of the family Asteraceae (compositae) and can also be found in other angiosperm families. They are described as the active constituents of a variety of medicinal plants used in traditional medicine for the treatment of various diseases. They are known to possess wide variety of biological and pharmacological activities such as antimicrobial, cytotoxic, anti-inflammatory, antiviral, antibacterial, antifungal activities, effects on the central nervous and cardiovascular systems as well as allergenic potency. Their wide structural diversity and potential biological activities have made further interest in the field of Pharmacy and Pharmacology. The present study was designed to isolate the STLs from *chrysanthemum cinerariefolium (pyrethrum)* and Sesame lignans and to investigate their potential synergistic effect on interleukin- 7 (IL-7) expression and cancer immunotherapy. The activity observed can be attributed to the presence of the pharmacologically important group of phytochemicals present in the plant extracts. Synergistic activities of pyrethrum STLs and Saseme lignanans extracts towards cancer cells. The extracts inhibited the growth of the cancerous cells.

The study reveals the significant potentials of STLs and lignans and may be used as an alternative for the management of cancer. *In vivo* IL-7 cytokine transcription increases in the intestinal mucosa. CD4+ cells levels increase in the peripheral blood an indication that it can be employed as a Nutraceutical in management of cancer patients. STLs and lignanas stimulates IEC cells to produce increased IL-7 Cytokine responsible for resultant adaptive immunity and cancer immuno surveillance.

5.0 Recommendation

The combination of the two extracts showed improved proliferative activities compared to single extracts. This indicates a probable use of pyrethrum and Saseme in cancer immunotherapy. Further studies are needed to isolate the bioactive compounds from the crude extracts which could be used as makers in the standardization of formulations from the two plants for cancer treatment and management. *Invivo* studies using pure isolated compounds which could better predict the potential application of the plant extracts and their bioactive compounds in immunotherapy are proposed. Elucidation of the mechanism of action of the plant extracts and pharmacologically active compounds is recommended.

6.0 Ethics approval and consent to participate

Ethical approval was sought from Kenya Medical Research Institute (KEMRI); Scientific and Ethics Review Unit (SERU) before conducting the study (**Approval number KEMRI/SERU/ CTMDR/ 042/ 3501).** Standard experimental protocols were followed as per the guidelines of SERU

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APPENDIX 1

Test for Alkaloids

2 ml of each crude extract was put in a test tube, followed by addition of 1% hydrochloric acid and the mixture heated gently. A few drops of Mayer's reagent were added and observations made. Formation of a precipitate delineates presence of alkaloids.

Test for Flavonoids 2 ml of each crude extract was put in a test tube and 5ml of dilute ammonia added. 2 ml concentrated sulphuric acid was then added to the mixture of each test tube and shaken. Presence of a yellow colour indicates the presence of flavonoids.

Test for Terpenoids

2 ml of each crude extract was put in a test tube. 2 ml of chloroform was then added and the mixture vortexed. The samples were then evaporated to dryness and about 2 ml of concentrated sulphuric acid added in each test tube and heating done for 2 minutes. A greyish colour indicates the presence of terpenoids.

Test for Saponins

5 ml of each crude extract was put in a test tube and 5 ml of water added. The contents were then mixed. Observation of a stable form shows the presence of saponins.

Test for Tannins

2 ml of each crude extract was added to the test tubes and the contents heated to boil. About 1% of $FeCl_3$ was then added drop wise in each test tube and observations made. A brownish colouration indicates the presence of tannins.