Saikosaponin B2 Prevents Against BAP-induced Lungs Cancer in Mouse Model by Regulating Oxidative Stress

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Acknowledgement

First and foremost, I would like to thank my greatest teacher of all: God. I know I am here and that I am able to write all of this for a reason. I will do my best to never forget what a great fortune I have had in just being here and that it comes with a lesson and a responsibility.

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Jannat Imran

Dedication

I would like to dedicate this thesis to my brother, Muhammad Abubakar Imran, as he inspired me to be strong in every path of life, his sudden demise changed me from head to toe, he always wanted to see me grow and to achieve so much, it all happened because of him, he inspired me with his true character, I am a better person now and I hope that I meet him in life hereafter (Ameen)'

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LIST OF ACRONYMS

IARC	International Agency for Research on Cancer
BAP	Benzo(a)pyrene
PAHs	Polycyclic aromatic hydrocarbons
PMA	Phorbol 12-myristate 13-acetate
PKC	Protein kinase C
DMBA	7,12-Dimethylbenz[*a*]anthracene
CP	Cyclophosphamide
ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
LFT's	Liver function test
CAT	Catalase
SOD	Superoxide dismutase
GSH	Glutathione
GST	Glutathione-S-transferase
MDA	Malondialdehyde
LPO	Lipid peroxidation

TABLE OF CONTENTS

Abstract

Cancer of the lungs is the second most frequent form of cancer overall and has the greatest mortality rate because to its severe pathological abnormalities. In lung cancer, abnormal cell proliferation causes the alveolar duct to clot. Lung cancer is difficult to treat due to its clinical and biological heterogeneity, which necessitates the development of new therapeutic approaches or the refinement of existing ones. To investigate this, saikosaponin b2 was administered to mice with lung cancer caused by B(a)P. (SSb2). BAP is considered a group one carcinogen by the International Agency for Research on Cancer (IARC). Therefore, B(a)P may play a role in producing lung cancer. Albino balb/c mice were given B(a)P on alternate days over a 4-week period to induce cancer, with DMBA given weekly to encourage tumor growth. Beginning in the third week, SSb2 was given daily for twenty days. The drug's potential anti-cancer effects were studied by examining hepatic biomarkers, lung histology, and oxidative stress indicators. Increases in SOD, CAT, GST, and GSH levels accompanied by decreases in MDA levels and enhanced B(a)P detoxification after SSb2 treatment, which also improved liver function. Histopathology data showed that as compared with the B(a)P alone group, SSb2 reduced inflammation, membrane blebbing, and alveolar structure began returning to normal.

Keywords: Oxidative stress; saikosaponin B2; bezo(a)pyrene; lung cancer

Chapter 1

1. Introduction

Cancer of the lungs is the leading killer on a global scale. It's the second most frequent form of cancer overall. There will be 228,150 new cases of lung cancer diagnosed in both sexes in the United States in 2020 [1]. In 2019, 76,650 men and 66,020 women will lose their lives to the disease. Lung cancer is more common in men than women since smoking is the primary cause of this disease [2]. A major factor in the development of lung cancer is oxidative stress, which is triggered by secondhand smoke. Approximately 50% of people who smoke will die. An estimated 7.2 million people worldwide are current smokers, with an additional 1.2 million being exposed to secondhand smoke every year and ultimately succumbing to their disease. Worldwide, 22.3% of the population uses tobacco products; 36.7% of men and 7.8% of women. Polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene (BAP) have large molecular weights. Although BAP is not naturally present in tobacco, it is created during smoking [3]. Each cigerrate's smoke contains between 3.36ng and 28.39ng of BAP. Specifically, the BAP concentrations found in cigarette smoke are between 22.92ng and 26.27ng [4]. BAP has been designated as a group one carcinogen by the International Agency for Research on Cancer (IARC) [5]. This poison is discharged into the air and consumed or inhaled by humans after being partially burned. As it travels through the human body, it undergoes a transformation that makes it even more cancerous. Cytochrome P450 converts 7,8-diol-9,10-epoxide-benzo[a]pyrene [6]. Eventually, this can result in inflammation, cell proliferation, DNA alterations, and carcinogenesis [7]. The mortality rate from lung

cancer may rise in the coming year. Lung cancer therapy options include chemoprevention and other cutting-edge methods [8]. Multiple experimental, epidemiological, preclinical, and clinical investigations testify to the efficacy of several chemopreventive drugs [9]. Their hazardous side effect has limited their application. Finding less harmful and more effective agents, whether they be synthetic or natural, is an urgent matter [10]. Because they are less toxic, have no long-term negative effects, and produce superior outcomes, plant-based treatments are increasingly being employed to treat diseases like cancer. Alkaloids, carbs, terpenoids, glucosides, phenolics, and steroids are all examples of natural products [11]. The root extract *Radix Bupleuri* contains a high concentration of saikosaponin B2 (SSb2). This compound is derived from the Chinese medicinal herb Bupleurum chinense DC. The Chinese have been using Bupleurum chinense DC for medicinal purposes for almost two thousand years. Glycoside saikosaponin b2 has a pentacyclic triterpene. Research suggests that SSb2 helps fight cancer. Studies have shown that the cancer drug SSb2 can kill B16 melanoma cells. Differentiation was seen at lower SSb2 concentrations, while apoptosis was seen at higher ones. Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), prevents the apoptosis and differentiation caused by high and low SSb2 concentrations, respectively. The results of this study provide further evidence that high levels of SSb2 cause apoptosis and have anti-cancer properties through suppressing (PKC) [13].

The current research focuses on regulating oxidative stress as a means of treating lung cancer caused by BAP using SSb2. No prior research has ever attempted something like this. There is a lot of research and data on Saikosaponin A and Saikosaponin D. There

hasn't been a lot of research into SSb2 to see if it has any anti-cancer or anti-oxidative qualities, even though it is a novel drug.

1.1.Objectives

- To check the activity of saikosaponin B2 in mice model.
- Treatment of lungs cancer by modulating oxidative stress.
- To check the cytotoxicity of saikosaponin B2 on mice model.

Chapter 2

2. Literature Review

2.1. Cancer: a leading cause of death

Diseases in which aberrant cells develop and spread uncontrollably are called cancers. In this event of spread of cancer to other organs (and is not stopped), the patients will likely die. Many environmental factors, such as tobacco use, chemical exposure, and radiation, and even some innate predispositions all contribute to the development of cancer (Inherited mutations, hormones, immune conditions and random mutations). Cancer's root causes are many, intricate, and poorly understood at best. Dietary variables, some illnesses, a lack of physical activity, obesity, and environmental contaminants are all known to raise the risk of cancer. Cancer is the greatest cause of mortality worldwide, and these elements may all have a role in its initiation or promotion within the human body [14].

According to the International Agency for Research on Cancer (IARC), 0.18 million people in Pakistan are diagnosed with cancer each year, 0.11 million people die from cancer, and 0.32 million people in Pakistan have had cancer at some point within the past five years [15]. Pakistan has the highest breast cancer rate in the Asian region [16]. An estimated 1 in 9 Pakistani women may develop breast cancer at some point in their lives. The death rate from breast cancer in Pakistan is extremely high [17]. When both sexes are considered together, lip and mouth cancer accounts for 15.9% of all malignancies [18]. A rise in the prevalence of severe lung tumors (SLT) has been linked to the rising use of smokeless tobacco products like areca nut [19]. More than 30 chemicals, both relatively

non- and highly toxic, make up SLT [20]. In 2022, the predicted number of new cases of cancer worldwide was 1,918.030, and the number of deaths from the disease was 322,090. Approximately 130,180 people will lose their lives to lung cancer this year, while the global incidence rate has risen to 236,740. Males were more likely to get lung cancer than females [21]. Surgery, radiation, and chemotherapy are now the only options for treating cancer, but each has downsides and often fails to completely eradicate the disease.

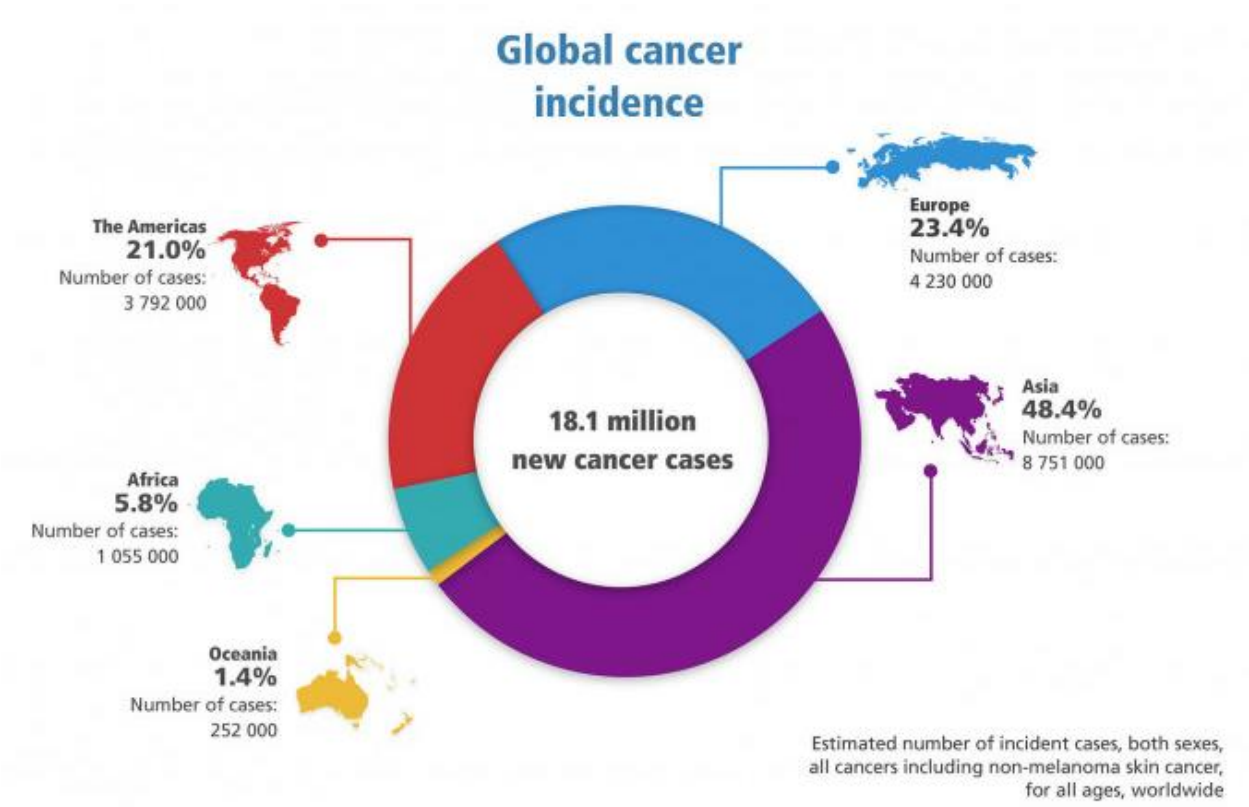

Figure 1: Global cancer incidence, [1]

Cancer's six defining characteristics are a collection of biological acquirables by human malignancies over the course of its complex multi-stage evolution. The hallmarks provide a framework for understanding the intricacies of cancer. Maintenance of proliferation signals, avoidance of growth suppressors, resistance to cell death, induction of

immortality by replication, induction of angiogenesis, and activation of invasion and metastasis are all examples. Inflammation stimulates several signature activities and genomic instability creates the genetic variation that facilitates their acquisition. Reprogramming of energy metabolism and escaping immune destruction are two developing hallmarks of potential generality that have been added to this list as a result of conceptual advances over the past decade [22].

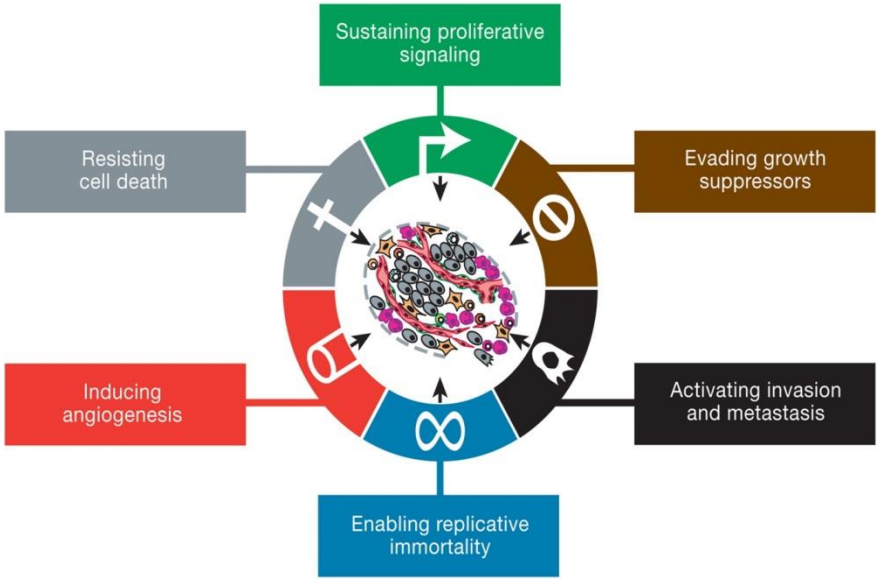

Figure 2: Hallmarks of cancer, [10]

1.1.1. Lung's cancer- Most common type of cancer

When aberrant cells in the lungs begin to proliferate uncontrollably, this is known as lung cancer. As a result, these aberrant cells cannot perform the duties of healthy lung cells and cannot mature into lung tissue. Tumors formed from the aberrant cells eventually impede normal lung function. Although it is more common in men than women, prostate cancer is the second most common form of cancer in women. Depending on how the cancer cells in the lungs look under a microscope, there are two main categories of lung

cancer. Treatment options for lung cancer are determined by the specific subtype of the disease that has been diagnosed.

- **Small cell lung cancer**: Heavy smokers are virtually guaranteed to develop small cell lung cancer.

- **Non-small cell lung cancer**: Several distinct but functionally related forms of lung cancer are together known as non-small cell lung cancer. Squamous cell carcinoma, adenocarcinoma, and big cell carcinoma are all forms of lung cancer that are not tiny cells.

For both smokers and bystanders, tobacco smoking is a major risk factor for developing lung cancer. In addition to smokers, non-smokers are at risk for developing lung cancer [23].

2.2. Benzo (a) pyrene: number one carcinogen

Polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene (BAP) have large molecular weights. BAP is found in cigarette smoke [3]. BAP has been designated as a group one carcinogen by the International Agency for Research on Cancer (IARC) [5]. This poison is discharged into the air and consumed or inhaled by humans after being partially burned. To make matters worse, cytochrome P450 in the human body converts it to the even more cancerous 7,8-diol-9,10-epoxide-benzo[a]pyrene [6]. Because of this, inflammation, cell proliferation, DNA alterations, and carcinogenesis occur [7]. PAHs are rarely found as a single component but rather as part of a more complicated combination. The EPA has included benzo(a)pyrene in its list of priority pollutants [24]. In nearly every in vivo experimental animal model system, it is tumorigenic. Induction of integrated genotoxic and nongenotoxic epigenetic changes is linked to the carcinogenic

activity of benzo[a]pyrene. More specifically, benzo[a]pyrene exposure causes the extensive and selective formation of anti-7, 8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) adducts at the major hot-spot mutation codons 157, 248, and 273 in the human tumor suppressor P53 gene, and at codon 14 in the human KRAS oncogene. Amplification of the synthesis of the genotoxic benzo[a]pyrene-DNA adducts stated above is considerably facilitated by CpG methylation at these codons.

Furthermore, DNA methylation, both globally and at the level of individual genes, is affected by the presence of BPDE-DNA adducts because of the interference they cause with DNA methyltransferases. Therefore, with exposure to benzo[a]pyrene, it is common to see hypermethylation of genes involved in cancer defense, such as cyclin-dependent kinase inhibitor 2A (CDKN2A; p16INK4A), retinoic acid receptor 2 (RAR2), hypermethylated in cancer 1 (HIC1), and glutathione-S-transferase [3].

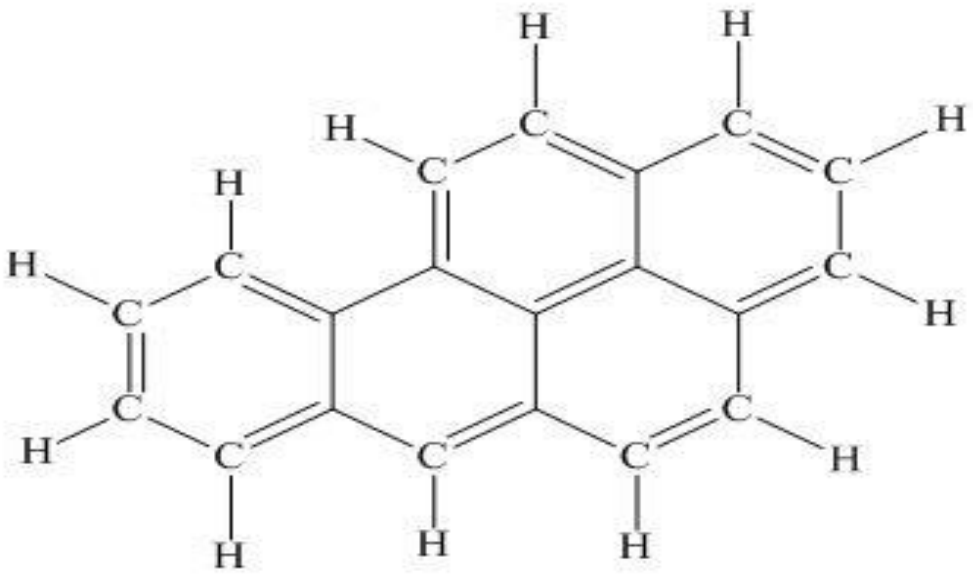

Figure 3: Chemical Structure of Benzo (a) pyrene [5]

2.3. Treatment of cancer: a complicated study

Despite the prevalence of cancer treatments, none have yet proven helpful in curing the disease, typically due to the development of resistance or high costs associated with therapy. Multiple approaches have been developed to combat the disease. The type and stage of cancer you have will determine the therapy options available to you.

Certain cases of cancer may only require a single round of treatment. On the other hand, most patients undergo a combination of treatments, including surgery, chemotherapy, and radiation therapy.

2.3.1. Surgical Treatment

When used to treat cancer, surgery involves a method in which the malignancy is physically removed from the patient's body. Medical doctors who have completed specialized surgical training are called surgeons. During surgery, surgeons will make incisions in your body using a variety of sharp instruments, including small, thin blades known as scalpels. It is common practice in surgery to make incisions into layers of tissue and even bone. These incisions often require surgery and require time to heal, causing discomfort and slowing recovery. Because of the anesthetic, the surgical procedure will not hurt. Substances that dull the senses or impair awareness are what this term alludes to. There are four further categories of surgery:

- Lasers
- Light-Based Medicine
- Cryosurgery\s
- Hyperthermia

There are two types of surgeries:

Open surgery involves making one major incision to remove the tumor, some surrounding healthy tissue, and potentially some lymph nodes.

A minimally invasive procedure is one in which the surgeon just makes a few minor incisions rather than one large one. She makes a few tiny incisions and slides a long, thin tube with a camera into one of them. A laparoscope is the name for this instrument. The camera placed within the patient allows the surgeon to view what she is working on by projecting images from the inside of the body onto a monitor. By inserting surgical instruments into the other incisions, tumor can be removed along with some healthy tissue [25].

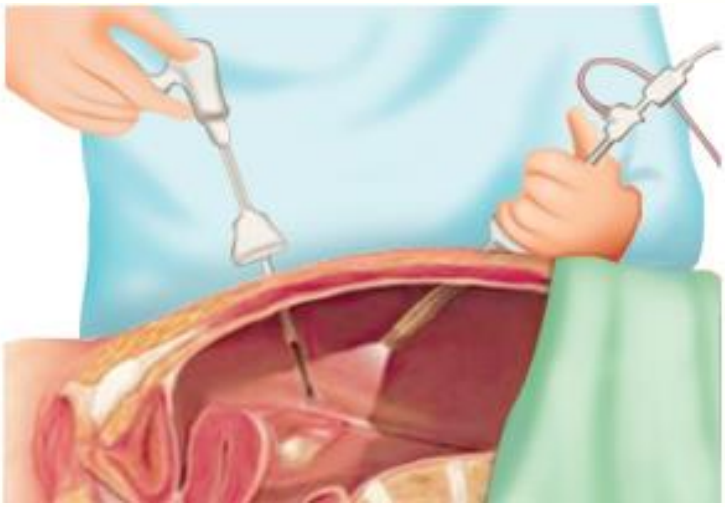

Figure 4: Surgical procedure, [25]

2.3.2. Radiation Therapy

Radiation therapy, often known as radiotherapy, is a method of treating cancer that makes use of radiation to eradicate cancer cells and reduce the size of tumors. Radiation is utilized in x-rays at safe levels to provide images of the inside of the body, such as those of the teeth or fractured bones. Radiation therapy can kill or halt the growth of cancer cells by disrupting their DNA, but only at very high dosages. Unrepairable DNA damage

causes cancer cells to cease dividing or die. Dead damaged cells are metabolized and flushed out of the body.

Radiation therapy takes time to eliminate cancer cells. Cancer cells DNA must be severely degraded over the course of several days or weeks of treatment before they die. After radiation therapy is finished, cancer cells continue to die for weeks or months. Radiation therapy can be broken down into two basic categories: external beam and internal [26].

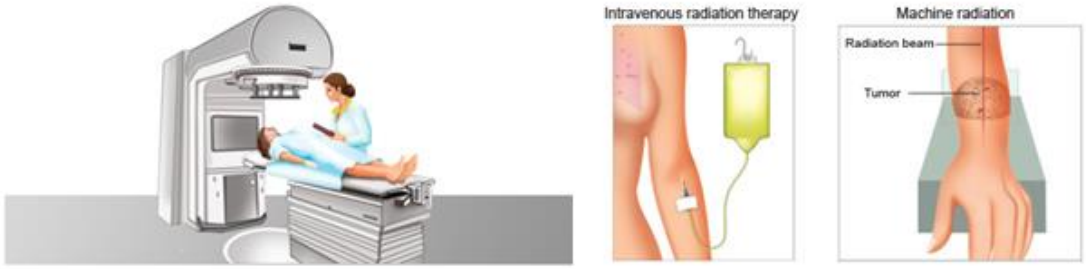

Figure 5: Radiation therapy, [26]

2.3.3. Chemotherapy

Chemotherapy, or chemo, is a treatment for cancer that makes use of chemicals to eliminate malignant cells. Chemotherapy is effective because it inhibits or slows the proliferation of cancer cells. There are two main applications for chemotherapy:

Treat cancer: Cancers can be cured, the disease's progression halted, or the risk of recurrence reduced by chemotherapy.

Ease cancer symptoms: Tumors that are painful may be shrunk with chemotherapy.

It is important to note that chemo is employed to treat many kinds of cancer. Chemotherapy is sometimes the sole option for patients. But chemotherapy is typically combined with other cancer drugs. Whether and how far your cancer has progressed, as

well as any other health issues you may have, will determine the course of therapy you need. Cancer cells that divide rapidly aren't the only ones that chemotherapy can kill; it can also kill or halt the growth of healthy cells that divide rapidly. The cells that make up your mouth and intestines, as well as the cells responsible for hair growth, are good examples. Damage to healthy cells can lead to unwanted side effects like mouth sores, vomiting, and hair loss. Chemotherapy's side effects tend to improve or vanish once the treatment is complete [27].

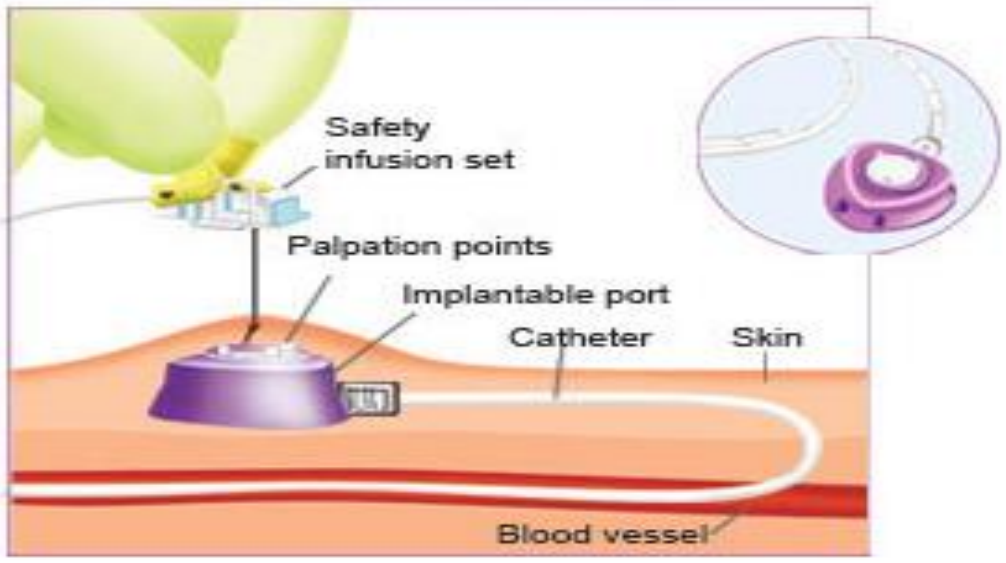

Figure 6: Chemotherapy Treatment, [27]

2.3.4. Targeted Therapy

By interacting with specific molecules essential to the growth of cancer cells, medicines and other substances used in targeted therapy prevent the disease from spreading. Some cancer treatments, known as targeted cancer therapies, work by interfering with molecules in charge of tumor growth. Potentially more successful than conventional therapies, these chemicals target cancer-specific alterations at the molecular and cellular

levels. They may also cause less damage to healthy tissue than conventional treatments like radiotherapy and chemotherapy.

Targeted therapies are effective because they disrupt the normal cycle of cancer cell reproduction in a variety of ways. Certain proteins are used by each cancer cell to establish an internal and external network of communication that fosters proliferation. Cancer cells can be helped to cease growing by targeted medicines that interfere with these signals. By activating the immune system to recognize and destroy cancer cells or by initiating programmed cell death, targeted medicines may potentially promote cancer cell death. The term "biotherapy" is often used to refer to this method of treatment [28].

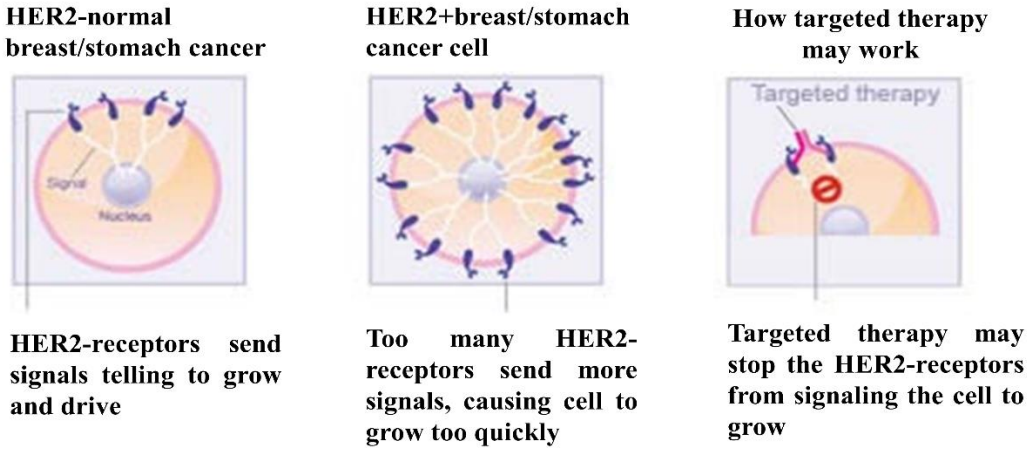

Figure 7: Targeted therapy for cancer treatment, [28]

2.4. Saikosaponins

The plant Bupleurum chinensis DC was first mentioned in Shennong's Herbal, one of the first Chinese materia medicamonographs. According to the fundamental principles of traditional Chinese medicine, the perennial herbaceous plant known as *Radix Bupleuri*

(from the *Umbelliferae family*) may alleviate symptoms such as fever, relieve liver-qi stagnation, and boost yang-qi by restoring internal organ balance. Because of this, it is useful for treating intermittent fever and chills brought on by infectious external factors. As a treatment for shaoyang illness, it is significant due to its ability to eradicate pathogenic substances from both the outer and inner layers of the body. The most powerful of them is saikosaponin d (SSd), followed by saikosaponin a (SSa) [29]. So far, scientists have isolated and characterized all 43 known saponins. Although the saponins in bupleurum root may be extracted by processing, this is seldom done with the ground component [30]. Saikosaponins may be responsible for the vast bulk of *R. bupleuri's* biological activities (SS). Saikosaponins are a class of oleanane chemicals found as glucosides in a broad variety of plant species. Saikosaponins, for instance, have been credited with biological actions against a wide range of conditions like hepatitis, nephritis, hepatoma, inflammation, infection, and immunological modulation, and they have been isolated from medicinal plants like Bupleurum spp., Heteromorpha spp., and Scrophularia scorodonia. In the lab, the sedative, analgesic, anti-inflammatory, antibacterial, protecting, anticancer, and antiviral actions of saikosaponins (a, b, c, and d) have been demonstrated. Saikosaponins are involved in a wide variety of processes i.e. ability to prevent seizures, efficacy in treating depression, the fight against alzheimer's, protection against cancer, hepatotoxicity and liver cancer prevention, immunostimulatory properties, assists in reducing inflammation, efficacy against viruses, inhibition of andipogenic Growth, efficacy in preventing asthma attacks, toxic hormone-like action

2.4.1. Antiepileptic activity

Despite the prevalence of epilepsy, for which no effective treatment is available, the neurological disorder continues to afflict many people. The triterpenoids saponin (SSa) extracted from B. chinensis DC has been demonstrated to have substantial antiepileptic properties in several in vivo models of epilepsy. It has been reported that SSa effectively terminates SREDs and CEEBs in a hippocampal neuronal culture (HNC) model of AE and SE, with an IC50 of 0.42 mm for AE and 0.62 mm for SE, respectively. Analyzing the effects of saikosaponins on the electroencephalography (EEG) of schizophrenic rats might provide light on the effectiveness of these compounds as an epileptic treatment. Six groups of 10 rats each were created from a total of 80-week-old normal SD rats: control, epileptic model, lamotrigine, and saikosaponin (low, medium, and high dosages). The EEG and onset of seizures were monitored in the other five groups after penicillin was used to induce epilepsy. Saikosaponins showed promise in reducing epileptic episodes in mice [32]. The astrocytes of the hippocampus responded well to SSa treatment in newborn mice. SSa dramatically attenuated proliferation, cell proliferation, and GFAP expression in Glu-activated astrocytes. Convulsions, epilepsy, and other neurological disorders may be treated with SSs due to their dramatic influence on the CNS, which is likely accomplished by SSa's capacity to prevent Possible to employ various activation of rat hippocampal astrocytes [33]. Previous studies [34] have demonstrated that SSA has an anticonvulsant effect via reducing sustained sodium current (INAP), which may be related to the increase in INAP seen during spontaneous limbic seizures. The hippocampus neuron culture (HNC) model of acquired seizures and the HNC model of status epilepticus both show concentration-dependent suppression of sporadic recurrent

epileptiform discharges (SREDs) by SSA (IC50=0.42 M). [35] When compared to phenytoin, SSA's (1 M) effectiveness in completely suppressing spontaneous, repetitive epileptic discharge is equivalent (50 M). Evidence shows that SSA has an anticonvulsant effect due to its ability to inhibit N-methyl-D-aspartic acid channel current and INAP [34]. A single dose of SSA, from as little as 0.1 M up to 4 M, eliminated the convulsions. The anticonvulsant activity of SSA47 is due to its ability to inhibit the epileptiform discharges of CA1 neurons in the hippocampus, which are generated by 4-aminopyridine (4AP) [36]. In addition, SSA is like other anticonvulsant medications (such as carbamazepine) in that it may reduce seizure severity by more than 30 minutes in the 4AP convulsion model, showing a longer lasting effect [36].

2.4.2. Anti-cancer activity

The capacity of SSs to prohibit tumor cells from connecting to one another, to halt the cycle of tumor cell formation, to limit tumor cell proliferation, and to cause tumor cell death are only some of their many known anti-tumor activities. One possible method by which SSA, SSD, and SSE inhibit tumor growth is by decreasing solid tumor cell adhesion [63]. There is evidence to show that SSA activates caspase-2 and caspase-8 in hepatoma cells, which may increase apoptosis [64]. Because of its steroid component, SSD, which has a structural resemblance with glucocorticoids, may have anti-tumor activities and inhibit Na+-K+-ATP enzymatic activity. 95 SSD further exacerbates the situation by inhibiting TNF-induced NF-B activation, which in turn regulates the expression of target genes involved in cellular proliferation, invasion, vasculature, and survival. SSD seemed to prevent TNF-induced invasion of H1299 carcinoma cells at a dosage of 10 mM, demonstrating that SSD's inhibitory action on cancer cell invasion is

selective and independent of direct cytotoxicity [65]. Researchers observed that SSD (10, 20 M) reduced the growth of the human lung cancer cell line A549 in a dose- and time-dependent manner [66]. The apoptotic pathway controlled by p53 and TNF receptor superfamily members Fas (FasL) [66] has been proposed as the mechanism by which SSD inhibits A549 cell proliferation. P53 target genes play an important role in apoptosis and cell cycle arrest. However, the validity of several genes, including p21.26, bcl-2, and bax, is being questioned. By inhibiting the G1 cell cycle checkpoint, increased p21 protein levels inhibit cell growth [67]. Catenin kinase 2 (CDK2), along with other members of the silk catenin kinase family, is essential for controlling the G1/S transition. Treatment of SMMC7721 cells with 3 g/mL SSD [69] under hypoxic and hypoxia conditions have the potential to drastically promote cell death. The SMMC7721 hepatocellular carcinoma cells are rendered more radiosensitive and eventually experience apoptosis after being treated with SSD. As proven by mitochondrial failure as the primary mechanism by which SSD triggered apoptosis in DU145 human prostate cancer cells [70], treatment with SSD decreased DU145 cell growth at low, medium, high, and ultrahigh cell densities. By suppressing Wnt (Wingless/ Integrated)/-catenin signaling and preventing the generation of -catenin and its upstream target genes, SSD (10, 15, or 20 M) may promote cancer cell death. This occurs independently of whether neurotensin receptor 1 or epidermal growth factor is altered. SSD treatment at 1-5 g/ml significantly increases the therapeutic index to Rapamycin [72] in the MCF-7 (human breast cancer cell line). SSD at 5 mm and SSA at 10 mm showed no impact on A375. The proliferation of S2 cells was not affected by SSC at any dose, not even 20 M. According to the study results, solid-state dnase is the best approach to treating melanoma [73]. SSD

nanoparticles cause cell death by destroying mitochondria. There is some speculation that its anti-melanoma impact may be attributed to an increase in cytochrome c levels and the synthesis of caspase 9. [73] Possible contributors include p38 and p53 activation. Medications used to treat liver cancer are more effective when SSB2 (64 M, 128 M) is present, since it inhibits the multidrug inhibitory drug transporters Pgp, MRP1/Mrp1, and MRP2/Mrp2. Patients diagnosed with hepatocellular carcinoma who have a mutation in SSB2 are less likely to acquire treatment resistance. When treating cancer, SSB2 should not be used with platinum-based medicines. One of the most common unwanted effects of cytotoxic chemotherapy medicines is neutropenia (CCIN). In vitro and in vivo studies showed that SSD at a dosage of 6.12 mg/kg aided in the production of neutrophils with antimicrobial capabilities, supporting its use in the treatment of CCIN. Network pharmacological analyses of signaling pathways have uncovered therapeutic targets for CCIN. The proposed mechanism was tested using Western blotting for further reliability. SSD was shown to increase the expression of PU.1 and CEBP, which thus facilitated neutrophil differentiation [74]. SSD, a natural regimen, has been shown to be helpful in restoring microbicidal neutrophils and lowering CCIN-associated infection via activating CBL-ERK1/2. The findings of the research provide strong support to the treatment approach under consideration. Doxorubicin (DOX) is a powerful cancer drug, but it has substantial side effects on the heart due to the oxidative damage it induces. Due to its ability to inhibit oxidative stress generation through the p38 MAPK signaling pathway, SSD (10 mg/kg) significantly protected cardiomyocytes from DOX-induced cardiac injury. Subsequent studies [75] found that 1 M SSD increased H9c2 cell proliferation and decreased DOX-induced cell death. Although SS has anticancer properties, how total

Bupleurum sulphate extracts (TBSE) affect colon cancer is not understood. TBSE (50ug/ml) strongly up-regulated Bax, Caspase3, Caspase9, cleaved Caspase3, and cleaved Caspase9, while dramatically down-regulating PI3 K and Akt (P 0.01). Too far, few drugs that specifically target chemo sensitivity have been successfully implemented into cancer treatment. SSD may inhibit the malignant phenotype of HCC cells in vitro and in vivo by reversing hypoxia's effects, particularly its effects on energetic sentrin/small proteolysis modifier (SUMO)-specific proteolytic enzymes 5 (SENP5) in a time- and dose-dependent manner. At 6 M, SSD showed promise in inhibiting the malignant phenotype of H. pylori, according to preliminary results. These findings improve molecular explanations and provide new insight on the role of SSD in the context of reported cisplatin resistance in liver cancer.

2.4.3. Anti-hepatotoxic activity

Preventative effects of SSA, SSD, SSB1, SSB2, and SSC after D-galactosamine hydrochloratemine administration in rats (5 mg/kg i.p. for 4 days) (D-GalN) was accessed. It has been found that both SSA and SSD significantly mitigate the hepatic damage induced by D-galactosamine hydrochloratemine (D-GalN). SSD was much more effective than SSA in reducing the levels of mitochondrial enzyme activity and cytochrome P450, two markers of drug metabolism, in rats. Neither SSB1, SSB2, nor SSC had any effect on enzyme activity [78]. Tumor necrosis factor alpha (TNF-alpha), interleukin 6 (IL-6), and nuclear factor kappa B (NF-kappa B) p65 expression in rat liver tissue is reduced after chronic intraperitoneal injection of SSD at dosages of 1.0, 1.5, or 2.0 mg/kg/d. SSD at concentrations of 0.5, 1, and 2 M was observed to protect HL-7702 hepatocytes from CCl4-induced damage. Total superoxide dismutase and MDA

production are both reduced by SSD, which protects HL-7702 cells from CCl4-induced acute hepatocyte damage [79]. The levels of bone morphogenetic protein-4 (BMP4) are regulated by SSA (5 M), which in turn suppresses the production of alpha-smooth muscle actin (-SMA) and the activation of hepatic stellate cells (LX-2 cells). Accordingly, SSA and SSD may be used in a dose-dependent way to treat liver illnesses characterized by elevated BMP4 expression. Liver tissue from mice that had been given SSA (5,10, or 20 mg/kg i.p.) was protected against the toxicity of LPS (60 mg/kg) and D-GalN (800 mg/kg). The protective benefits of SSA against dose-dependent LPS/D-GalN-induced liver damage [81] may be due to its capacity to decrease the NF-kappaB signaling cascade, and therefore, inflammation. SSA provides protection in part through increasing expression of the X receptor alpha in the liver (LxR). However, oestrogen receptor alpha (ER) expression was not found in primary cultured rat stellate cells [81]. OS-induced activation of ER-active hepatic stellate cells may be inhibited by 5 M SSD's ability to abrogate the ROS/MAPK signaling pathway [82]. Li et al. [82] demonstrated the amelioration of diet-induced chronic liver disease with SSA or SSD at doses of 5, 10, and 20 mg/kg. (NAFLD). The expression of genes involved in glycolipid metabolism, such as lipases E and F, is regulated by SSA and SSD, as shown by an analysis of lipid and transcript data. The translation of FASN and ACACA was much reduced by SSD, while the expression of ACOXL and CPT1A was greatly increased; this had the effect of greatly decreasing fatty acid synthesis and greatly enhancing fatty acid breakdown. Bioinformatics analyses have implicated master transcription factors including phosphoinositide 3-kinase-activated receptor alpha in mediating the protective effects of SSA and SSD (PPAR). Chang et al. [83] found that SSD's anti-inflammatory and

antioxidant capabilities made it a potent hepatoprotective agent in cases of liver damage. Intake of food, body weight, and hepatic antioxidant properties enzymes like catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were found to increase, while hepatic cyclooxygenase-2 (COX-2) and serum aspartate transaminase (ALT) and aspartate aminotransferase (AST) levels decreased. Both nitric oxide synthase and nuclear factor kappa B, two inflammatory genes, had their mRNA expression suppressed by SSD (iNOS). Oxidative stress and ER stress markers were measured in the blood, including phosphorylated eukaryotic initiation factor 2 subunit (p-eIF2), activating signalling pathway 4 (ATF4), and cAMP-dependent protein kinase (C/EBP) (CHOP).

2.4.4. Immunomodulatory activity

The immunomodulatory effects of SSs were studied, and although SSA, SSF, and 23-O-acetyl saikosaponin-a were all proven to have some impact, SSD was shown to be the most effective [84]. After receiving SSA (2, 10 mg/kg/day, i.g.), male BALB/c mice may have lower levels of ovalbumin-specific immunoglobulin IgE/IgG1 [85]. At 5 and 10 M, SSA increases the mortality of activated T cells without affecting the rest of the immune system, as shown by the work of Sun et al. [86]. The mitochondrial membrane potential is broken down and cytochrome c leaks out into the cytoplasm when SSA concentrations are high enough [86]. SSA may induce apoptosis and G0/G1 phase arrest in T cells through a mitochondrial pathway [86]. As a result of stimulation by biochanin A or phorbol 12-myristate 13-acetate, T cells express early (CD69) and late (CD71) markers, both of which are significantly downregulated by 10 and 20 mm SSD (PMA). Possibility of involvement of CD69 in immune-mediated inflammatory diseases. SSD inhibits ERK phosphorylation in PMA-activated murine T cells but inhibits phosphorylation of IB and

JNK. When stimulated, SSD regulates the PKC pathway by activating PKC, JNK, and NF-B.

2.4.5. Anti-inflammatory activity

The anti-inflammatory effects of SSD are the greatest among the SSs [88]. SSD may be useful in reducing many inflammatory responses [37, 38], including capillary leakage, the production of inflammatory cytokines, leukocyte migration, and collagenous proliferation. The inflammatory cytokine production is controlled by the nuclear factor-kappa B (NF-kappa B) signaling pathway. It was shown by Lu et al. [37] that both SSA (3.125 M12.5) and SSD (3.125 50) inhibited LPS-induced RAW264.7 macrophages' production of inducible nitric oxide synthase, cyclooxygenase-2, and proinflammatory cytokines. LPS and the NF-kappaB signaling pathway could operate through the same mechanism. Survival of RAW264.7 cells was significantly decreased by SSA (12.5100 M) [89]. When macrophages are stimulated by LPS, what events take place? Although SSA may boost the production of generally pro cytokines, it may also reduce the production of pro-inflammatory cytokines [89]. This impact is achieved by modulating the signaling pathways MAPK and NF-kappaB. The human acute monocytic leukemia cell line THP-1 has been shown to be inhibited in their ability to produce inflammatory cytokines by SSA (50 M) [90]. SSA (520 mg/kg/day, i.p.) has been shown to strongly reduce cigarette smoke (CS)-induced NO, TNF-, and IL-1 production [91, 92]. Myeloperoxidase (MPO) and malondialdehyde (MDA) are produced in lung tissue in response to CS, and SSA greatly suppresses the synthesis of these enzymes [91, 92]. In a dose-dependent manner, the expression of nuclear factor E2-related factor 2 (Nrf2) (Nrf2 may modulate the inflammatory response) and HO-1 was increased in mice administered

SSA (5, 10, or 20 mg/kg intraperitoneally 1 h before LPS treatment) [91]. By blocking the NF-B signalling pathway and limiting NF-Bp65 and IB phosphorylation in response to LPS, activated Nrf2 may be responsible for SSA's anti-inflammatory impact. The immunological response is modulated by the chemokine receptor LxR. However, LxR acts as an antagonist to NF-B activation. 14 days of SSA treatment at dosages of 5, 10, or 20 mg/kg/d substantially reduced IL-1 levels and inhibited TNF- and NF-B activation generated by dextran sulphate sodium (Pdp) in a study by Zhou et al. [93]. SSA (2 mg/kg /day and 10 mg/kg /day) [85] inhibits T helper 2 (Th2) and T helper 17 (Th17) cytokines by downregulation of the IL-6 signal transduction, Message transduction and transcriptional activator 3 (STAT3)/retinoid-related orphan nuclear receptor t (ROR-t), and NF-B pathway. In the Ali et al. study, SSA (1, 5, 10 mg/kg/day) was given for 7 days before 5-fluorouracil (5-FU) injection. It has been shown that SSA has a remarkable impact on lowering pro-inflammatory mediators like TNF-, COX-2, IL-1, and IL-6, and that it decreases apoptotic indicators like phosphorylated c-Jun N-terminal kinase (p-JNK) or caspase-3 [94]. The levels of reactive oxygen species (ROS) are reduced, kinases p38 and jNK are deactivated, and NF-kappaB activation is blocked in studies using SSD. SSD was infused intragastrically at a rate of 8 milligrammes per kilogramme of body weight per day in order to reduce the inflammation of the intestines caused by DSS. SSD has been shown to elevate IL-10 mRNA levels [95], according to the available research. Inflammation may be mitigated with the use of IL-10, a cytokine. As a result, SSD inhibits the synthesis of inflammatory cytokines such TNF-alpha, IL-1, and IL-6. The total SS isolated from Radix Bupleuri, the majority of which is composed of SSA, SSB2, SSC, and SSD, has a significant anti-inflammatory effect in mice with formalin-induced

inflammation [96]. Regulation of nicotinic acid, nicotinamide, and arachidonic acid metabolism contributes to SS's anti-inflammatory effects on the plasma metabolome. Compared to lesser dosages, HD-SS (4.68 g/kg) reduced inflammation in animals [96]. When it came to lowering inflammation produced by paw edoema, SS was just as efficient as aspirin [96]. In a mouse model of LPS-induced inflammatory bone loss, Wu et al. [97] investigated the therapeutic potential of SSD and showed that SSD dramatically decreased LPS-induced inflammation bone loss in vivo. A study looking into the mechanism of action of SSD discovered that at 2 millimolar concentrations, it suppressed the proliferation and bone resorption of osteoclasts generated by Receptor Activator of Nuclear Factor B Ligand (RANKL) in vitro.

2.4.6. Anti-viral activity

HIV, measles, influenza, herpes simplex, and Streptococcus hydrophila viral proliferation may be inhibited by SSA, SSB2, SSC, and SSD, according to several hypotheses. Cheng et al. discovered that the IC50 for SSs varied from 0.25 to 25 M, with SSB2 having the greatest activity (IC50 = 1.7 0.1 M) against a wide spectrum of viruses. Among the SS, SSB2 is the most effective in halting the spread of HCV. The elimination of viral particles and the prevention of virus attachment and infection of cells may be possible thanks to SSB2 [12]. To demonstrate that SSB2 (10, 100 M) decreased HCV infectivity without impacting cell survival, we employed Huh-7.5.1 cells. Since SSB2 efficiently decreases HCV viral entrance into cells and RNA replication, it is likely that it either kills the virus or modifies the structure of the glycoprotein in such a manner that the virion is rendered noninfectious. Multiple viruses (including measles virus, dengue virus, herpes simplex virus type I, and unenveloped reovirus) may be stopped in their tracks by SSB2,

according to research. The inhibitory concentration (IC50) of SSC for inhibiting HBeAg secretion was 11 g/ml, while the IC50 for inhibiting HBV DNA expression was 13.4 g/ml, demonstrating its anti-HBV efficacy. HepG2 cells that are grown in parallel. 2.15 cells treated with 90 SSC (240 mg/mL) showed downregulation of the host transcription factors hepatocyte nuclear factor 1 and hepatocyte nuclear factor 4, upregulation of IL-6 expression, and suppression of HBV pgRNA production. Viruses like measles and herpes might be neutralised by a 5 M [98] SSD. SSD may contribute to the rupturing of the envelope by interacting with sialic acid residues of the viral glycoprotein. Extensive research confirms the cytotoxic effects and replication-inhibiting properties of type I SSs against influenza. Nepasaikosaponin K, saline sodium nitrate (SSN), and sodium selenite are potent antiviral medications because they attack the virus in a very precise way (SSH). It has been suggested that the enterovirus A71, which causes hand, foot, and mouth disease, might induce autophagy (EV-A71). Cells were protected from EV-A71-induced mortality when RNA replication was rapidly inhibited at 15 and 30 microM SSD [85].Following rapamycin-induced autophagy, the viral protein EVA71 was shown to be synthesised at a higher rate, but the autophagy-related protein ATG5 was synthesised at a lower rate. Evidence like these supports the idea that SSD and SSA might be used to protect against EV-A71. Acute respiratory illness associated with viral pneumonia (2019-nCoV) has also emerged as a consequence of this year's novel coronavirus (2019-nCoV). Research in the laboratory and on animals has shown that SSD has antiviral properties, and that these properties are effective against a broad range of respiratory viruses [26].

2.4.7. Anti-Andipogenic

Obesity is a disorder of lipid metabolism that may be influenced by a person's genes, medications, eating habits, and other aspects of their environment. Abnormal buildup of fat in the adipose tissue is the key distinguishing hallmark of this complex illness. Adipogenesis is the process through which immature adipocytes grow into cells that may aid in fat storage. To determine if SSA and SSD could be useful in the fight against obesity, Lim et al. [99] studied their effects on rat 3T3-L1 adipocytes. Both SSA and SSD are clearly detected in the range of 0.938-15 M that inhibits lipid production without affecting cell viability. Adiponectin synthesis and the expression of PPAR, CCAAT/enhancer binding protein, sterol regulatory element binding protein, and SREBP-1c were suppressed by SSA and SSD. Lipogenic genes such as fatty acid synthase (FAS), lipoprotein lipase, and fatty acid binding protein 4 (FABP4) were also inhibited as a result of the decrease of these transcriptional factors (LPL). Furthermore, phosphorylation of adenosine structural and functional protein kinase (Akt) and its substrate acetyl-CoA carboxylase (ACC) was enhanced by SSA and SSD, but phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 was decreased. However, both SSA and SSD had no effect on c-Jun-N-terminal kinase (JNK) (JNK). Such studies provide credence to the hypothesis that SSA and SSD inhibit adipogenesis through modulating AMPK and MAPK signalling pathways. Since this is the first study to evaluate the anti-adipogenic effects of SSA and SSD, more research is required in animals and humans to demonstrate the SSs' potential as therapeutic agents for obesity [85].

2.4.8. Anti-asthmatic activity

Intravenous administration of SSA to rats at doses ranging from 1 to 10 mg/kg decreased the occurrence of asthma episodes in the rodents, as observed by Park et al. SSA has a mild anti-allergic or anti-asthmatic action by limiting the activity of histamine and decreasing histamine production in mast cells, hence lowering bronchoconstriction. Tobacco smoke bitterness (SSB) is a direct agonist for the bitter receptor TAS2R14, which has anti-inflammatory and bronchiectasis-preventative effects. Further research is required to ascertain whether these drugs are effective in preventing asthma attacks. As a reference point, we found that chloroquine, at 100 mM, 50 mM, and 25 mM doses, greatly reduced IgE-induced hexosidase release from mast cells. At concentrations of 10.0 M and 5.0 M, respectively, SSB prevented IgE-induced mast cell degranulation [65].

2.4.9. Hormone-like activity

It has been said that menopausal symptoms, among other gynecological issues, may be alleviated with the use of *Radix Bupleuri*. 130 There are structural similarities between estradiol and SSD. Due to its ability to bind to the oestrogen receptor, it has a low to moderate phytoestrogenic effect. Treatment with SSD at concentrations between 0.01 and 10 microM significantly impacted MCF-7 cell growth and cell cycle progression. SSD loses its proliferative capabilities when combined with the anti-estrogen ICI-182780. This suggests that SSD may have an estrogenic effect on MCF-7 cells. 54 Through an ER-mediated mechanism, increasing concentrations of SSD (from 10 nM to 10 M) induced oestrogen response element (ERE)-luciferase activity [65]. As an oestrogen receptor agonist, SSD was a useful tool in the study of female reproductive biology. There is some evidence that SSD may trigger a selective activation of ER, resulting to increased levels

of both the mRNA and protein forms of this hormone. Adrenocorticotropin (ACTH) and corticosterone blood levels are raised when SSA is administered to a live organism. Furthermore, corticotrophin releasing factor (CRF) neurons are strongly affected by SSD (0.2, 2.0, and 20 g/kg, i.c.v.). By increasing CRF gene expression [79], SSD may increase CRF synthesis, which in turn increases anterior pituitary ACTH production and proopiomelanocortin gene production.

2.5. Saikosaponin B2

Terpenoids like SSb exist naturally. Melanoma patients may benefit from saikosaponin b2 (SSb2), a component of SSb that has been shown to have anticancer effects. High concentrations of SSb2 (60-100 M) trigger apoptosis in B16 melanoma cells, but low doses (5 M) are more likely to promote differentiation than death during a 30-day time span [20, 21]. Protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) strongly attenuates SSb2-induced apoptosis and differentiation at low and high concentrations, indicating that SSb2 exerts its anti-cancer action via down-regulating PKC activity. Since a lot of SSb2 is needed to induce cell death, this is a big downside. Thorough spectroscopic and chemical investigation may be used to identify the structures of saikosaponins. Structure-activity connections may now be investigated using an unique spectroscopic method (SAR). Results from SAR studies showed that SSa, SSd, and SSb all have potent anti-cancer activities. In cancer treatment [100], the selectivity and cytotoxicity of saikosaponins may be determined by the 13,28-epoxy bridge, where C-28 may be a methylene, hydroxymethylene, or carbonyl group, as indicated by structure-activity relationship (SAR) data. Recent SAR studies have demonstrated that

the effectiveness of saikosaponins in treating cancer depends on the spatial orientation of the hydroxyl group and the kind of sugar unit.

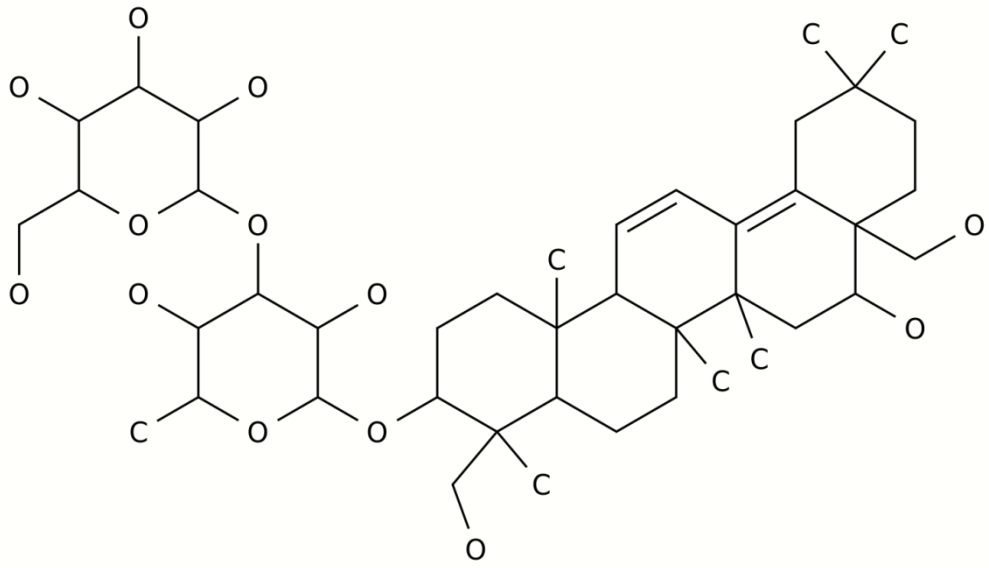

Figure 8: Chemical structure of Saikosaponin B2, (Liang-Tzung Lin et al, 2014)

Qing Ma et al. found that SSb2 inhibited STAT3 activity and the synthesis of VASP, MMP2, and MMP9, hence reducing MCF-7 cell proliferation and migration. In addition, the results indicated that SSb2 may prevent the spread of breast cancer cells. The results of this research, which were only evaluated on the MCF-7 line, need to be replicated in additional breast cancer cell lines. This study [101] sheds light on a novel molecularly focused therapeutic strategy for patients with breast cancer.

According to Liang-Tzung Lin et al. [12], SSb2 has the potential to be studied as a preventative medicine before, during, and after a liver transplant. In addition, it has been shown that a high concentration of SSb2 induces cell cycle arrest at G1 phase and death in B16 melanoma cell lines through a likely PKC pathway. Treatment of B16 melanoma cells over time with a low dose of SSb2 produces differentiation and inhibits melanogenesis while having no effect on cell proliferation [52].

2.5.1. Mechanism of action

Saikosaponins treat diseases in a wide range of ways. There is a complex web of processes at work in SSb2 that regulates its pharmacological effects. Even though SSb2 is involved in pro go along, where it reduces oxidative stress by lowering apoptosis, and in anti-inflammatory paths, which are linked to neurological pathways, it still inhibits multidrug resistance via pgp mediators, causing apoptosis and increasing oxidative stress [36].

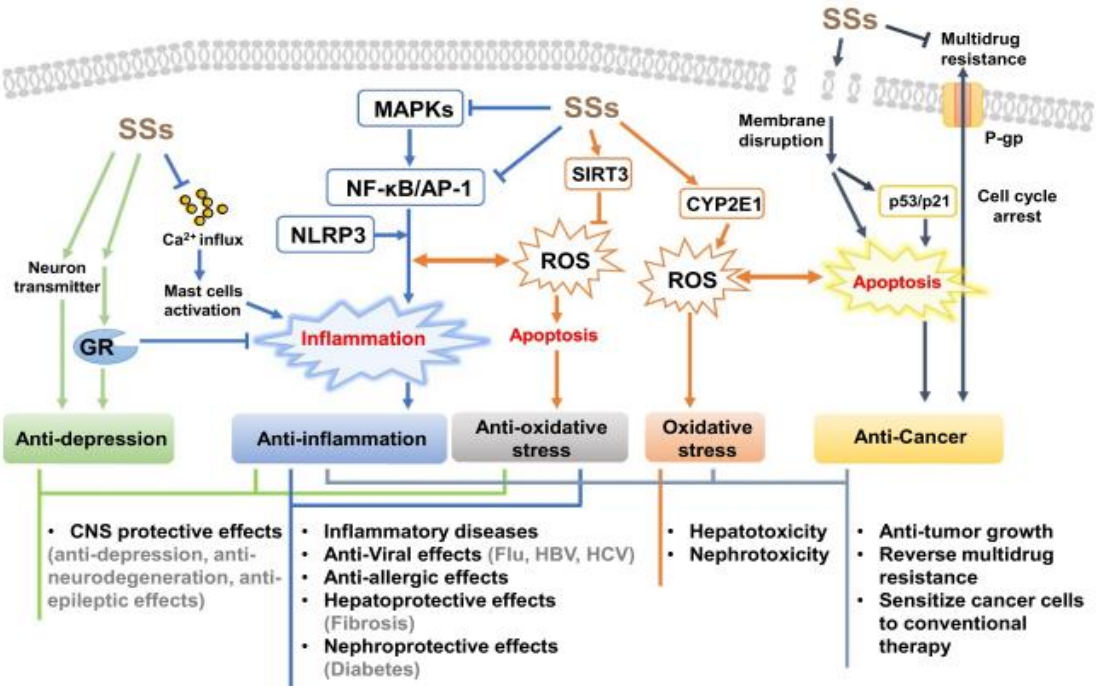

Figure 9: Putative pathways involved in SSs bioactivities, [36]

2.5.2. Role in oxidative stress

Oxidative stress has been related to a wide range of diseases and conditions, including ageing, cancer, reactive arthritis, cardiovascular disease, rheumatoid arthritis, neurological issues, and narcotic-induced liver and kidney damage. Animal models of

depression, atherosclerosis, diabetic nephropathy, and chemical-induced liver damage have revealed that RB-containing herbal formulations such Da-Chai-Hu-Tang, XiaoChai-Hu-Tang, and Chai-Hu-Shu-Gan-San have antidepressant, anti-atherogenic, anti-diabetic, and anti-hepatotoxic properties. Both SSa and SSd, when present as purified monomers, have been shown to protect cells from chloroform- and CCl4-induced peroxidation and liver damage by restoring normal hepatic superoxide dismutase activity, boosting hepatic antioxidant defence capacity, removing ROS, and reducing lipid peroxidation. Supporting our previous findings, further studies show that SSd efficiently protects the hepatocyte cell line HL-7702 cells against CCl4-induced massive oxidative stress and further reduces NLRP3 inflammasome-mediated inflammation. Finally, SSd mitigated oxidative stress caused by heat and high glucose by decreasing ROS production and increasing antioxidant enzyme expression/activity in renal tubular cell lines. The buildup of reactive oxygen species (ROS) and the MAPK/NF-B pathways triggered in response to ROS are both reduced by SSd, protecting kidney function against cisplatin's nephrotoxicity. Neurons are protected by SSd against H2O2-induced oxidative damage, MPP+-induced peroxidation and cytotoxicity in SHY5Y cells caused by downregulation of MAPK signalling and overexpression of SIRT3. As a bonus, SSd prevented cell death and oxidative stress in wounded lung tissue that was caused by a breathing machine. However, new studies show that SSa and SSd boost ROS formation and oxidative stress, which renders certain cancer cell lines more vulnerable to cisplatin. Our previous research shown that a relatively high dose of the SSs combination was related to enhanced lipid peroxidation and impaired hepatic antioxidant responses, both of which led to the development of acute liver damage. Despite their apparent contradiction, these

findings demonstrated that the effects of SSs on oxidative stress varied with dose and cell type [102].

Chapter 3

3. Materials and methods

3.1. Materials

Benzo(a)pyrene (BAP) and 7 12-dimethylbenz(a)anthracene were purchased from Sigma-Aldrich(USA). Saikosaponin B2 was purchased from European Pharmacopoeia Standard. Cyclophosphamide (CP) was purchased from Uniphos Chemicals (India). All the chemicals and reagents used in the present experiment were of analytical grade. BAP was dissolved in olive oil. DMBA and SSb2 were dissolved in DMSO. Cyclophosphamide was dissolved in distilled water.

3.2. Animals

20 male albino mice (Balb/c) were purchased from Atta-ur-Rehman School of Applied Biosciences Lab Animal House National University of Science and Technology H-12, Islamabad. Animals were 6-8 weeks of age (weighing 25-32 g). All the experimental activities were performed in Atta-ur-Rehman School of Applied Biosciences Lab Animal House National Institute of Science and Technology H-12, Islamabad according to recommendation of institutional bioethical committee. During study animals were housed under standard environmental conditions i.e. controlled temperature, pathogen free environment, 12h light-dark cycles and free access to water and food. Animals were subjected to acclimatization period of 1 week. Animals were housed in standard home cages. All animal experiment was performed according to guidelines of the National Institute of Health (NIH) “Principles of Laboratory Animal Care.” Similarly, the experimental protocols mentioned in the current guidelines for the laboratory animals

care and ethical recommendation issued for the commencement of pain experiments in conscious animals were followed.

3.3. Experimental Design

Mice were divided into four groups with five mice per group. This grouping was based on the mode of treatment that the mice will undergo.

Table 1: *Grouping of mice*

Sr.no	Groups	Size	Label
1.	**Group 01**	**05**	**Control**
2.	**Group 02**	**05**	**BAP+DMBA**
3.	**Group 03**	**05**	**BAP+DMBA+SSb2**
4.	**Group 04**	**05**	**BAP+DMBA+CP**

Group 1: animals in this group were given normal water and feed. Group 2 (B(a)P/olive oil+DMBA/DMSO): animals in this group were given 20mg/kg B(a)P dissolved in olive oil 3 days/week for 4 weeks and 1.5mg/kg DMBA dissolved in DMSO once/week for 3 weeks intraperitoneally (i.p). Group 3 (B(a)P/olive oil+DMBA/DMSO+SSb2/DMSO): animals in this group were given 20mg/kg B(a)P dissolved in olive oil 3 days/week for 4 weeks and 1.5mg/kg DMBA dissolved in DMSO once/week for 3 weeks intraperitoneally (i.p). During 3rd week of disease induction animals were given intraperitoneal injections of 10mg/kg SSb2 dissolved in DMSO for 20 days consecutively. Group 4 (B(a)P/olive oil+DMBA/DMSO): animals in this group were treated with 20mg/kg B(a)P dissolved in olive oil 3 days/week for 4 weeks and 1.5mg/kg DMBA dissolved in DMSO once/week

for 3 weeks. During 3rd week of disease induction animal were given intraperitoneal injections (i.p.) of 50mg/kg of CP dissolved in distilled water for 14 days consecutively.

Figure 10: Mice is given intraperitoneal injection

3.4. Euthanization and sample storage of mice

Mice were sacrificed at the start of 6th week using chloroform anesthetization. Before euthanization body weight of mice were recorded then they were euthanized through abdominal incision and whole lungs and livers from all groups were dissected and weighted. Lungs were cut into two halves. First half was stored in 10% formalin and other half was washed with PBS and stored at -80 degrees. Blood samples were collected via cardiac puncture in serum tubes.

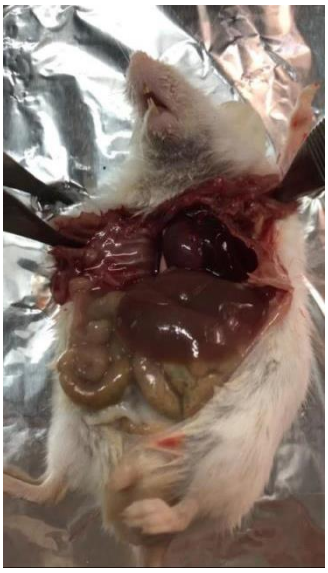

Figure 11*:*Euthanization of mice

3.5. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and adenosine monophosphate (AMP) activities

Blood samples collected in serum-tubes were given to Metropole laboratories private limited (MPL), Islamabad and results of ALT, AST and AMP were obtained.

Figure 12: Blood collected in serum tube after euthanization

3.6. Histological Examination with H&E staining

First half stored in 10% formalin solution were used for slide preparation. Slides of lungs of H&E stain were prepared and organ embedded paraffin plates were prepared from Ali pathology lab, Islamabad. Pictures of slides were taken under a light microscope with magnification 100x.

3.7. Tissue homogenate Preparation

Second half of lungs stored at -80 degrees were used for homogenate preparation. At the time of homogenate preparation, lungs were thawed and homogenized in PBS by a tissue homogenizer at 1000rpm for 5 minutes. Homogenates were centrifuged at 8000g at 4 degrees for 10 minutes and supernatants were collected.

3.8. Determination of lung GSH, GST, SOD, and catalase concentrations

Antioxidant enzyme levels in mouse lungs were analyzed by homogenizing lung tissue. Lung homogenate catalase concentrations were determined with a conventional technique described by Aebi et al. This test relied on catalase's ability to break down H2O2 as its foundational concept. The microplate reader was set to detect a shift in absorbance at 240 nm. Lung homogenate GSH levels were determined using the gold standard technique described by Ellman et al. free thiol groups reacted with DTNB of GSH to produce a yellow chromophore, which was used as the basis for this test. The resulting hue was detected in a microplate reader at a wavelength of 412 nm. Lung homogenate GST concentrations were determined using the gold standard technique described by Warlhom et al. This assay's fundamental concept relied on tracking the rate at which GSH was coupled to CDNB. The microplate reader tracked the absorbance shift at 344 nm [103]. The levels of SOD in lung homogenate were measured using the conventional technique

described by Lowry et al. This test relied on autooxidation background in the absence of SOD as its fundamental premise. The microplate reader was set at 525 nm to detect the absorbance shift [104].

3.9. Lipid peroxidation Assay

Malondialdehyde (MDA) was measured by lipid peroxidation of lung homogenate, as described by Ohkwa et al. A microplate reader was used to measure the absorbance shift at 532nm [105].

3.10. Statistical Analysis

The results were reported as means and standard deviations (S.D.). The statistical analysis was performed using Graph Pad Prism version 8.0.1 (software). Significant differences between groups were determined using one-way analysis of variance and unpaired Student's t-test. It was decided that statistical significance was achieved at a p value of less than 0.05.

Chapter 4

4. Results

Present study shows the effect of SSb2 on B(a)P induced lungs cancer in mice by regulating oxidative stress.

4.1. Effect of SSb2 on B(a)P induced lungs cancer on body, lungs, and liver weight

Whole-body weight of lungs cancer induced mice reduced by 1.5 folds .Treatment with SSb2 and CP helped in recovery of final body weight of lungs cancer induced mice as shown in Fig. 1(A). Relative lungs and liver weights of lungs cancer induced mice increased.Lungs weight of lungs cancer induced mice significantly increased. Treatment with SSb2 helped in recovery of lungs weight towards normal as shown in Fig.1(B). Weight of liver of lungs cancer induced mice increased 1.5, 1.2 folds respectively. Treatment with SSb2 and CP helped in recovery of liver weight towards normal..

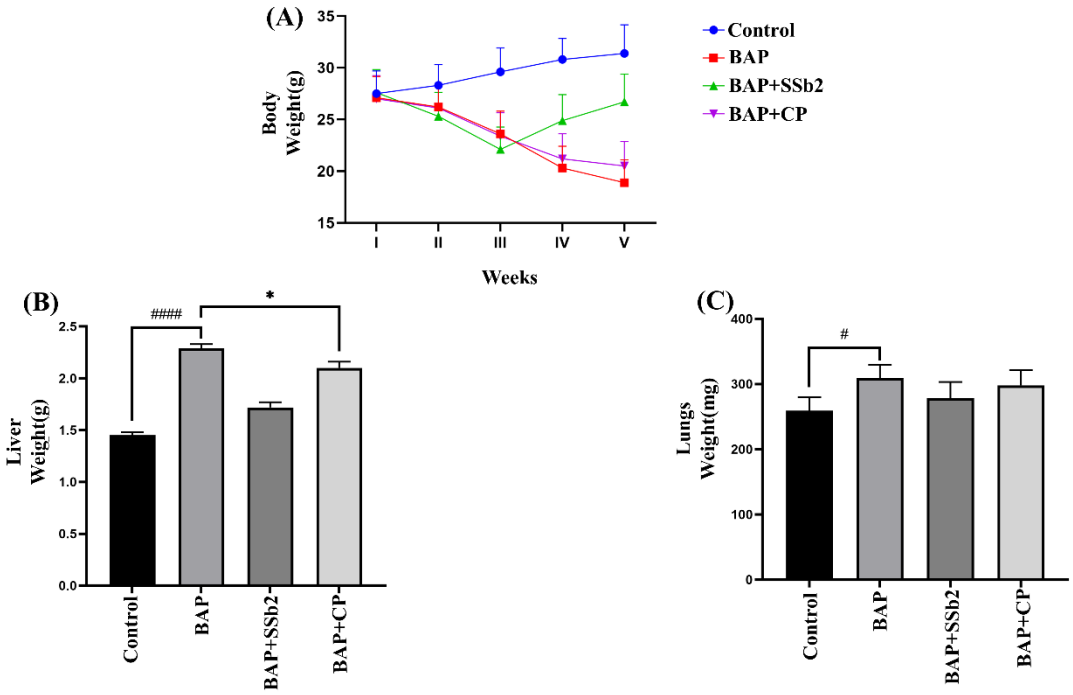

Figure 13: Comparison of weekly body weight, lungs and liver weight. Panel A depicts mean ± SD body weight (n=5) of Balb/C mice before dissection. Treatment of lungs cancer induced mice with SSb2 significantly recovered body weight to normal. Panel B&C describes the comparison (mean ± SD) of lungs and liver weight respectively. The intergroup comparisons was done with one-way ANOVA. $p<0.05$ was taken as significant and was represented as(*). All the groups were compared with BAP group (diseased). Difference between control and BAP group was represented as (#). Statistical difference of $p<0.05$ was taken as significant difference, was represented as (#).

4.2. Restoration of hepatic biomarkers ALT, AST & ALP after treatment with SSb2

Hepatic biomarkers ALT, AST &ALP started reducing back to normal after treatment with SSb2. Fig.2 (A, B, C) shows that after induction of treatment hepatic biomarkers increased significantly 1.5, 1.3, 1.4 folds respectively. They started coming back to

normal after treatment with SSb2 1.1, 1.3, 1.1 folds respectively. Treatment with CP also showed slight reduction in levels of hepatic biomarkers.

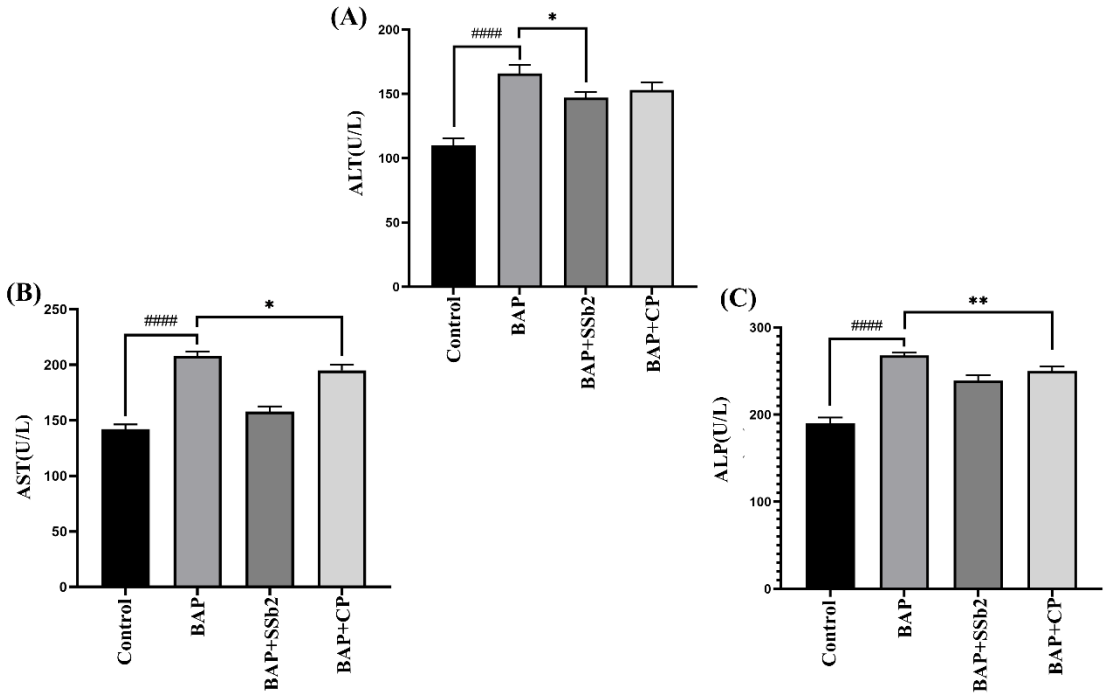

Figure 14*:* Comparison of hepatic biomarkers A. ALT, B AST & C ALP in blood serum of Balb/c mice. Treatment with SSb2 started reducing levels of hepatic biomarkers. Values are shown as mean ± SD (n=5). Results were interpreted using one-way ANOVA. P<0.05 was taken as significant and was represented as (*). All the groups were compared with BAP group (diseased). Difference between control and BAP group was represented as (#). Statistical difference of p<0.05 was taken as significant and represented as (#).

4.3. Effect of SSb2 treatment on oxidative stress markers

Oxidative stress markers: Catalase**,** GSH, GST**,** SOD and MDA started coming back to normal when treated with SSb2. Fig.3 shows that after induction of lungs cancer oxidative stress markers started increasing. Catalase and GSH value increased 1.8 folds,

GST value increased 1.9 folds, SOD value increased 1.6 folds, and MDA value increased 1.5 folds. After treatment values of oxidative stress markers decreased significantly.

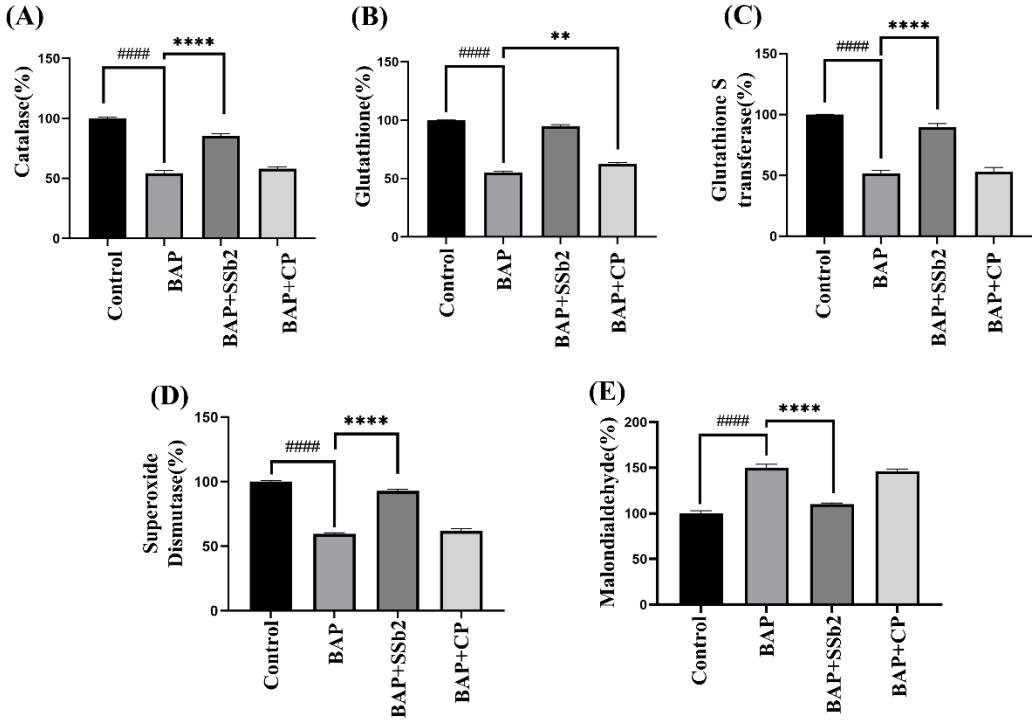

Figure 15*:* Comparison of oxidative stress markers A Catalase B GSH C GST D SOD E MDA in lung homogenate of Balb/c mice. Values are shown as mean ± SD (n=5). Results were represented in percentages. Results were interpreted using one-way ANOVA. P<0.05 was taken as significant and was represented as (*). All the groups were compared with BAP group (diseased). Difference between control and BAP group was represented as (#). Statistical difference of p<0.05 was taken as significant and represented as (#).

4.4. Effect of SSb2 on lungs histopathology of BAP challenged mice

Modifications occurred in lungs histology in experimental models is represented in fig 4. Fig 4 (BAP+DMBA) shows alterations occurred in BAP challenged mice after disease induction, this shows injury in alveolar pattern, inflammation and architecture loss as

compared to normal animals that shows intact alveolar pattern, no inflammation and intact architecture. (BAP+DMBA+SSb2) shows the alterations in lungs histopathology after treatment with SSb2 which shows normal architecture with no inflammation but shows injury in alveolar pattern to some extent. However, treatments with CP shows that inflammation infiltration and alveolar injury occurred to some extent with curtail alveolar injury as compared to BAP challenged mice.

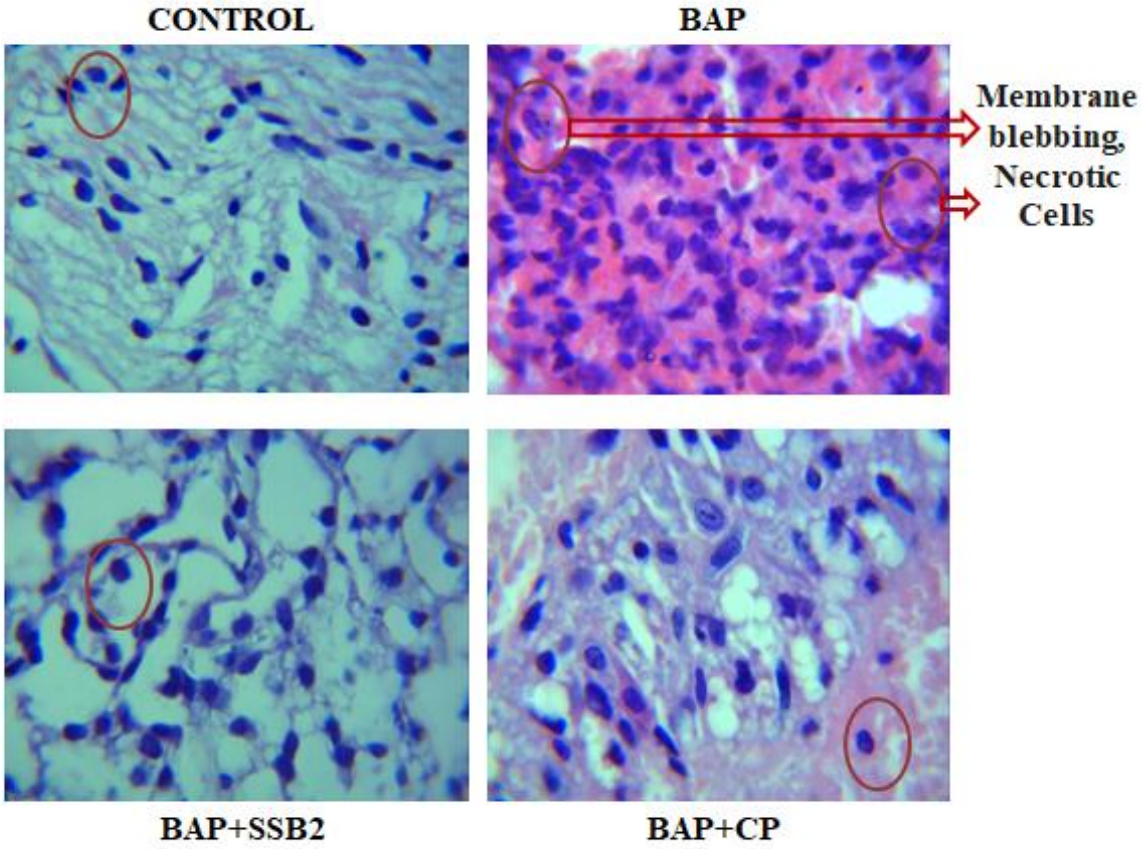

Figure 16*:* Effects of SSb2 on lungs histology in BAP-induced lungs cancer. H&E staining method was used to check the alterations in lungs of mice model. BAP-induced mice displayed loss in normal architecture with inflammation and abnormal cell growth and loss of alveolar pattern. The SSb2 treated mice showed no inflammation and intact

architecture and alveolar pattern which is near to normal. SSb2 treated mice showed better results as compared to CP treated mice.

Chapter 5

5. Discussion

Lungs are one of the most vital organs in our body which is responsible for proper functioning of our respiratory system and also controls gaseous exchange i.e. oxygen/carbon dioxide from our body to environment and environment to our body during respiration. This means that lungs are main targets of carcinogens like B(a)P and air contaminated with environmental pollutants [106]. In the origin of lungs cancer B(a)P has been proved to have to major role. About 73% of lungs cancer cases are identified at last stages when small number of treatments are available. To overcome such scenarios more studies have been done on progression and treatment of lungs cancer [107]. Only lungs cancer treatment available at present is chemotherapy which cause more harm than good to patient as it has side effects like immunosuppression and haemopoietic tissue toxicity [108].

Currently, tactics like oxidative stress regulation through antioxidative agents from plant origin are being used for treatment of lungs cancer which is commonly known as chemoprevention. Lungs cancer prompted through B(a)P can be tackled effectively through chemoprevention [109]. This study investigates the efficiency of saikosaponin B2 against B(a)P induced lungs cancer in mice model. The compound worked by regulating oxidative stress in body of mice.

Saikosaponins are the major active compound found in dry roots of *Radix Bupleuri* which is extracted from a Chinese plant called *Bupleurum chinense*. There are many subtypes of saikosaponins but most common are SSa, SSd, SSc, SSb1, SSb2. All these saikosaponins show antiviral, anti-inflammatory, anticancer, anti-oxidative properties. Previously so

much work has been done on SSd and SSa to check their activity against cancer or viruses. Less work has been done on SSb2 [99]. Previously SSb2 activity was checked on B16 melanoma cell lines and it was reported that SSb2 induced apoptosis and G1 cycle arrest probably through following protein kinase pathway at higher concentration while its long term treatment at lower concentration induced melanogenesis with cell proliferation and cell differentiation of B16 melanoma cells [102]. Its activity on cell culture derived HCV has also been checked where it inhibited the entry, replication and translation of virus [12]. SSb2 activity on human coronavirus 229E cell lines has also been checked which showed SSb2 showed no cytotoxic effect on cell viability and it has more anti- HCOV-229E activity as it possibly worked by interfering with early stages of viral replication [98]. No work has been previously done to check the anti-cancer property of SSb2 on animal model as it showed great results with melanoma a cell line that is why in the present study its effect was checked on lung cancer induced mice model.

In the present study, SSb2 showed impressive results. Overall bodyweight of the mice having lungs cancer decreased (Fig.1A) as cells started rapidly dividing in their body so the energy which is otherwise stored in body also invested in cell division. Treatment of mice with SSb2 helped in recovery of body weight to considerable extent. Organ weights during in-vivo experiment increased which indicated cell proliferation leading to hypertrophy in these organs (Fig.1B-C). Observed hypertrophy was due to infiltration of malignant cells in these vital organs i.e., lungs and liver. As a result, these organs may enlarge and increase in weight.

Slight improvement in hepatic biomarkers was observed (Fig.2A-C) which showed that SSb2 showed slight cytotoxicity as all the drugs are broken down in liver, but this can be solved through using nanotechnology or by using any other drug as an adjuvant with SSb2. As shown in (Fig.2A-C) cytotoxicity hepatic biomarkers i.e., AST, ALP and ALT increased which showed that B(a)P was highly cytotoxic to liver along with inducing lungs cancer.

Improvement in oxidative stress markers i.e., CAT, SOD, GSH, GST and MDA was observed (Fig3.A-E) which showed B(a)P treated mice showed increase in MDA level. MDA is an end product of LPO increase in MDA levels indicates lipid peroxidation. LPO is a reactive molecule which can cause tumorigenesis by reacting with DNA [110]. These toxic radicals act as arbitrate in tissue lipid peroxidation. CAT, SOD, GSH and GST mitigate the level of tissue lipid peroxidation. Increase in MDA level in B(a)P treated mice showed more lipid peroxidation than the mice treated with SSb2. CAT is responsible for breakdown of H2O2 into water and oxygen while SOD is responsible for the conversion of superoxide ions into H2O2. Decrease in level of SOD and CAT indicated accumulation of toxic superoxide and H2O2 radicals in lungs of B(a)P treated mice while increase in their level after treatment with SSb2 showed downregulation of such toxic radicals. GSH is a major non-enzymatic antioxidant which is responsible for regulation of redox reaction by combating ROS specie production. Decrease in GSH level indicated overuse of GSH during cellular combat against ROS and increase in case of SSb2 treatment indicated decrease in oxidative stress by combating ROS. GST binds epoxides with GSH which is discharged as mercapturic acid. Decrease in GST level in

lungs of B(a)P treated mice during present study showed that toxic epoxides accumulated in then while after treatment GST level started coming towards normal [111].

Conclusion

In conclusion, the present study with saikosaponin b2 showed calculated results in in-vivo experiments which shows profitable effect against B(a)P induced lungs cancer in mice. Henceforth, these results indicated that saikosaponin b2 is a promising chemo preventive agent for treatment of lungs cancer. However, in future further research should be done on the type of pathway it uses for being a chemo preventive agent and its cytotoxicity should be mitigated by using nanotechnology.

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