Chemical Investigation and Antiproliferative screening of extracts from *Stevia rebaudiana* (Bertoni)

A Thesis submitted to the Central University of Punjab

For the award of

Master of Pharmacy

In

Pharmacognosy and Phytochemistry

By

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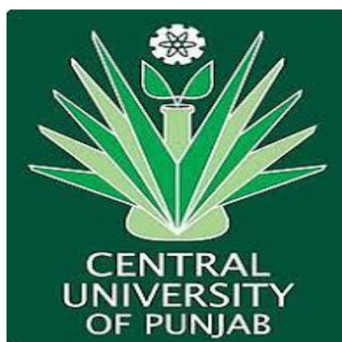

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June, 2018

DECLARATION

I declare that thesis entitled **"Chemical Investigation and Antiproliferative screening of extracts from *Stevia rebaudiana* (Bertoni)"** has been prepared by me under the guidance of Dr. Vikas Jaitak, Assistant Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

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ABSTRACT

Cancer is the uncontrolled development of abnormal cells in the body. It is considered as the leading public health problem in both developed and the developing countries. As no drug of cancer is establish to be completely efficient and safe as anticancer therapy and is responsible for the prolonged toxicity and also causes various side effects. Chemoprevention of cancer by natural products is beneficial, as these compounds have the nominal side effects and short of toxicity compared to the synthetic compounds. The phenomena of Carcinogenesis is very complex and includes so many signaling pathways. Thus Phytochemicals are measured as the right candidates for developing the anticancer drug. The study for developing more potent candidates which can obstruct or slow down the expansion of the cancer cells without causing any side effects from these phytochemicals are still in progress and Many new phytochemicals and its derived analogs have been recognized as potential candidates for anticancer therapy among these one of the potent plant is Stevia rebaudiana. The leaves of Stevia rebaudiana tends to possess zero calories, and consists mainly of ent kaurene diterpene glycosides generally recognized as steviol glycosides. Stevioside is the main sweet component found in S. rebaudiana. studies suggest that the stevioside along with

other associated compounds including rebaudioside A, steviol, and isosteviol tends to have therapeutic benefits including anticancer activity. Taking in consideration the above mentioned factors we have investigated the Anticancer potential of extracts of S. rebaudiana. Four extracts was prepared using petroleum ether, chloroform, ethyl acetate and aqueous methanol. T47D cell line have been used to evaluate the anticancer potential using MTT assay. AD-2 that is chloroform extract showing IC_{50} value of 7.79µg/ml. Moreover IC_{50} value of AD-4 that is aqueous methanol was also comparatively better and found to be 9.53 µg/ml and AD-1 that is petroleum ether had shown IC_{50} value of 9.58µg/ml. Thus, various extracts have shown good Antiproliferative activity and S. rebaudiana can be further investigated for its anticancer potential. Furthermore docking study on estrogen receptor–alpha, androgen receptor and aromatase receptor discovered that the phytochemicals of the plant have good binding affinity towards all the three mentioned receptors and can be suitably customized to search its anticancer potential. Moreover unfavorable ADME profile can be overcome by structure modification. Thus on the basis of in-vitro and in-silico data we can conclude that S. rebaudiana extracts have promising anticancer potential. further isolation of compounds have been done successfully and total four compounds have been isolated. two compounds ASP-2 and ASP-4 have been successfully characterized and found to be Stevioside and Rebaudioside A respectively which are already known compounds.

(Aditi Saxena) **(Dr. Vikas Jaitak)**

Dedicated

To

My Loving Family

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List of Abbreviation

Sr. No.	Full form	Abbreviation
1.	Ductal carcinoma In-situ	DCIS
2.	National cancer institute	NCI
3.	Estrogen receptor	ER
4.	Sex hormone binding globulin	SHBG
5.	Steviol glycosides	SGs
6.	Stevia rebaudiana	SR
7.	Phosphophenol pyruvate carboxy kinase	PEPCK
8.	Tumor necrosis factor	TNF
9.	Interlukin	IL
10.	Nitric oxide	NO
11.	Standard precision	SP
12.	Extra precison	XP
13.	Grid based docking with energetics	GLIDE
14.	Absorption,distribution,metabolism and excretion	ADME

CHAPTER 1.0

INTRODUCTION

1 INTRODUCTION

The word “cancer” for the first time was coined by Hippocrates, known as father of western medicine, who connected Greek words “carcinoma” and “Karakinos” to express tumor. Cancer is considered as the hazardous disease in which the cells keeps on dividing without any control that can also attack to other close tissues. In normal condition the process which takes place to protect the secure condition of tissues is meiosis and apoptosis whereas the procedure which is involved in Carcinogenesis is consists of multi mechanism (Figure 1)(Safarzadeh et al.,2014).

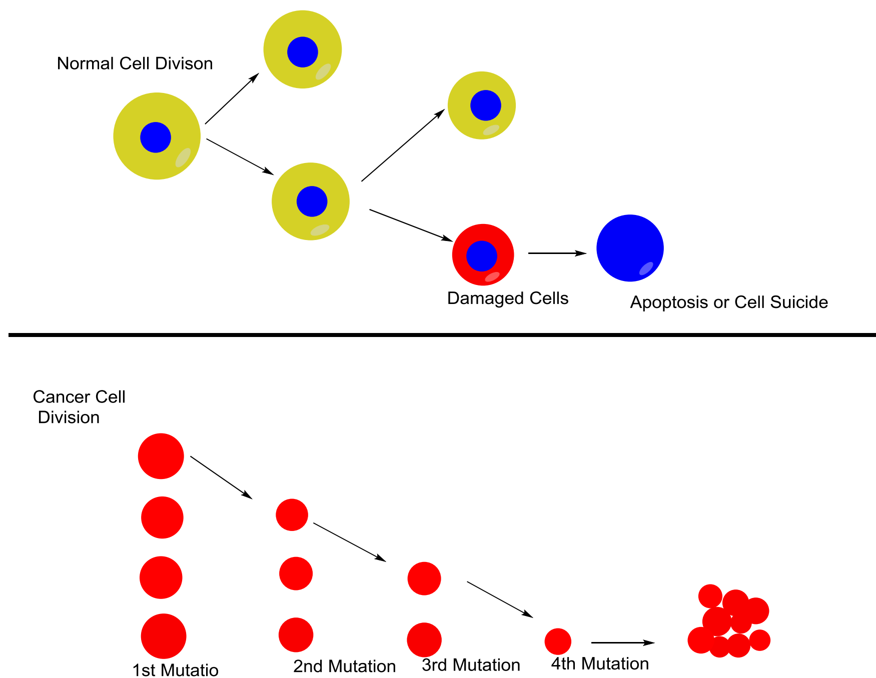


Figure 1.1: Difference between normal cell and cancer cell.

Cancer is considered as the foremost public health problem in both developed and developing countries. More than 9.7 million cases are detected each year and 6.7 million people died from cancer and everyday, around 1700 Americans died of this disease and approx 20.4 million people are living with cancer in the world today and almost 1 in 3 people were diagnosed with cancer in the UK and 1 in 4 died from this deadly disease. It has been predicted by WHO that the new cases of cancer will reach 15 million until 2020 (Tavakoli et al., 2012). Cancer is the second most important cause of death in the United States after the cardiovascular disease where

every one person among the four dies due to cancer (Manju et al., 2017). The substances which are responsible to cause cancer are known as carcinogens. The main reason behind the cause of cancer is mutation which make changes in DNA and loses the control over the growth of the cells (Manju et al., 2017) . Likewise some other factors which causes cancer includes external factors including radiations, smoking, tobacco, toxins in drinking water, foodstuff, atmosphere, certain metals and transmittable agents and internal factors like hereditary mutations, body immune system and hormonal issues are highly responsible for this deadly disease (Iqbal et al., 2017). Today there are more than 100 different types of cancer are known and are usually named by the organ or type of cell from where they start like a cancer that begin in the colon is called colon cancer (Zaid et al., 2017). According to National Cancer Institute (NCI) classification, different types of cancer are classified as follows(Safarzadeh et al., 2014).

• Carcinoma: Cancers resulting from epithelial cells. It includes most of the regular cancers, mainly in older adults like breast, prostate, lung, pancreas, and colon cancer.

• Sarcoma: Cancers resulting from connective tisssue like bone, cartilage, fat, nerve each of which develop from cells originating in mesenchymal cells exterior to the bone marrow.

• Lymphoma and leukemia: these both types of cancer results from the cells tht are involved to make blood. Leukemia is the most general type of cancer mainly in children accounting for almost 30%.conversely, in case of adults both types of cancer can develop.

• Germ cell tumor: these types of Cancers are resulting from pluripotent cells, mainly present in the testicle or the ovaries .

• Blastoma: These types of Cancers are resulting from undeveloped "precursor" cells or developing tissue. Its mainly occur in the children compare to adults.

Among these the lung cancer is reported the top listed in male followed by breast cancer in female (Zhou et al., 2017)

1.1 BREAST CANCER

Breast cancer (BC) is the most commonly diagnosed cancer and is the leading cause of death among women worldwide(Tabatabaei et al., 2016). It is a malignant tumor that gets started in the cells of breast. The most common Signs of BC include a lump in the breast, changes in shape and size of the breast, dimpling of the breast skin, release of liquid from the nipple without squeezing ,pain in the breast that last forever, swelling in the armpit area and at the site of collarbone and a red scaly patches in the skin. Breast cancer most commonly develops in cells lining the milk ducts and the lobules that supply the ducts with milk (Kabel et al., 2015).Similar to other cancers, there are various factors that can elevate the risk of receiving breast cancer like female sex, obesity, short of physical exercise, consumption of alcohol, hormone replacement therapy in menopause, high exposure to ionizing radiation, early age at the time of first menstruation and old age(Gøtzsche et al., 2013). High exposure of estrogen damage the DNA and causes genetic mutation which can result into the BC. In addition to this few people acquire defects in the DNA and mutation in BRCA1, BRCA2 and P53 genes due to hereditary reasons as compare to other peoples. Thus the person who have the family history of ovarian or breast cancer are at increased risk of BC(Kamińska et al., 2015).

1.1.1 STATISTICS

In high wages countries like the United States, about 232340 ladies has been diagnosed and 39620 has been died from breast carcinoma in 2016(Siegel et al., 2018). In 2017, an estimated 252,710 new instances of invasive breast cancer has been diagnosed among ladies and 2,470 instances has been diagnosed in men. Moreover, 63,410 cases of in situ breast carcinoma were diagnosed among ladies and Around 40,610 ladies and 460 men died from breast cancer in 2017(DeSantis et al., 2017) and In 2018 more 1,735,350 new cancer cases and 609,640 cancer deaths are expected to occur in the United States(Siegel et al., 2018). It has been reported that in an American lady, the danger of rising breast cancer in their entire lifetime is 12.38% or 1 out of 8(Nowsheen et al., 2017).

1.1.2 TYPES OF BREAST CANCER

BC can be categorized as invasive or non-invasive.

Ductal carcinoma in situ (DCIS) is a non-invasive breast cancer moreover it is also recognized as stage 0. In the DCIS, the unusual cells are controlled in the milk ducts of the breast and do not broaden into the neighboring breast tissue. Whereas incase of Invasive breast cancer abnormal cells do multiply from their original site that can be either the milk ducts or the lobules that is the sacs that make breast milk to the neighboring breast tissue. It might also have multiply to the lymph nodes.(Horlings et al., 2013). All the invasive breast cancers and DCIS are tested for hormone receptors. steroid hormone are mainly responsible for this deadly disease specially estradiol, which plays an significant function in the development of breast cancer, and a majority of the human breast cancers begin out as estrogen dependent and expresses the estrogen receptor (ER)(Clarke et al., 2003). From the literature it has been confirmed that the elevated levels of endogenous estrogens and androgens and minor levels of sex hormone binding globulin (SHBG) are linked with higher danger of postmenopausal breast cancer (Hormone et al., 2013)These breast cancers can be treated with hormone therapy such as tamoxifen and aromatase inhibitors. Nearly all the invasive breast cancers are hormone receptor-positive(Abeshouse et al., 2015).

1.1.3 ROLE OF ESTROGEN, ANDROGEN AND AROMATASE IN BREAST CANCER

The steroid hormone, estradiol, has the very significant function in the development of BC, and mostly the human breast cancers found were estrogen dependent and express the estrogen receptor (ER). Estrogen mediates its biological effect by binding to one of the structurally and functionally different ERs that is ERα and ERβ (Saha Roy et al., 2012). Over expression of estrogen caused the risk of breast cancer as much clear mechanism is not known. In normal condition where the level of estrogen is maintained the chance of getting breast cancer is very low, but mutation in the certain metabolites of estrogen that when bind to DNA causes the hydroxylation of estrogen and induce the quinone and semiquinone formation(Santen et al., 2015). The quinone form of estrogen binds with DNA and produce DNA adducts and begins

the transcription of proteins which is responsible for the cause of breast cancer. Semiquinone intermediate are free radicals which can bind to oxygen and produce superoxide radicals and alter the structure of DNA (Figure1.2.)(Santen et al., 2015). Aromatase receptor play an important role in breast carcinogenensis. The aromatase enzyme is the important enzyme for the biosynthesis of estrogen. This enzyme is also known as estrogen synthase. It induces aromatization of androgens to estrogens hence responsible for the overexpression of estrogen(Yamamoto et al., 2014). From the literature it has been confirmed that about 60% of BC state this enzymes with elevated levels of mRNA expression and activity when compared among the non-malignant tissue. It catalyzes the final steps of estrogen biosynthesis androgens to estrogens particularly conversion of testosterone to estradiol and androstenedione(Group et al., 2015). Androgen receptor is expressed in 60-70% of breast cancer and plays a dual role with estrogen. Inhibition of Androgen receptor is mainly responsible for the down regulation of ERK signaliing therefore androgen receptor induce its effect by genomic and non genomic pathways. In the genomic pathway androgen bind and directly control the expression of gene in DNA and in case of non genomic signaling where nucleus receptor gets signals with other proteins interactions. Androgen receptor is responsible for the breast cancer mainly due to irregulation of growth hormone and cytokines. this combined signaling contribute majorly for the BC(Iacopetta et al., 2012).

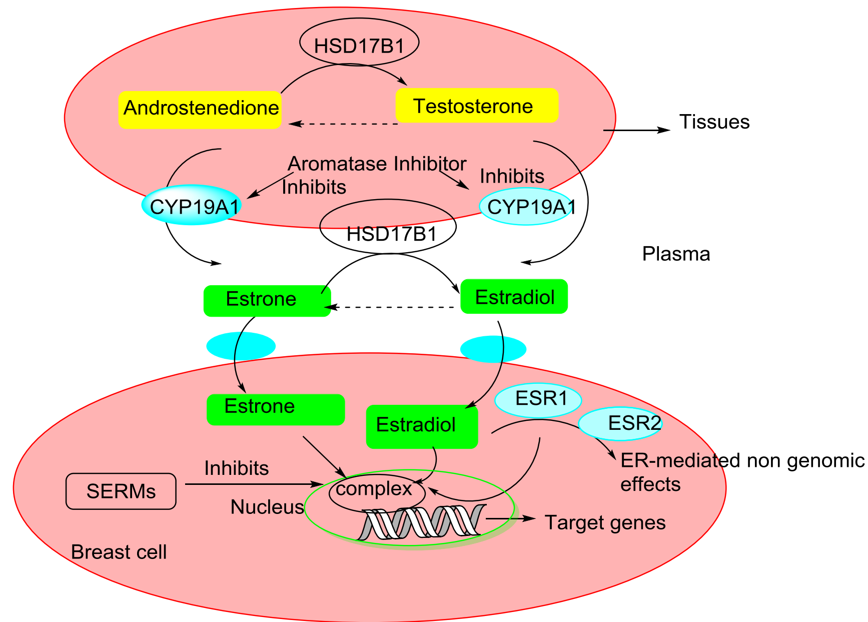


Figure1.2: Role of Estrogen, Aromatase and Androgen in Breast Cancer

1.1.4 THERAPIES FOR BREAST CANCER

The main treatments used for cancer are chemotherapy, radiotherapy and surgery(Solowey et al., 2014). A successful anticancer drug should destroy or injure the cancer cells devoid of causing unnecessary damage to the normal cells. But this ideal process by the anticancer drug is very complex, or maybe impossible to achieve and is the main reason for the unpleasant side effects from which the patients have to suffer when under-going treatment (Manju et al.,2017). the main Chemotherapeutic agents includes cytostatic and cytotoxic drugs which have revealed excellent outcome either alone or in the grouping with additional cancer therapies. Some main chemotherapeutic agents includes the topoisomerase inhibitors like- Irinotecan which shows the side effects including neutropenia, sensory neuropathy, and diarrhoea and doxorubicin whose having the high risk of causing cardiotoxicity. The alkylating agents used for curing cancer includes oxaliplatin, melphalan, carboplatin, cisplatin and cyclophosphamide but shows the side effects including cardiovascular toxicity, nephrotoxicity, gastrointestinal toxicity,

pulmonary and hematologictoxicity (Pegram et al., 2004). In addition to this the main drawback about these drugs is the cancer cells resistance to these drugs as they go through mutation like ABCA4 and ABCA12 are the Drug resistant genes which are over-expressed in human MCF-7 breast cancer cells correspondingly when the drug docetaxel was used for the treating the cancer (Iqbal et al., 2017)

As there are many drugs available for the treatment of cancer but it also have number of side effect thus it is very important to look for novel anticancer agents which have superior efficiency and minor side effects. In this case Natural compounds are considered as the good sources for developing new remedies for the treatment of various diseases(Aung et al., 2017).

1.1.5 USE OF MEDICINAL PLANTS TO TREAT BREAST CANCER

Medicinal plants and herb have been used from ancient times to treat human chronic diseases including cancer much before the invention of modern drugs(Manju et al., 2017). At present about 60% of drugs used for the treatment of cancer are obtained from natural products in which the plant kingdom has been the most important source(Solow ey et al., 2014).

Plants have various active compounds which work synergistically for giving the therapeutic benefits and bringing down the dangers of side effects so that no other supplemental therapy is required to control the cancer debility. Thus it has made very important to uphold the use of natural Ayurvedic therapies for curing various types of cancers and imply an incorporated approach for the management of tumor and for its treatment(Shukla et al., 2015).Some important plants used for the treatment of BC as well as for other cancers are mentioned in the Table 1.1. (Safarzadeh et al., 2014).

Table 1.1: List of important medicinal plant having major anticancer phytochemicals

Plant name	Family	Phytochemicals	Specific cancer
Allium sativum	Liliaceae	Allin	Carcinoma of human(mammary) Gland
Aloe vera	Liliaceae	Aloesin, emodin	Anti-angiogenic activity
Linum usitatissimum	Linaceae	Cynogenetic glycosides	Breast cancer
Momordica charantia	Curcurbitaceae	Charantin, triterpenes, cucurbitane-type	Breast cancer, colon cancer
Stevia rebaudiana	Asteraceae	Labdane sclareol properties	Anti-tumorous and cytotoxic
Gymnema sylvestre	Asclepiadaceae	Gymnemagenol	Anticancer
Peganum harmala	Zygophyllaceae	Harmine	Breast cancer
Artemisia annua	Asteraceae	Artemisinin	Liver, breast and pancreatic cancer
Solanum nigrum	Solanaceae	Solamargine,solasonine	Liver, breast, lungand skin cancer
Vigna unguiculata	Fabaceae	Black-eyed-pea trypsin/chymotrypsin inhibitor	Breast cancer
Curcuma longa	Zingiberaceae	Curcumin	Liver, breast, lung, prostate, oesophagus, colon and skin cancer

Cicer arietinum	Fabaceae	Bowman-birk-type protease	Breast and prostate cancer
Hibiscus mutabilis	Malvaceae	Lectin	Liver, breast cancer
Centella asiatica	Apiaceae	Asiatic acid, Tamoxifen	Breast cancer

The therapeutic significance of the plants are due to the occurrence of chemical substances that generate a specific physiological action in the human body as mentioned above In the Table 1.1. Main other bioactive compounds of the plants used for the treatment of diseases include alkaloids, flavanoids, tannins and phenolic compounds. Also, several number of plant leaves tends to have antimicrobial principles such as tannins, essential oils and new aromatic compounds. In addition to that some preclinical studies have confirmed that the phytochemicals have great importance in the prevention of colorectal cancer and other cancers(Jayaraman et al., 2008). Thus Herbal medicines are refined natural compounds which can control the different phases of diseases at the same time by the different mechanisms (Shukla et al., 2015). Whereas the chemical medicines are the individual synthetic compounds that can be intrusive in an ideal condition by the single mechanism. Mainly the anticancer drugs used for the treatment of cancer whether it is synthetic chemicals or the natural products, tends to cooperate with the DNA or its precursors which produces the irreversible harm to DNA and restrain the synthesis of proteins. Thus, curing the cancer cells by using the mono-target chemical agent is not an efficient method. hence, on the basis of broad research conclusion, the phytochemicals and their resulting analogues are considered as the best option for the enhanced and less lethal for the cancer treatment(Singh et al., 2016). As there are number of plants whose phytochemicals are used today for the treatment of many diseases as well as for the cancer and among them one of the very important plant is Stevia rebaudiana belonging to the Asteraceae family in which the major sweet constituent in the leaves of the Stevia rebaudiana (Bertoni) is stevioside which is 300 times

sweeter as compare to sucrose and has just gained significance as a natural non-caloric sweetener(Siddique et al., 2016).Beside sweetness, stevioside along with other associated compounds including rebaudioside A, steviol, and isosteviol may also recommend therapeutic benefits, such as antioxidative, antihyperglycaemic, anti-hypertensive, antitumor, antidiabetic, anti-HIV(Paul et al., 2012). It is also reported that the isosteviol inhibited DNA polymerases and DNA topoisomerase II. Which are considered as the important cellular targets for the development of anti-cancer agents(Mizushina et al., 2005). Moreover Stevia leaf extracts and the presense of polyphenolic constituents have revealed the inhibitory effect on tumor commencement and its promotion (Heikal et al., 2008). Thus, information concerning the structural characteristics of stevioside-based compounds may provide valuable insight for the design of new anti-cancer agents.

CHAPTER 2.0

REVIEW OF LITERATURE

Chapter 2

Review of literature

Stevia rebaudiana (SR) is considered as the medicinal herb which has been utilized in the traditional Armenian medicine to lowering down the glucose, cholesterol and blood pressure levels and also adjust the immune function of the body.(Aghajanyan et al.,2017). It is originated from the northeast of Paraguay(Mathur et al.,2017). The initial botanical explanation of the plant was acknowledged by M. S. Bertoni In 1889. The plant was earlier recognized as Eupatorium rebaudianum Bert. in tribute of Rebaudi, the first chemist who studied the chemical distinctiveness of the substances extracted. Its name was afterward changed to the present one. The genus Stevia incorporates 230 species but only S. rebaudiana provides the sweet taste property. Some other related species include S. eupatoria, S. micrantha, S. plummerae, S. rhombifolia, S. serrata, S. salicifolia, S. viscida, S. commixta, S. origanoides, S. leptophylla, S. satureiaefilia, S. ophryphylla, S. selloi, S. nepetifolia and S. triflora (Ruiz-Ruiz et al., 2017). Stevia is about 200-300 times sweeter than the sugar; its sweetness effectiveness is alike to that of aspartame. Stevia is cultivated worldwide due to its function as a non-caloric sweetener. It has been utilized for many years in the treatment of diabetes in various countries as it have no toxic harm as well as no side effect (Yadav et al., 2011). It have great value as a economic medicinal plant since it have pharmaceutically active compounds all through the world. Stevia has been used a non-caloric natural-source having other remedial applications together with anti-cariogenic, anticancer, antioxidant and antidiabetic properties. Biotechnological techniques present novel approaches for its industrial production, propagation as well as conservation and management of Stevia (Karimi et al., 2017).

2.1. PHARMACOGNOSTICAL CHARACTERISTICS

Table 2.1: Taxonomical classification of *S. rebaudiana.*

Kingdom	**Plantae**
Subkingdom	Tracheobionta
Superphylum	Spermatophyta
Phylum	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Group	Monochlamydae
Order	Asterales
Family	Asteraceae(compositae)
Subfamily	Asteroideae
Tribe	Eupatorieae
Genus	Stevia
Species	rebaudiana

2.1.1 COMMON NAMES

Sweet leaf, sweet herb, honey leaf, Stevia, sweet leaf of Paraguay, caa-he-éé, kaa jheéé, ca-a-jhei, ca-a-yupi, azucacaa, eira-caa, capim doce, erva doce, sweet-herb, honey yerba, yaa waan, candy leaf, sugar leaf, sweet honey leaf, Eupatorium rebaudianum(Jayaraman et al., 2008; Marković et al., 2008).

2.1.2 GEOGRAPHICAL SOURCE

The genus Stevia, mainly Stevia rebaudiana is initially from Paraguay. The sweet feature of Stevia has been used by the Paraguayan Indians from several centuries. It is native to the northern regions of South America. Stevia is still found growing wild in the highlands of the Amambay and Iguacu districts which is the border area flanked by Brazil and Paraguay. It is predicted that almost 200 species of Stevia are native to South America; conversely, none of the other Stevia plants have acquired the similar

intensity of sweetness as the S. rebaudiana. It is developed commercially in many parts of Paraguay, Brazil, Israel, Uruguay, Thailand, Central America and China. it is also refined on a minor scale in Canada, Mexico, USA, some European countries and also with the Israel. In india it is mainly cultivated in Karnataka, Punjab, himachal Pradesh, Haryana, uttar Pradesh, west Bengal , Madhya Pradesh and tamil nadu. In Japan Stevia have been utilized for more than 50 years as a sweetening agent in a variety of foods and beverages(Brandle et al., 1998; Mandal et al., 2013).

2.1.3 PLANT MORPHOLOGY

Stevia rebaudiana is a herbaceous perennial plant belongs to the Asteraceae family. The plant reaches upto 65cm in height, having sessile, oppositely arranged lanceolate to oblanceolate, dark green colour leaves with toothed margins. The taste of leaves is sweet. The seeds of Stevia are commonly small, brown and black in colour and on maturation the seeds turn into black coloured fruits which are dispersed with the help of persistent pappus bristles as in the case of dandilon. The root system of Stevia rebaudiana is on the surface and with a little lateral root. The flower are little in size, white in colour, are bisexual and fashioned in clusters of 2 -6 florets, through sprouts that are initially tender and finally gets harden (Lester et al.,1999; Shaffert et al.,1994).

2.2. CHEMICAL COMPOSITION

2.2.1 Steviol glycosides

Steviol glycosides are the most important components of Stevia rebaudiana which is responsible for its sweetener capacity. It commonly contains glucose moieties which are joined with the aglycone part termed as Steviol **(1)**(Puri et al., 2011) . Isosteviol **(2)** is the oxidized product of steviol mainly famous for its therapeutic benefits including anticancer activity(Jaitak et al., 2008). The plant consists of more than 40 different steviol glycosides in varying concentrations(Oehme et al.,2017). The major steviol glycosides found are Stevioside **(3)** and Rebaudioside A **(4),** having the highest content in the plant. The percentage of stevioside found to be 6–10%, and of rebaudioside A is 2–4% and the other minor glycosides is up to 0.1-1%.

Stevioside is 250-300 times sweeter than sucrose whereas rebaudioside-A is 300-400 times sweetener than sucrose(Singla et al., 2016). the other steviol glycosides found in the plant are Rebaudioside B **(5)**, Rebaudioside C **(6)**, Rebaudioside D **(7),** Rebaudioside E **(8),** Rebaudioside F **(9),** Rebaudioside G **(10),** Rebaudioside H**(11),** Rebaudioside I**(12),** Rebaudioside J **(13),** Rebaudioside L **(14),** Rebaudioside M **(15),** Rebaudioside N **(16),** Rebaudioside O**(17),** Rebaudioside R **(18),** Rebaudioside S **(19),** Rebaudioside T **(20),** Rebaudioside U **(21),** Dulcoside A **(22),** Dulcoside B **(23),** Rebusoside **(24),** steviolbioside **(25).** Structure of various isolated steviol glycosides is represented in Figure 2.1

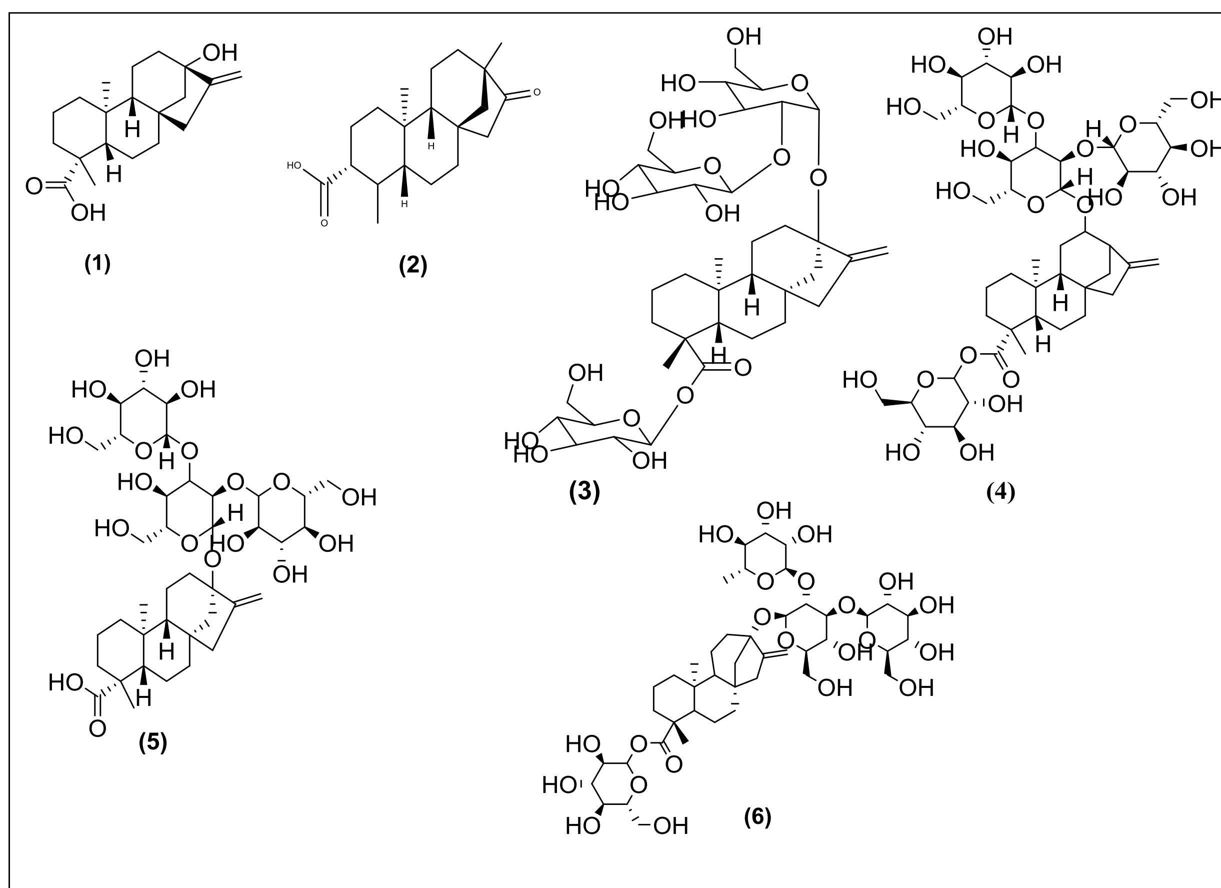


Figure 2.1: Chemical structure of steviol glycosides

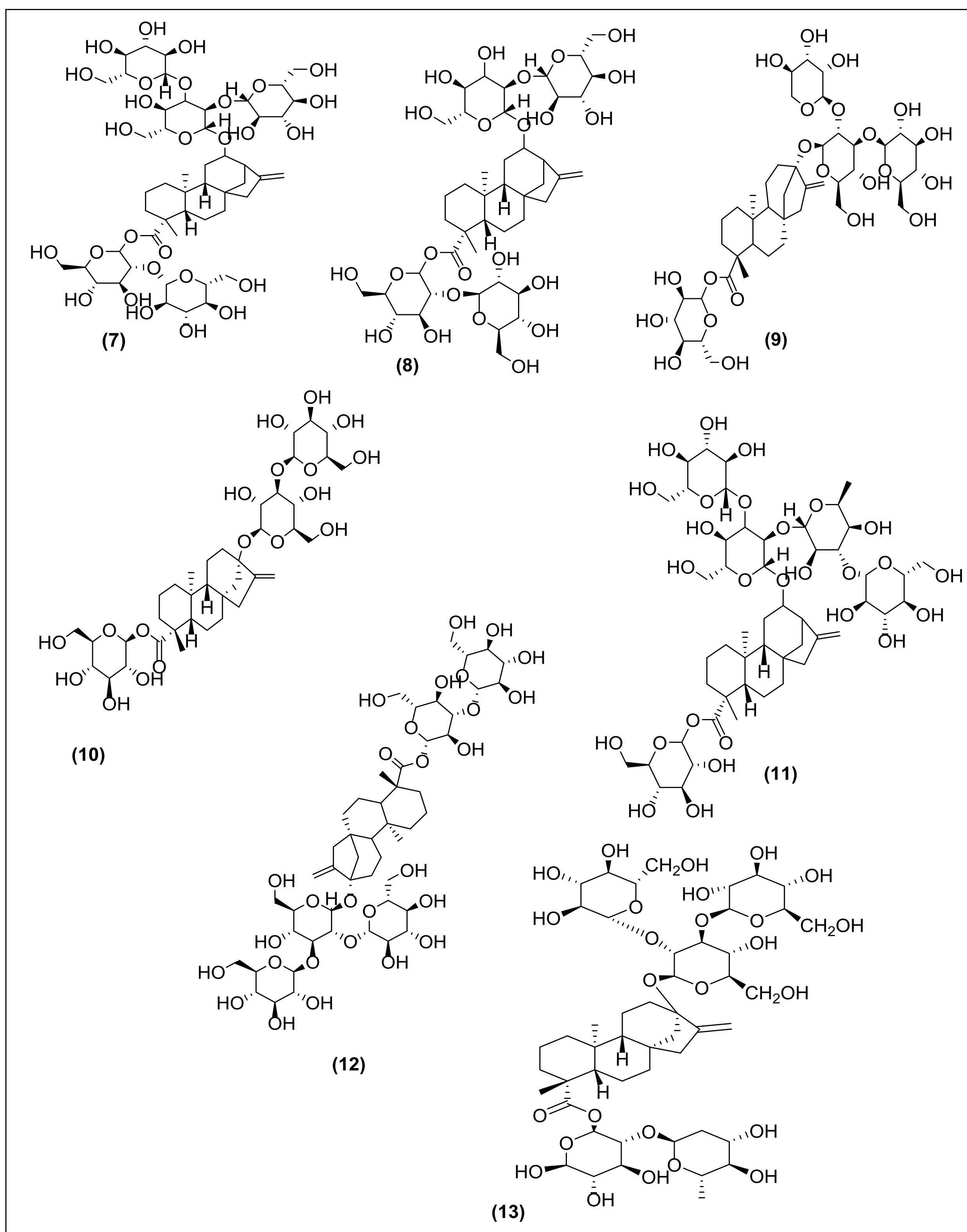


Figure 2.1: Continue

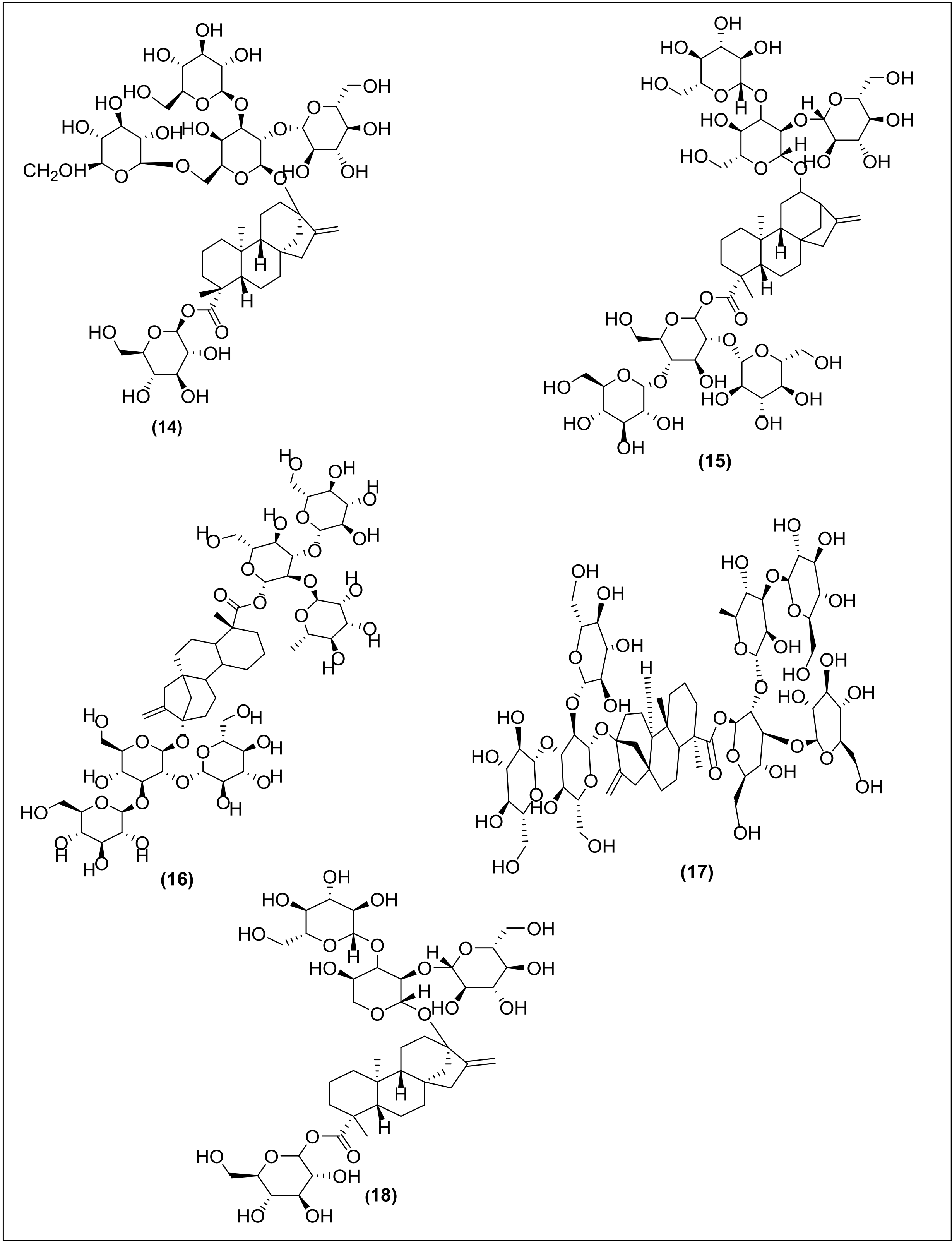
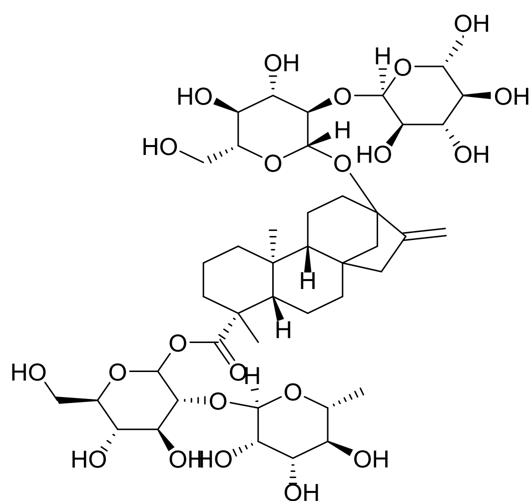
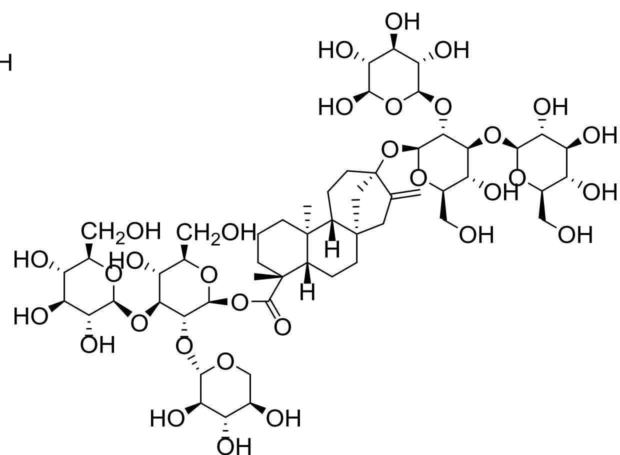
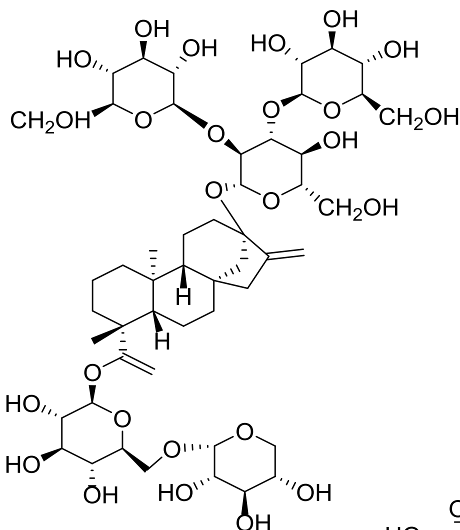
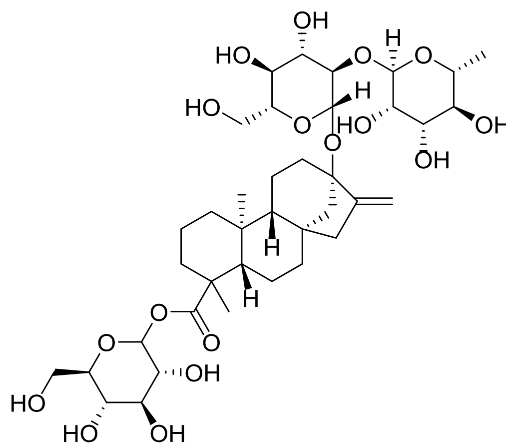


Figure 2.1: Continue









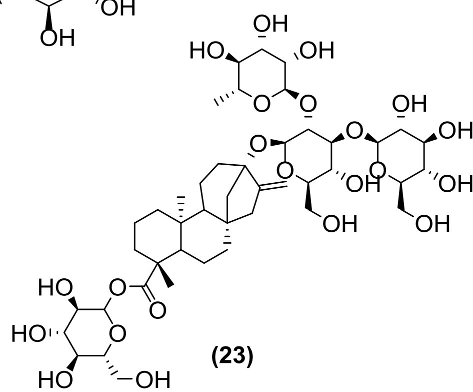


Figure 2.1: Continue

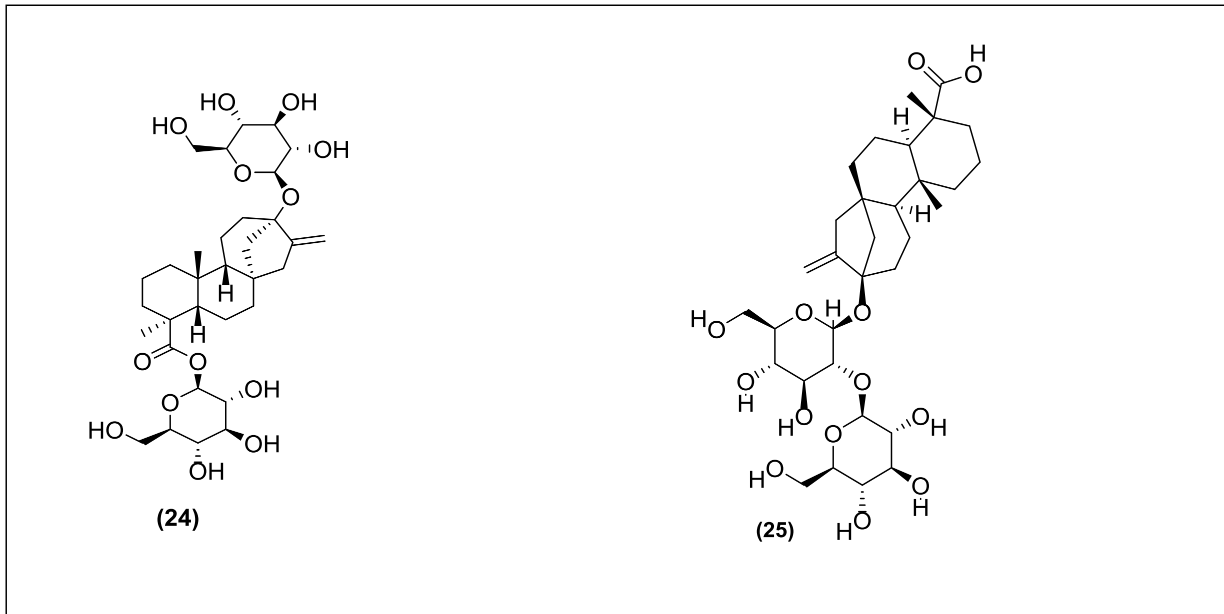


Figure 2.1: Continue

2.2.2 Non-glycosidic diterpenes

Labdane-type diterpenes comes under this category of constituents of S .rebaudiana. the various compounds of this group are austroinulin **(26)** jhanol **(27),** Sterebins **(28-40),**(Ibrahim et al., 2007). The chemical structure of these non –glycosidic diterpenes has been mentioned in Figure 2.2

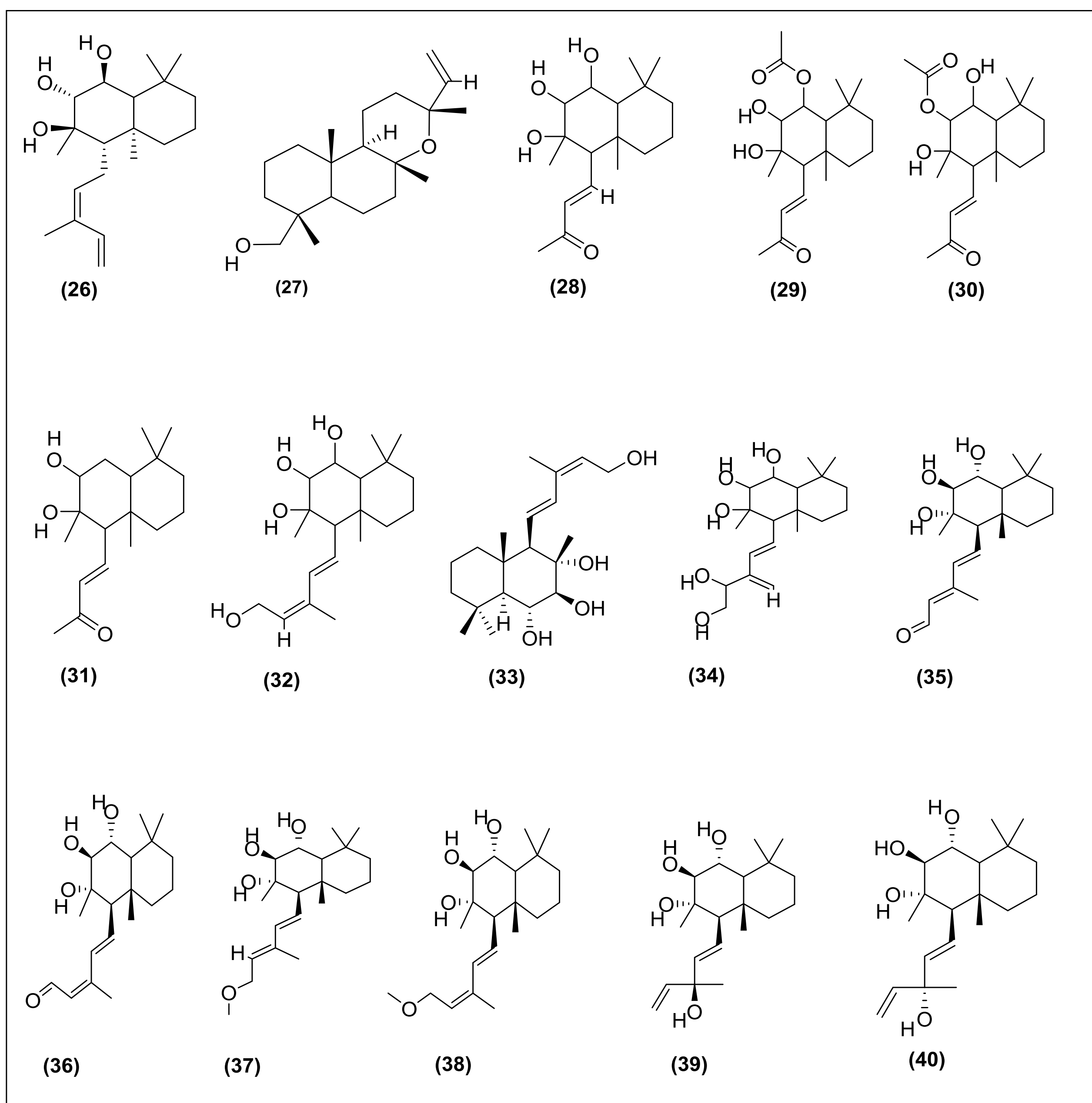


Figure 2.2: Chemical structure of Labdane-type diterpenes

2.2.3 Flavonoids

The plant contains numerous number of flavonoids including quercetin **(41),** apigenin 4-O-glucoside **(42),** apigenin **(43),** Kaempferol 3-O-rhamnoside **(44),** luteolin 7-O-

glucoside **(45),** quercetin 3-O-rhamnoside **(46),** quercetin 3-O-glucoside **(47),** apigenin 7-O-glucoside **(48), r**utin **(49),** centaureidin **(50)** (Wölwer-Rieck et al., 2012). The chemical structure of these flavonoids has been shown in Figure 2.3

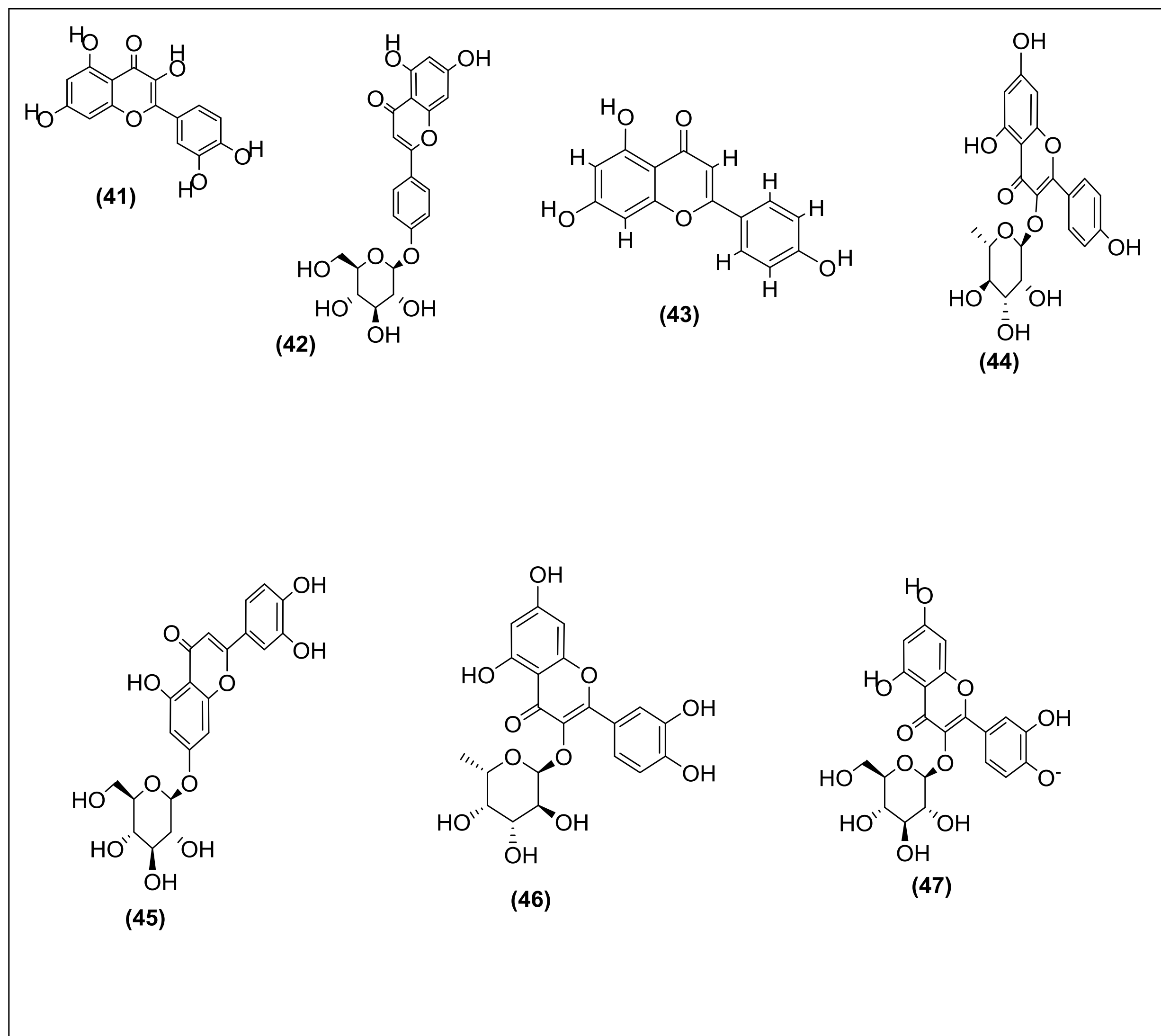


Figure 2.3: Chemical structures of flavonoids

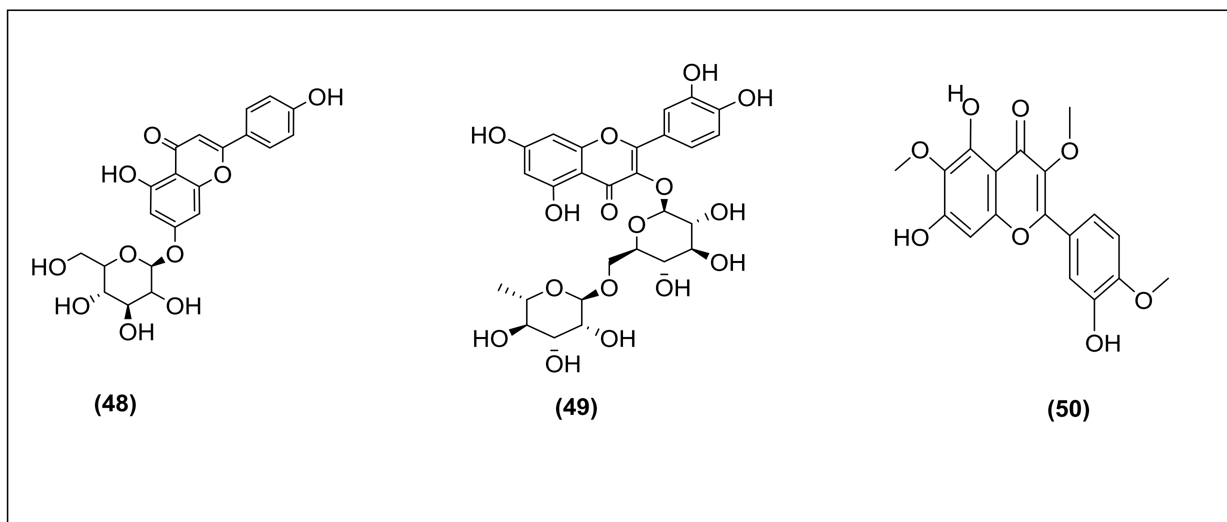


Figure 2.3: Continue

2.3. BIOSYNTHETIC PATHWAY OF SGS

Steviol glycoside molecules come under the diterpene group of compounds which tends to have huge number of biological functions in plants. The Production of these diterpene group of compounds have the common primary biosynthetic pathway. Poly –isoprene is the basic backbone of all the diterpene compounds. Isoprene is a simple organic compound having the following chemical structure (Figure 2.4).

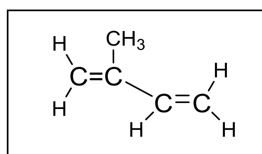


Figure 2.4: Chemical structure of Isoprene

The structure of poly-isoprene is mentioned in Figure 2.5.

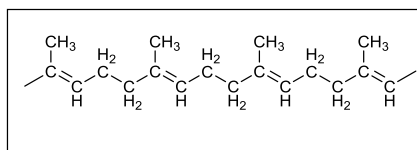


Figure 2.5: Chemical structure of poly-isoprene

structure of Geranylgeranyl diphosphate is meintioned below (Figure 2.6) it is the main precursor for numerous compounds found in the plants including the steviol glycosides.

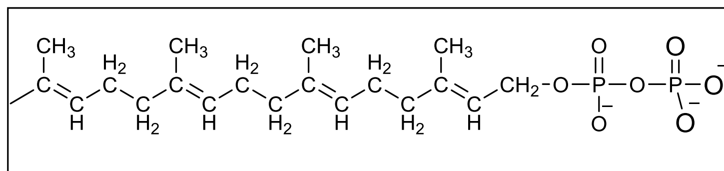


Figure 2.6: Chemical structure of Geranylgeranyl diphosphate

Biosynthesis of steviol glycosides consist of two stages. first stage includes the Geranylgeranyl diphosphate or GGDP synthesis from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Geranylgeranyl diphosphate is the initial point for various biologically essential diterpene compounds present in the plants. In the second stage, steviol glycosides are formed by the conversion of Geranylgeranyl diphosphate by following multiple steps. The two main precursors that is isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are produced by a very important pathway in plants known as 2-C-methyl-D-erythritol-4 phosphate pathway or MEP pathway. The MEP pathway is described below with the formation of GGDP(Brandle et al., 1998). The steps followed in this pathway can be catalyzed only in the presence of some definite enzymes. In case if these specific enzymes are absent, the biochemical reactions cannot be completed.

STEP 1

In this step Pyruvate and Glyceraldehyde 3 phosphate are combined to form Deoxyxyulose 5 phosphate which further gets phosphorylated by the Cytidine triphosphate. This is the beginning of the MEP Pathway (Figure 2.7).

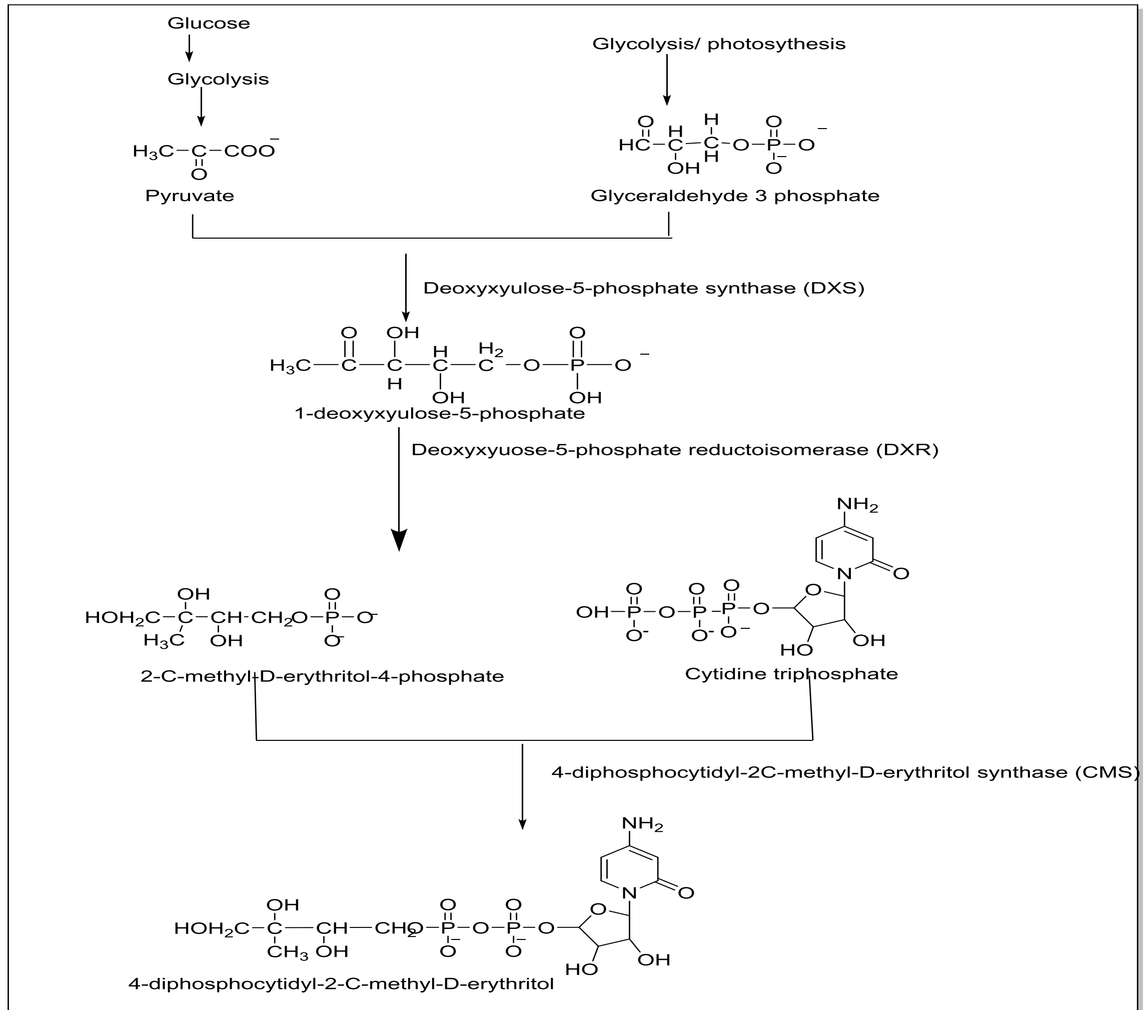


Figure 2.7: Formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol

STEP 2

Further phosphorylation by Adenosine Triphosphate (ATP) followed by formation of 2-C-Methyl-D-Erythritol-2,4-Cyclodiphosphate (Figure 2.8).

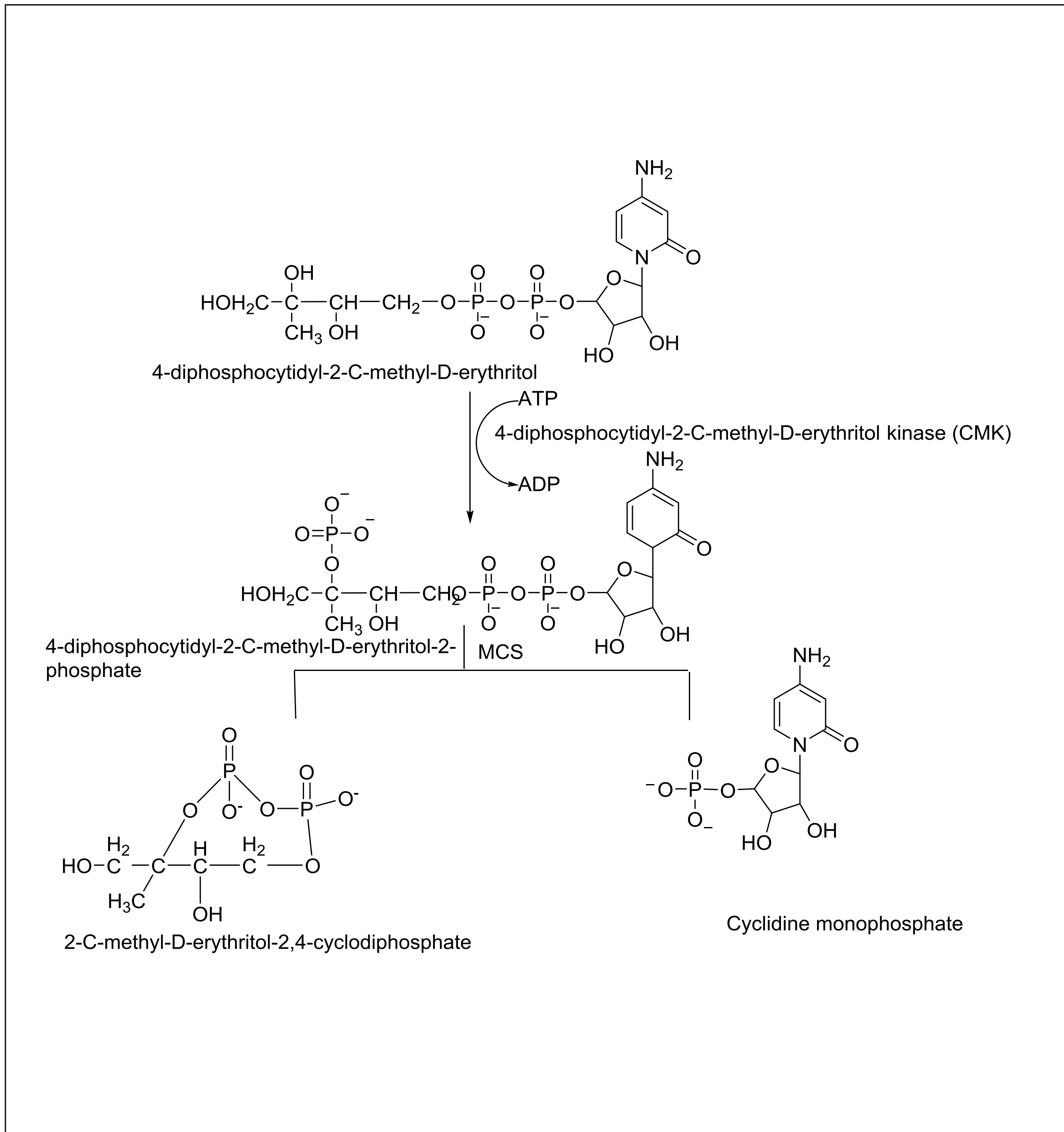


Figure 2.8: Formation of 2-C-Methyl-D-Erythritol-2,4-Cyclodiphosphate

STEP 3

In this step Isopentenyl Diphosphate and Dimethylallyl Diphosphate are formed. These are known to be the precursors for variety of isoprenoid compounds found in the plants. This is the last step of MEP pathway (Figure 2.9).

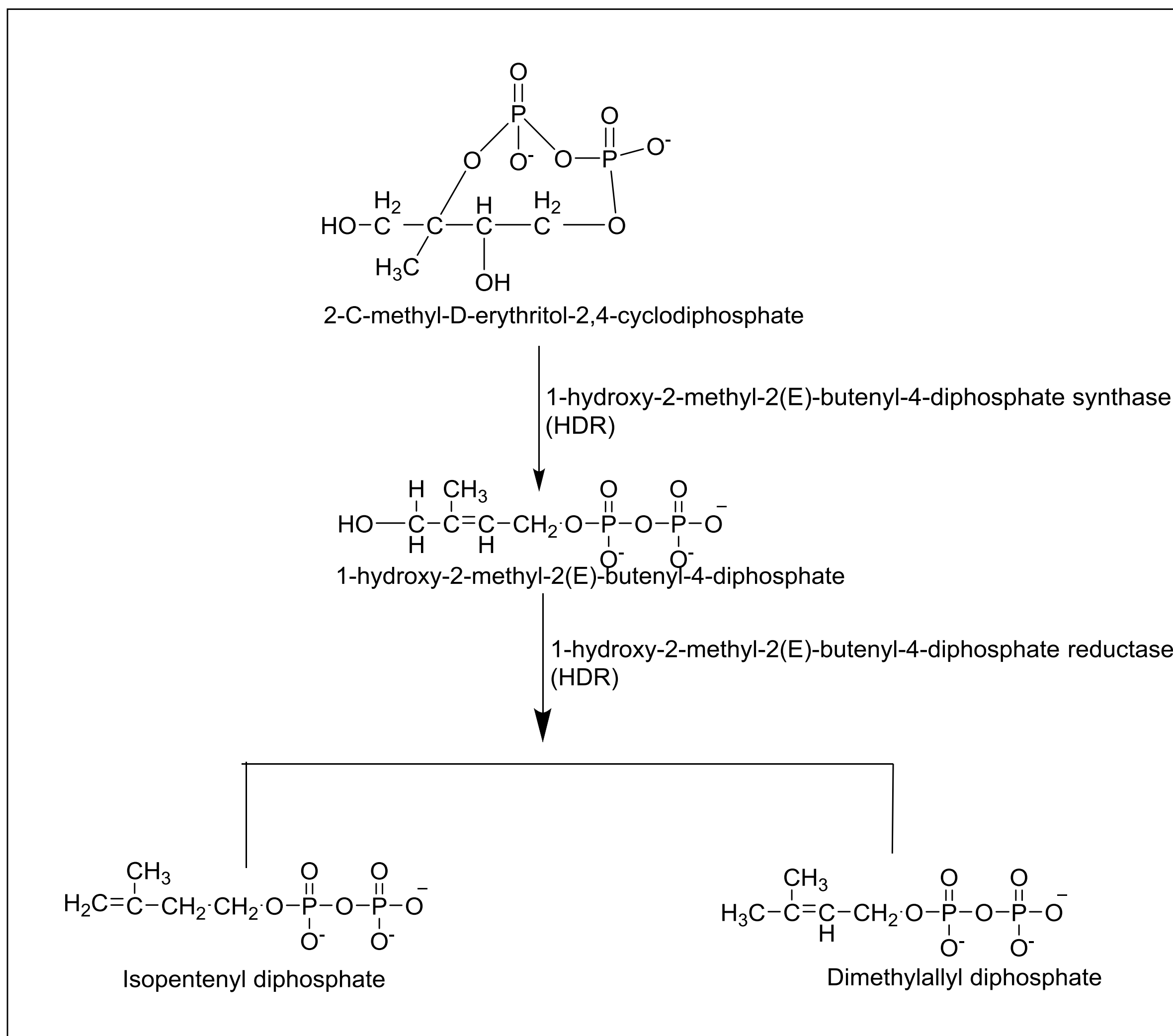


Figure 2.9: Formation of Isopentenyl Diphosphate and Dimethylallyl Diphosphate

STEP 4

In this step Copalyl diphosphate is form by the Geranylgeranyl diphosphate formation and its cyclization. This is the beginning of the late stage Steviol glycoside synthesis (Figure 2.10).

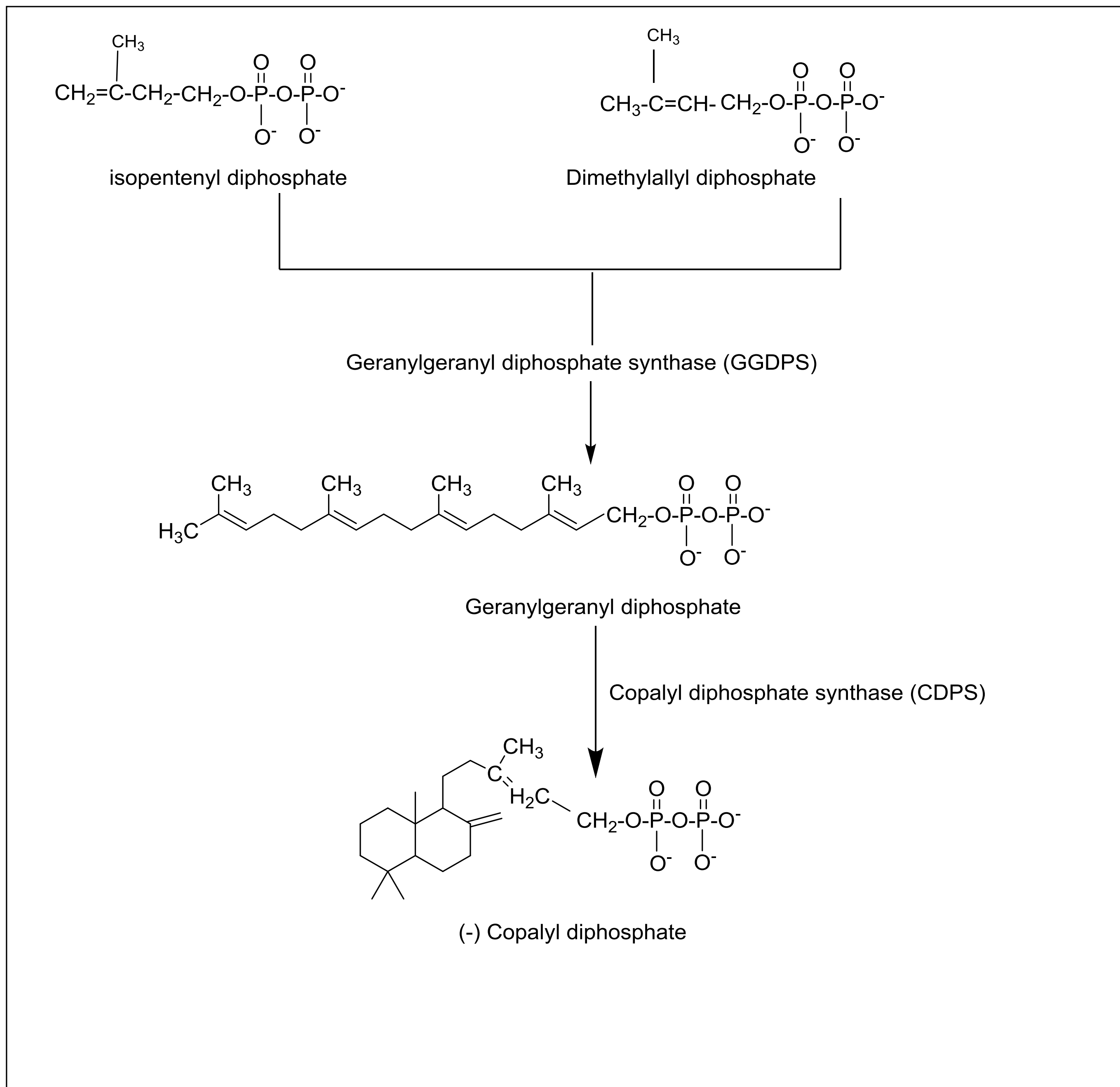


Figure 2.10: Formation of Copalyl diphosphate

STEP 5

In this step, ionization dependent cyclization takes place and Kaurene is produced formed from Copalayl Diphosphate which then gets oxidized to kaurenoic acid. The next step involves the formation of steviol by the hydroxylation of kaurenoic acid. From this step steviol glycoside biosynthetic pathway takes a distraction from gibberellin biosynthesis pathway (Figure 2.11).

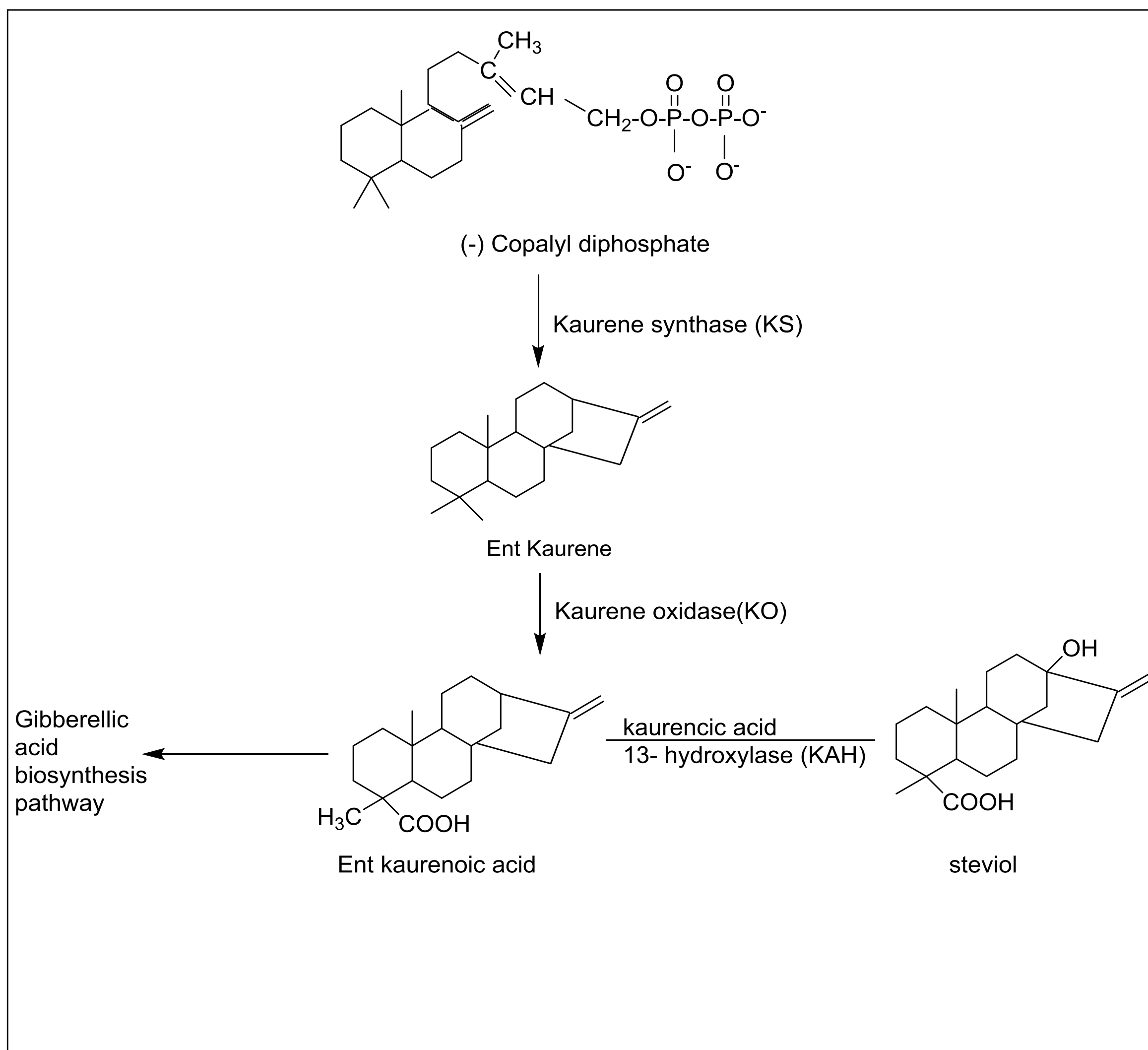


Figure 2.11: Formation of steviol

STEP 6

In this step various steviol glycosides are form as the steviol gets glycosilated by the Glucosyl Transferase enzymes. (Figure 2.12)

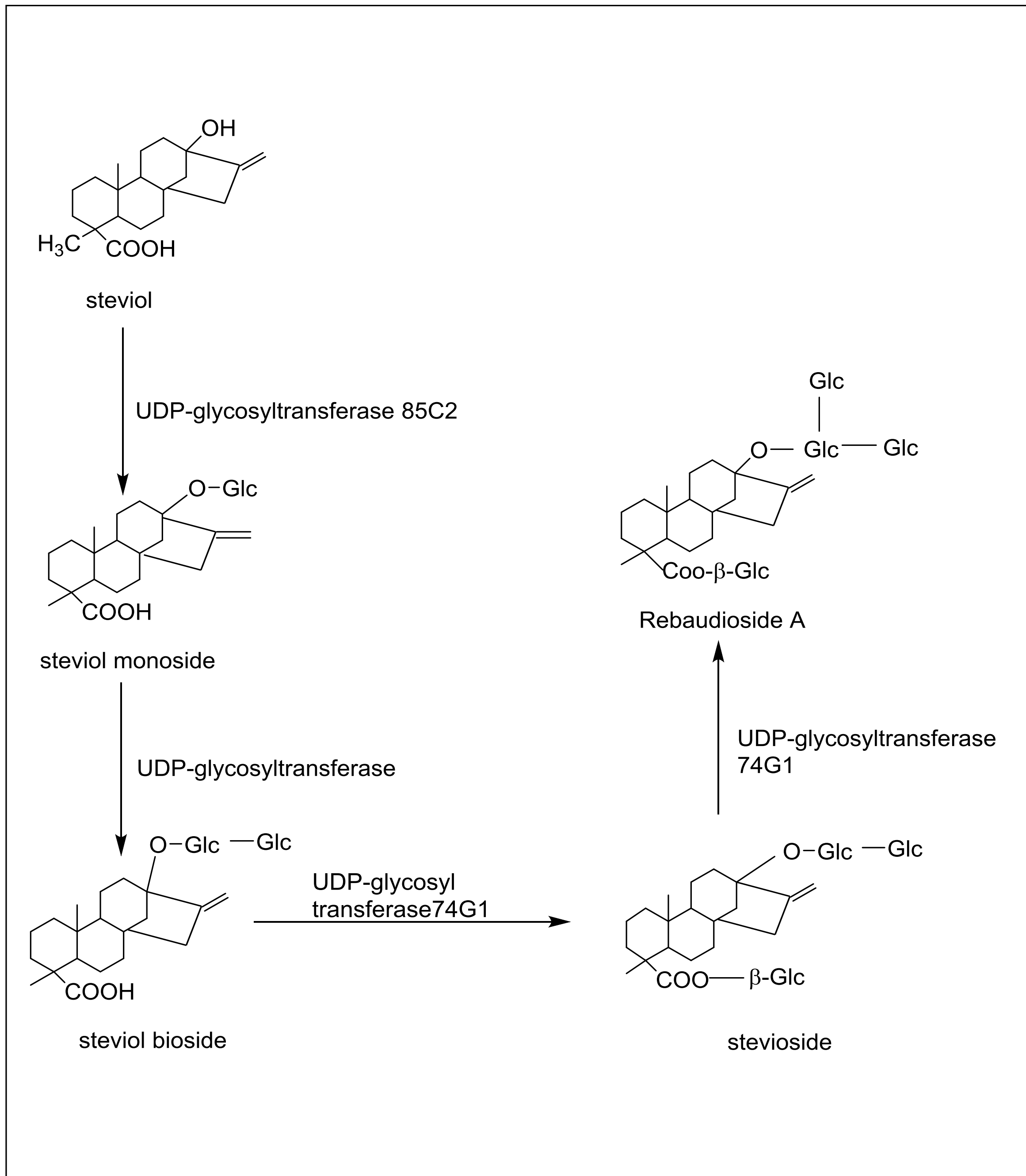


Figure 2.12: Formation of various steviol glycosides

2.4 Pharmacological properties of *S. rebaudiana*

2.4.1 Anti-hyperglycemic effect

Stevia rebaudiana extracts has been used from the ancient times for treating the diabetes as it increases the sensitivity and secreation of insulin(Chatsudthipong et al., 2009).The effect of stevioside as Anti-hyperglycemic was found to be mediated by its effect on phosphophenol pyruvate carboxy kinase (PEPCK) which is the rate-limiting enzyme intended for gluconeogenesis and consequently controls the glucose production in the liver. It was observed that stevioside repress the PEPCK gene expression and lastly reduces gluconeogenesis. In addition stevia powder is more proficient in down-regulating PEPCK in comparison to stevioside which specify that except stevioside various other chemical compounds can be present which are responsible for down-regulating PEPCK(Chatsudthipong et al., 2009). In the study it is found that the production of insulin is enhanced by the action of Rebaudioside A in the pancreatic islet cells of mouse. Also stevioside promotes the glucose-activated insulin secreation, devoid of any disturbance in case of fasting insulinemia (Mathur et al., 2017).

2.4.2 Antimicrobial activity

It has been reported that Stevia inhibits growth and reproduction of bacteria which is responsible for gum disease and tooth decay thus showing anti-microbial activity.It was also further found that the major cariogenic organism, Streptococcus mutans, experiences growth repression and less acid secreation when grown on media containing stevoside in comparison to media containing the fructose, glucose or the sucrose(Mathur et al., 2017).

2.4.3 Anti-inflammatory and immunomodulatory activity

Various SGs have shown important effects on pro-inflammatory cytokines. 1 mM dose of stevioside moderately amplified the creation of tumor necrosis factor (TNF-α), interlukin (IL-1β) and nitric oxide (NO), in un-stimulated human THP-1 cell by interacting through toll-like receptor-4. Therefore stevioside can be valuable to

healthy individual as it is accomplished to develop the innate immunity(Boonkaewwan et al., 2013). In the study the effects of stevioside and steviol as anti-inflammatory was studied on epithelial cells of colon. Moreover Stevioside has been found to reveal inhibitory effects on the contraction of smooth muscles of intestine whose inhibition deals with the hyper-motility related diarrhea. It has been onserved that the steviol along with its analogs has antidiarrheal effect as it inhibits cAMP regulated Cl^- secretion in T84cells(Mathur et al., 2017).

2.4.4 Anti-diarrheal activity

The anti-diarrheal potential of SGs was reported by Pariwar and his co-workers . According to the information stevioside and its foremost metabolite, steviol, were establish to influence the ion transport in various types of tissues including kidney, pancreas and intestine. Moreover short-circuit current measurements by them in the study showed that steviol and its analogs isosteviol, dihydroisosteviol and isosteviol 16-oxime inhibits forskolin-induced chloride secretion in a dose-dependent manner and have IC_{50} values of 101, 100, 9.6, and 50 mM, respectively. Parent compound stevioside was found to be free from this effect. In the same study apical current measurement indicated that dihydroisosteviol besieged the cystic fibrosis transmembrane regulator. Inhibitory action of this compound was found reversible and was not linked with changes in the intracellular CAMP level. Pariwar and co-workers further recognized that it did not affect calcium-activated chloride secretion and T84 cell viability. In-vivo studies using a mouse closed-loop model of cholera toxin-induced intestinal fluid secretion showed that intra-luminal injection of 50 mM dihydroisosteviol reduced intestinal fluid secretion by 88.2% devoid of altering fluid absorption, thus indicating that dihydroisosteviol and related compounds could be a new class of cystic fibrosis transmembrane regulator inhibitors that may be helpful for additional development as anti-diarrheal agents(Brahmachari et al., 2011).

2.4.5 Anti-hypertensive effect

Stevia and stevioside extract property as a antihypertensive could be due to their effects on the plasma volume. Stevioside administration by I.V causes natriuresis, diuresis and increased renal plasma flow but glomerular filtration rate (GFR) is

unaffected(Melis, et al., 1992). It is reported that the stevioside causes vasodilation therefore reduces the total peripheral resistance as it inhibits the influx of Ca^{2+} in the vascular smooth muscle(Chatsudthipong et al., 2009). However stevioside has no influence on vasopression induced vasoconstriction when the medium is free of Ca^{2+}. Thus it is clear that it causes vaso-relaxation by inhibiting the influx of Ca^{2+}(Ruiz-Ruiz et al., 2017).

2.4.6 Anticancer activity

From the literature it has been confirmed that the stevioside have the cytotoxic ability. it mediates the apoptosis in MCF-7 human breast cancer cell line by the induction of reactive oxygen species (ROS) through mitochondrial pathway. By the intracellular ROS generation it conveyed the apoptotic signal(Gupta et al., 2017).Hence it was concluded that the stevioside increases the expression of apoptotic proteins such as Bax, Bcl-2 and Caspase-9 By the induction of the ROS-mediated mitochondrial permeability transition(Gupta et al., 2017). It is also reported that the Stevioside have inhibited tumor promotion by the TPA in the mouse skin cancer(Karimi et al., 2017). In addition isosteviol inhibits DNA polymerase and human topoisomerase II which are prominent targets of anticancer drugs(Chatsudthipong et al., 2009). Chaiwat reported the effects of steviol and stevioside on the human colon carcinoma cell lines by the MTT method. The cell viability in Caco-2, HT29 cells and T84 decreases when the concentrations of stevioside was 2–5 mM and that of steviol was 0.2–0.8 mM (Brahmachari et al., 2011). It is also mentioned that the Stevia leaf extracts and the presense other constituents such as its polyphenolic constituents have the inhibitory effect on tumor commencement and its promotion (Heikel et al., 2008). As a result SGs have revealed major anticancer activity which can be more explored for investigating the better anticancer compounds.

CHAPTER 3.0

Aim and Objectives

Chapter 3
Aim and Objectives

Work has been conducted by following these objectives

- Isolation and characterization of secondary metabolites from S. rebaudiana leaves
- In-vitro study of different extracts of S. rebaudiana (Bertoni).
- In-silico Study of reported secondary metabolites from S. rebaudiana.

CHAPTER 4.0

Rational

Chapter 4
Rational

- Therapies available for treating the cancer cause maximum side effects such as bone marrow depression, neutropenia, cardiotoxicity, mouth soreness, hairfall, nerve changes and also affects the normal cells which is danger for the person suffering from it and ultimate can cause the death of the individual, so the research for drugs having minimum side effects is necessary.

- Drugs obtained from natural products are considered safe as it have minimal side effects and the chances for curing the disease is much higher and today almost 60% drugs available for curing cancer are obtained from natural products therefore plant kingdom is considered the main source.

- From literature review, we observed that Stevia rebaudiana which is a very famous plant for its sweet taste and having zero caloric value mainly used for the treatment of diabetes is also having many other therapeutic benefits including Antiproliferative activity and as mentioned in literature various cell line including HT-29, MCF-7 the Antiproliferative action of the phytochemicals from S. rebaudiana has been observed.

- Keeping in view the above mentioned facts about the medicinal value of the plant present study was conducted to further explore the phytochemical and medicinal aspect of Stevia rebaudiana using In-vitro and In-silico approach.

CHAPTER 5.0

Material and Method

Chapter 5

Material and Method

5.1 Chemical and Instruments

Solvent methanol and ethyl acetate (laboratory grade) was procured from Finar limited India, petroleum ether (laboratory grade) was procured from SDFCL (sd fine-chem limited) Mumbai India. Solvent Chloroform laboratory grade was procured from Thomas baker chemicals Pvt. Ltd India. RPMI 1640 and DMEM, antibiotic solution, Phosphate buffer and bovine serum media were used to culture cancer cell lines. Sulphuric acid (91%) was purchased from Loba Chemie Pvt Ltd. Silica gel 60\120 for column chromatography was procured from SDFCL, glassware of fine quality were used and procured from Borosil JSGW, readymade TLC plates F254 from Merck were used, Rota vapor instrument LABINDIA was used. Laboratory grade reverse osmosis water R.O system was used. T-47 (Breast Cancer) cancer cell lines were selected for biological activity. These cell lines procured from National Cell Repository located at NCCS Pune. For visualizing the TLC plates procured UV chamber from Mac Company,the characterization of structure of isolated compound NMR (H1 and C13) 400 MHz were used. Isolation of compounds was done by flash chromatography procured from Biotage. Other instruments used for thesis work such as Incubator for incubation,oven, automatic cell counter, UV-Vis spectrophotometer, laminar airflow. For docking studies of reported phytochemicals and isolated compounds Maestro software 2015 procured from Schrodinger Company.

5.2 Procurement and Preparation of Plant material

Dried aerial part of plant S. rebaudiana was procured in the month of july 2013 from Green Valley farm situated at Pojewal (Garhshanker) in Punjab (Altitude 355m; Latitude 31.191769^{o}N, longitude 76.258774^{o}E) (figure 5.1).

Figure 5.1: *Stevia rebaudiana*

5.3 Preliminary phytochemical investigation of extracts

The powdered material (4.1kg) was subjected to successive extractions with petroleum ether, chloroform, ethylacetate and aqueous methanol to yield 60g, 64g, 67g, and 190g respectively. Now the qualitative chemical test of all the extracts of stevia rebaudiana was subjected to detect the presence of various phytoconstituents.

5.3.1 Test for Alkaloid: The samples are added with 3ml of picric acid saturated solution. Samples giving yellow precipitate shows presence of alkaloid.

5.3.2 Test for Fixed oil: On a clean filter paper add 2 drops of each sample. If it leaves a translucent spot then it is the presence of fixed oil

5.3.3 Test for Volatile oil: All samples are added with alcoholic solution of Sudan III dye. If the samples become red then volatile oil is present.

5.3.4 Test for Tannins: Add $FeCl_3$ to all the sample. Yellow colour give hydrolysable tannin whereas green colour give condensed tannin.

5.3.5 Test for Flavanoid: The samples are added with NaOH solution producing yellow colouration. On adding dilute acid if the mixture goes colourless then presence of flavonoid is confirmed.

5.3.6 Test for Glycoside: Part A: Add dilute H_2SO_4 in the samples then add 5% NaOH neutralizing it. To it add equal volume of Fehling solution 1 and solution 2. Red colour is produced.

Part B: Add DM Water to all the test tubes until it is diluted then add equal volumes of Fehling solution 1 and 2. Red colouration

Compare the redness if part A has more intensity than part B then we can say that there is presence of reducing sugar.

5.3.7 Test for Steroids and triterpenes: All samples are added with conc. Sulphuric acid. Yellow on top layer show steroid and green on bottom show triterpenes.

5.4 In *vitro* anticancer activity

Different Extracts of S. rebaudiana were prepared for evaluating its anticancer activity. For studying this activity MTT assay was performed using the T-47 D breast cancer cell lines. In this cell lines 7500 cells were taken in each well consisting of 96 well total in plate. All the prepared extracts were completely dried and stored in an eppendorf tube and weighed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a stock solution The tubes were submitted to animal cell culture laboratory in central university of Punjab. The eppendorf tubes were marked as AD-01 (Petroleum ether extract), AD-2(chloroform), AD-3 (Ethyl acetate), AD-4 (Methanol + water extract). In Animal cell culture laboratory T-47D cells are taken on petridish after thawing it from -80^oC to body temperature and then media is added after 24 hours adherence is formed and then splitting of adherent cells. The cells of the cell lines was treated with different extracts of the plant in triplicate of its concentration and the experiment was also repeated three times .This process continues until sufficient cells for 96 well plate can be used enough. In the 96 well plate Plant extract of different concentration is added along with serum free media and MTT. Violet colour formation is seen due to formazone ring formation with dehydrogenase

enzyme. The colouration is checked under 570 nm which is directly proportional to cell viability. Results were then plotted in graphs to calculate cytotoxic potential and IC_{50} values.

5.5 Isolation and characterization compounds

5.5.1 Isolation

On the basis of preliminary test performed and the in-vitro study aqueous methanol extract was selected for the isolation of compounds. The aqueous methanol extract (23.67g) was dissolved in methanol and 14g silica was added. The mixture was dried over rotavapor and further was fractionated with methanol: chloroform (50:50 v/v), 100% methanol and aqueous methanol to yield 10g,6g and 4g respectively and a TLC for all the three extracts was prepared. Progress of the reaction was monitored by TLC using solvent system ethyl acetate/ethanol/H_2O (8:2:1.2, v/v/v).The developed plate was dried in oven and spots were visualized by spraying with 5% sulfuric acid. Maximum spots were obtained with 100% methanol. The 100% methanol extract was subjected to flash column chromatography using a gradient elution of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 45, 50%, 55%, 60%, 65%, 70%, 75%, 80% chloroform: methanol to give 1,792 fractions. The analysis of all the fractions was performed by TLC using the mobile phase consists of ethyl acetate/ethanol/H_2O (8:2:1.2 v/v/v). Fraction 239-290 were combined and dried on the rotavapour yielding ASP-1 JMG (14mg). Fraction 423-443 were combined and dried on the rotavapour yielding ASP-2 JMG (22mg). Fraction 529-533 were combined together on the basis of a single spot on precoated silica gel $60F_{24}$ TLC plates .The combined fractions were dried on rotavapour yielding ASP-3 JMG (17mg). The Fractions 600-610 were combined together on the basis of single spot on precoated silica gel $60F_{24}$ TLC plate. The combined fractions were dried on a rotavapour to give 24 mg of ASP-4JMG.

5.5.2 Characterization of ASP-2

Melting point was found to be 197-199°C.

^{1}H NMR (400 MHz, DMSO): Showed three anomeric peaks at δ_{Hn} 4.87 (1H, d, J= 4Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) respectively.

^{13}C NMR peaks of ASP-2 in (PPM,100 MHz,DMSO): 14.92, 18.55, 19.91, 21.14, 27.95, 35.46, 37.44, 40.96, 41.93, 43.14, 43.53, 46.81, 48.65, 53.10,56.52, 60.51,60.73, 61.07, 69.44, 69.60, 70.34, 72.41, 75.11, 75.93, 76.09, 76.17, 76.72, 76.91, 77.45 , 78.44, 82.67, 84.71, 94.09, 96.27, 103.88, 104.66, 153.33, 175.73.

HRMS (ESI): m/z calc. [M+Na]$^{+}$ calculated for $(C_{38}H_{60}O_{18}Na)^{+}$ 827.3868 corresponding to molecular formula $C_{38}H_{60}O_{18}$.

5.5.3 Characterization of ASP-4

Melting point was found to be 244-246°C

^{13}C -NMR (100 MHz, DMSO): 14.89, 18.56, 19.79, 21.14, 28.04, 37.48, 38.70, 39.20, 40.03, 41.04, 41.64, 43.10, 43.20, 47.0, 53.23, 56.52, 60.56, 61.02, 61.34, 68.90 69.48, 70.01, 70.32, 72.44, 73.59, 74.35, 75.98, 76.49, 76.88, 76.98, 77.42, 78.46, 78.79, 78.88, 79.12, 85.40, 86.27, 94.45, 96.45, 102.47, 102.98, 104.0, 152.78, 175.63.

^{1}H NMR (400 MHz, DMSO): Showed four anomeric peaks at δ_{Hn} 5.64 (1H, d, J = 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz) respectively.

HRMS (ESI): m/z calc. for $C_{44}H_{70}O_{23}$ [M-H]$^{-}$ 965.5040 corresponding to molecular formula $C_{44}H_{70}O_{23}$.

5.6 *In-silico* activity

The computational study of molecular recognition is an important part of structure-based drug design. The molecular docking problem is commonly cast as a problem of finding the low-energy binding modes of a small molecule, or ligand, within the active site of a macromolecule, or receptor, whose structure is known. With the help of molecular docking environment, using Maestro software 11. The mechanism behind the antiproliferative activity of phytochemical from S. rebaudiana was determined. The targets selected for the study were estrogen receptor alpha, androgen receptor and aromatase receptor.

5.6.1 Ligand preparation

With the help of ChemBio Draw ultra 12.0 structures of all the phytochemicals (Figure 3.1) including the structures mentioned in chemical constituents in the review section were drawn and saved in SDF format. The molecules were changed from 3D structure to the 2D by using the Lig Prep version 2.5. These ligands were imported from workspace option then were subjected to ligand preparation using 'LigPrep' wizard application in Maestro 9.6 The Lig Prep developed a single, low energy, 3D structure with correct chiralities for the each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Lig Prep application of the Maestro operates OPLS 2005 force field.

5.6.2 Protein Preparation:

The PDB for the crystal structure of estrogen alpha (PDB Id 3ERT) has been obtained from RSCB protein data bank. It is mainly responsible for the breast cancer. crystal structure of human androgen receptor in complex with testosterone (PDB ID 2AM9) have also been downloaded from protein data bank and the crystal structure of human aromatase receptor in complex with androstenedione (PDB ID 3EQM)has obtained from protein data bank.The protein preparation wizard option was worn for preparation of protein structure with polar hydrogen. This procedure consists of two steps that is preparation and refinement. In this step, bond orders were consigned, all

hydrogen atoms were added ,bonds to metals were deleted and formal charges were set on the metal and the neighboring atoms and water molecules were deleted that were more than the 5Å specific distance. Also the reorientation of hydroxyl groups, water molecule and amino acids to the optimization of hydrogen bond network. The refinement process is the last step for protein preparation. This steps involves firstly optimization then add hydrogen only and minimization. Thus in this process the restrained impact of minimization of protein was taken and stearic clashes were revealed and made the protein ready to dock.

5.6.3 Receptor Grid generation

Generation of the grid at a exacting site in the protein is an important factor to perform docking study. It is not possible to perform Ligand docking without the generation of receptor grid. In case of 3ERT, 2AM9 and 3EQM the grid was generated at the position of co-crystallized ligand which already has to bound to protein. The ligand molecule has to be selected for grid generation. The length of ligands which are selected for docking were made to 36Å.

5.6.4 Glide Docking

GLIDE (Grid Based Ligand Docking with Energetics) software was used for docking procedure developed by Schrödinger. Three docking precision is available in GLIDE docking module such as HTVS (High throughout visual screening), standard precision (SP) and Extra precision (XP). HTVS is proposed for quick screening of very large numbers of ligands. It has controlled conformational sampling. SP is set as a default parameter and is used to screen thousands of compounds. Extra precision docking and scoring is more dominated and sharp procedure. Extra precision is developed to locate active compounds that binds to a particular conformation of receptors. Docking of reported phytochemicals and the inhibitor was done with extra precision. Extra precision descriptions were written, ligand was taken as flexible. Epik penalties were added to the docking score. Docking score was taken into consideration for comparing the results, more negative the docking score more potent the compound and indicates the good binding potential. Various components are present in GLIDE score like hydrogen bonds, hydrophobic contacts, Van der Waals interaction, and

Columbic interaction, Polar interaction in the binding site, Metal binding and Freezing rotable bonds.

5.6.5 ADME Study

For estimating the absorption, distribution, metabolism and excretion (ADME) properties of chemical compounds Qikprop module application present in Schrödinger suit 2013 is used. It predicts physically significant descriptions and evaluates the pharmacokinetics properties of ligands by retrieving the drug like properties. Using this module we have calculated ADME properties of chemical compounds of S. rebaudiana used in the study.

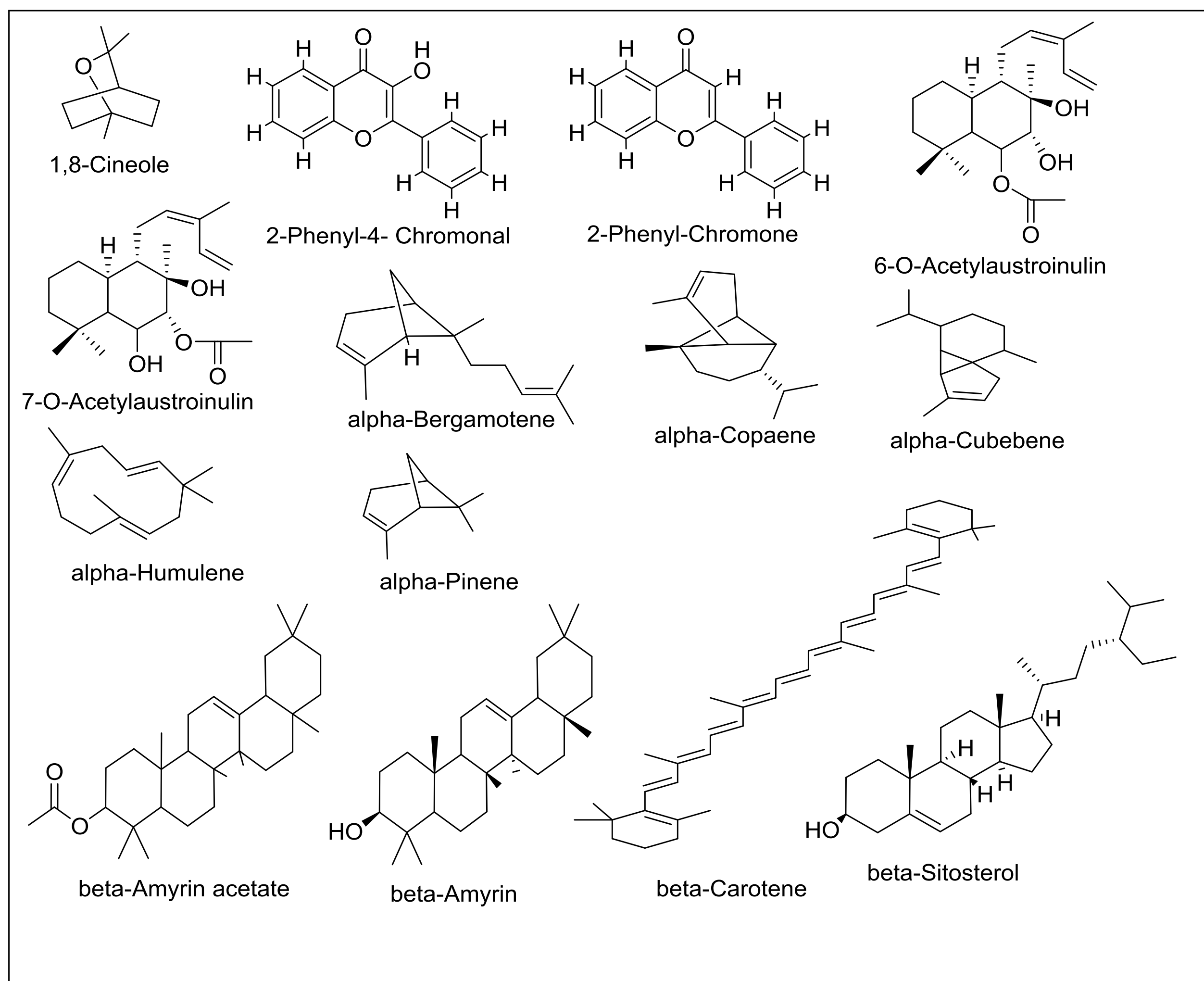


Figure 5.2: Ligands used in *in-silico* study

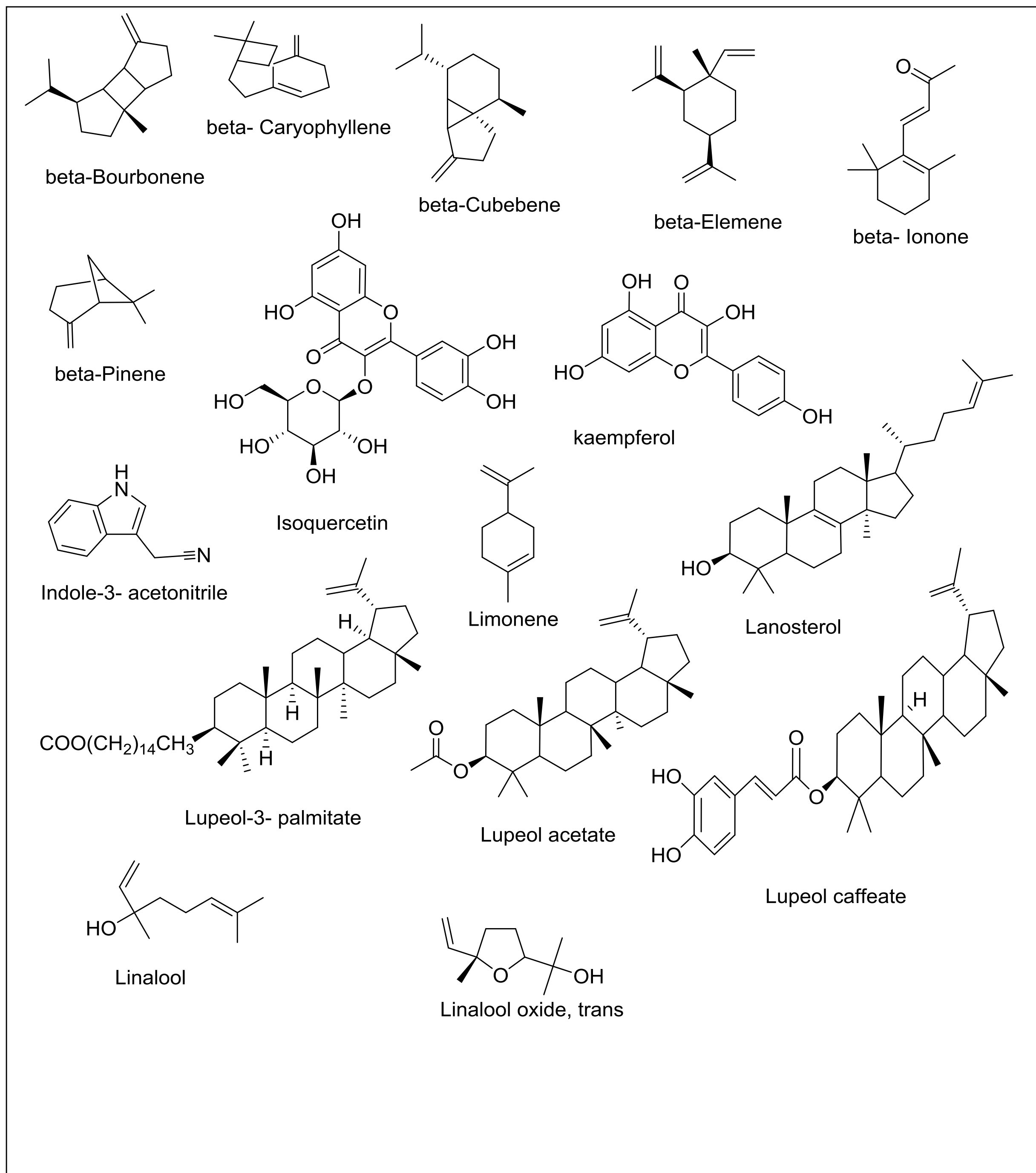
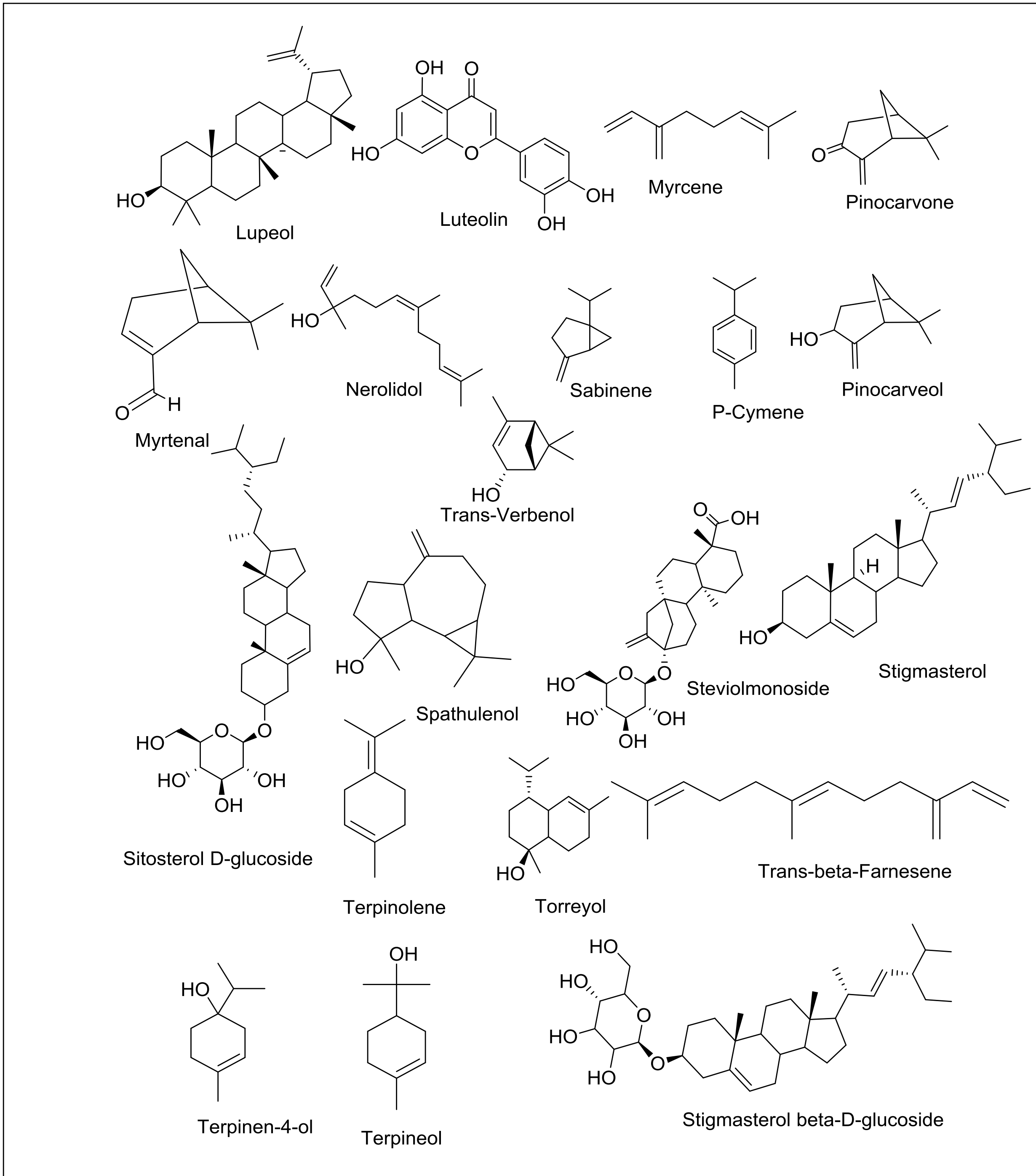


Figure 5.2: Continue



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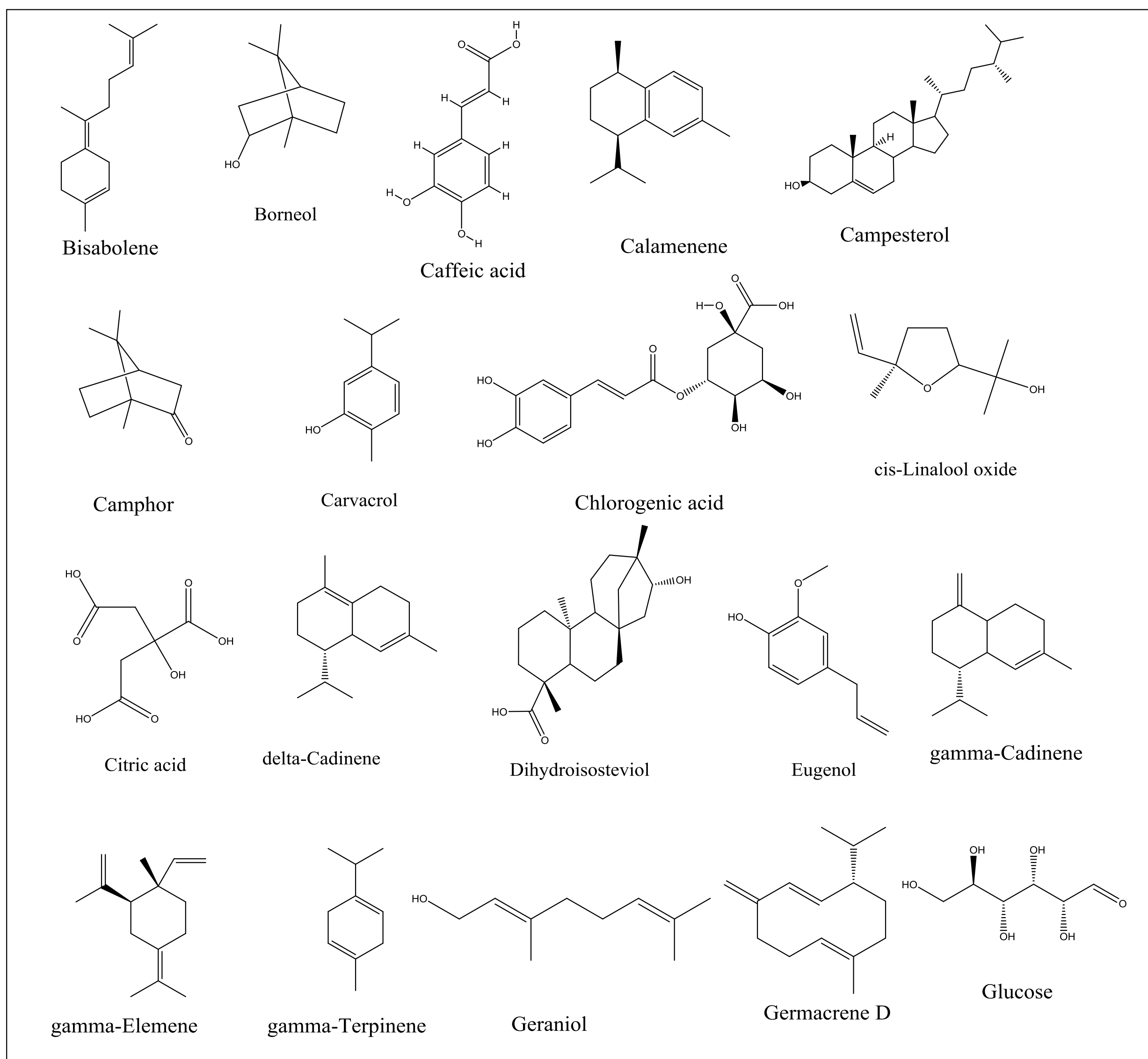

Figure 5.2: Continue

CHAPTER 6.0

RESULTS AND DISCUSSION

Chapter 6.0

Results and discussion

6.1 Preparation of Extracts

The powdered material (4.3 kg) was subjected to successive extractions (Figure 6.1) with petroleum ether, ethyl acetate, $CHCl_3$ extract, and aqueous methanol (20:80 v/v).

Figure 6.1: Powdered leaves of *S. rebaudiana* packed in Aspirator

Table 6.1: List of extracts prepared

Serial number	Extracts	Weight of extract
1	Petroleum ether extract	60g,
2	$CHCl_3$ extract	64g
3	Ethyl acetate extract	67g
4	Aqueous methanol extract	190g

6.2 Preliminary phytochemical investigation of extracts

Phytochemical test of Petroleum ether extract, $CHCl_3$ Extract, ethyl acetate and Aqueous methanol extracts of S. rebaudiana were subjected to tests detection for the presence of various phytoconstituents. General test were performed such as alkaloid test, fixed oil test, test for tannin, volatile oil test, Flavonoid Test, Glycoside test, Steroid test, Trirepenes test and test for saponin.

Table 6.2: Result of the test performed

Tests	Pet. Ether Extract	$CHCl_3$ Extract	Ethyl Acetate Extract	Aqueous Methanol Extract
Alkaloid Test	+	+	+	+
Fixed oil Test	-	+	-	-
Test for tannin	-	+	+	+
Volatile oil Test	+	+	+	+
Flavanoid Test	-	+	+	-
Glycoside test	+	+	+	+
Steroid test	+	+	+	-
Triterpene test	+	-	+	+
Saponin	+	-	-	+

From the phytochemical investigations of extracts of S. rebaudiana, $CHCl_3$ Extract extract and methanol- water exhibited that the most chemical constituents were present. So aqueous methanol extract was selected for further study and for the isolation of the compounds.

6.3 *In- vitro* study

For estimating the anticancer activity of extract of the S. rebaudiana MTT assay was performed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a

stock solution and subjected to in- vitro antiproliferative activity. MTT evaluates demonstrated results in varied range of concentration. In the MTT cell proliferation measurement of all proliferation rates and similarly, when metabolic events lead to apoptosis or necrosis, that reduction in cell viability. The MTT reagent yields get low. The T-47D BC cell lines confirmed that the chloroform extract has high activity against breast cancer cell lines having the IC_{50} value 7.79 µg/mL after that aqueous methanol shows the high activity against the cell line having the IC_{50} value 9.53 µg/mL and than the petroleum ether extract with the IC_{50} value 9.58µg/mL. Further IC_{50} values of aqueous methanol extract, petroleum ether extract, and chloroform extract were calculated and tabulated in the Table 6.3.

Table 6.3: IC_{50} values of various extracts as examined by MTT anti-cancer in-vitro assay

Sr. No.	Extracts	IC_{50} Value (µg/mL)
1.	AD-1 (petroleum ether)	9.58
2.	AD-2 (chloroform)	7.79
3.	AD-3 (ethyl acetate)	ND
4.	AD-4 (aqueous methanol)	9.53
5.	Bazedoxifene	18.29

6.4 Characterization of isolated compounds

6.4.1 Characterization of ASP-2

Compound ASP-2 was a crystalline white solid, produced a single black spot which was visualized by spraying with 5% sulfuric acid on a precoated silica gel $60F_{254}$ TLC

plate. Its m/z ratio indicated an ion peak at m/z $[M+Na]^+$ calculated for $(C_{38}H_{60}O_{18}Na)^+$ 827.3868 corresponding to molecular formula $C_{38}H_{60}O_{18.}$ The ^1H and ^{13}C NMR of compound ASP-2 showed three anomeric values at δ_{Hn} 4.87 (1H, d, J= 3.44Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) and δ ^{13}C 94.09, 96.27 and 104.66 respectively assigned to three glucose molecules.

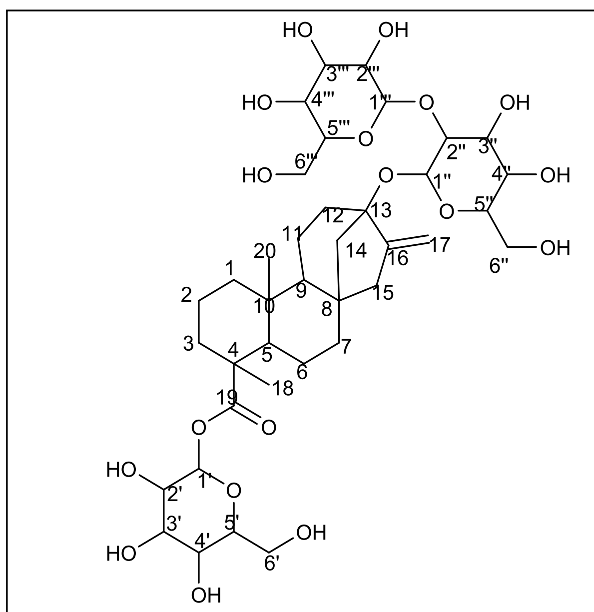

Figure 6.2: ASP-2

In ^{13}C NMR, δ_c at 14.92 (C-20) and δ_c 27.95 (C-18) indicates the presence of two methyl groups. δ_c value at 175.73 and δ_c 103.88 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively. Above mentioned observation confirmed that the compound ASP-2 is stevioside which is represented in Figure 4.2. stevioside is a SG Containing three glucose molecules attached with steviol moiety having melting point of 197-199°C. Further confirmation of structure was done on the basis of literature(Danieli et al., 1997).

6.4.2 Characterization of ASP-4

Compound ASP-4 was a crystalline white solid, produced a single black spot on a precoated silica gel $60F_{254}$ TLC plate. Its m/z ratio indicated an ion peak at m/z calc. for $C_{44}H_{70}O_{23}$ $[M-H]^-$: 965.5040 corresponding to molecular formula $C_{44}H_{70}O_{23.}$ The ^1H and ^{13}C NMR of compound ASP-4 showed four anomeric values at δ_{Hn} 5.64 (1H, d, J

= 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz). and δ ^{13}C 94.45, 96.45, 102.98 and 102.47 respectively assigned to four glucose molecule.

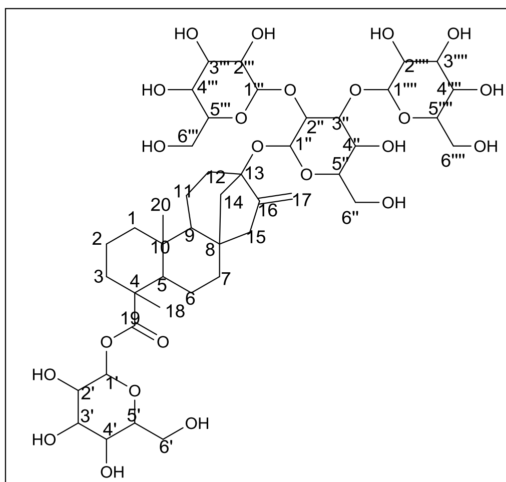

Figure 6.3: ASP-4

In C^{13} NMR, δ_c at 14.89 (C-20) and δ_c 28.04 (C-18) indicates the presence of two methyl groups. δ_c value at 175.63 and δ_c 104.0 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively and additional peaks for sugar moiety at C-3'' was obtained to be 102.98, 78.46, 74.35, 73.59, 70.01 and 61.02. Which confirms the presence of one extra glucose moiety compare to stevioside. Above mentioned observation confirmed that the compound ASP-4 is Rebaudioside A which is represented in Figure 4.3. Rebaudioside A is a SG Containing four glucose molecules attached with steviol moiety having melting point of 244-246°C. Further confirmation of structure was done on the basis of literature(Steinmetz et al., 2009).

6.5 *In silico study*

The preliminary screening for anticancer property of S. rebaudiana extracts by MTT assays using T47D cell line demonstrated that the extracts of S. rebaudiana assures the antiproferative activity specially showing the activity against the breast cancer, due to presence of phytochemical constituents. From the literature it is also confirmed that the constituents showing excellent dock score are obtained from aqueous

methanol extract (Muanda et al., 2011).Estrogen receptors like estrogen alpha play an important role in regulation of estrogen hormone in women. Irregulaties in these receptors causes to induce the breast cancer. Docking study experiment shown that phytochemical constituents S. rebaudiana regulate the estrogen receptors. Phytochemical constituents prevent the binding of tamoxifen with its receptor (Estrogen α). Androgen receptor also playing key role in the induce of breast cancer. Testosterone used as a standard inhibitor for androgen receptor. Aromatase receptor also initiated the breast cancer by play a key role in biosynthesis of estrogen. Aromatase convert the testosterone into estrone by aromatization. 4- Androstene-3-17- dione used as a standard inhibitor for the aromatase receptor. These inhibitor makes down regulation of the signaling pathway and these phytochemical constituents also acts as inhibitor of enzymes involved in the biosynthesis of estrogen hormones. The targets selected for the docking study are estrogen receptor alpha , androgen receptor and aromatase receptor. All these targets having a vibrant role in the breast cancer proliferation. anticancer drug candidates were identified by glide docking of compounds of S.rebaudiana on all the three receptors and the best compounds were selected on the basis of lowest docking score. Reported 123 compounds of S.rebaudiana were taken for the docking study and the topmost compound showing the best docking result for antiproliferation is mentioned in detail below with compare to the standard inhibitor taken and other compounds result are mentioned in the table.

6.5.1 Estrogen receptor-α

In Estrogen receptor alpha (PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -13.98, -7.84, -1.01 and -1.02 Kcal/mol respectively. The interaction pattern is represented in Figure 6.4 in which majority of amino acid residues interact with hydroxyl group and with the amino group of ligand by forming the hydrogen bond. The main residues showing interaction with ligand include GLU 353, ARG 394 and ASP351.

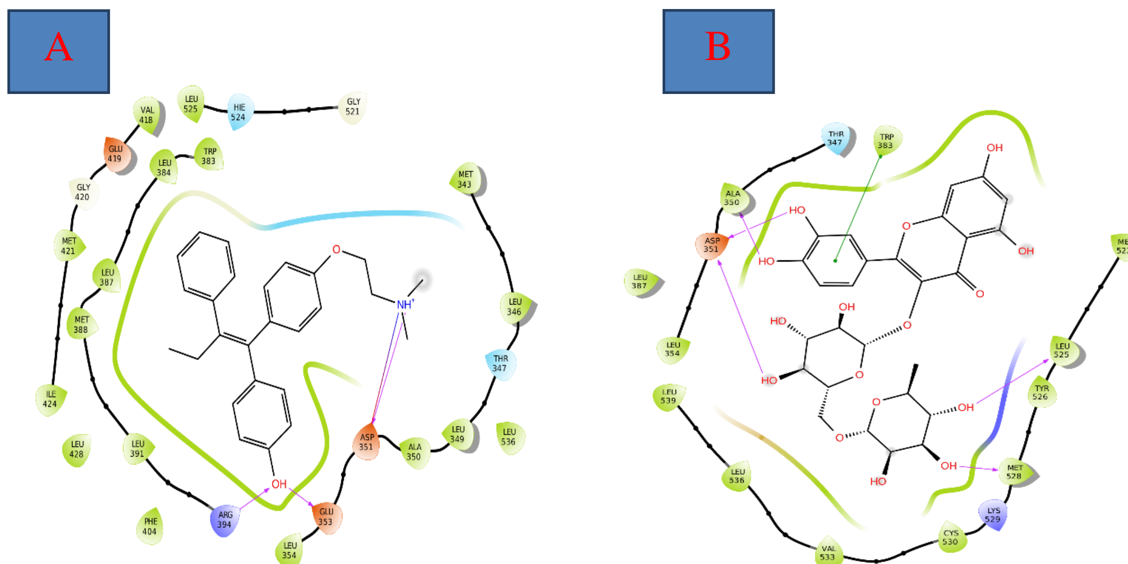

Figure 6.4: Interaction pattern of (A) 4-Hydroxytamoxifen and (B) Rutin with estrogen receptor- alpha (PDB ID 3ERT) showing best docking score

Next in the rank order according to docking score was Rutin which showed dock score, Lipophilicity, H bond and electrostatic energy of -10.44, -4.26, -4.34 and -1.3Kcal/mol respectively. The main interaction pattern included stacking interaction and Hydrogen bonding which is shown in Figure 6.4. TRP 383 showed π-π stacking interaction with ring A of ligand. ALA 350, ASP 351, MET 528 and LEU 525 amino acid residues showed hydrogen bonding of various hydroxyl groups of ligand. Thus on the basis of docking score top two compound was mentioned in case of estrogen receptor alpha which shows significant binding affinity for the receptor. The dock score, Lipophilicity, H bond and electrostatic energies of all the compounds includes in the study are shown in Table 6.4.

Table 6.4: Docking score of various phytochemical constituents of *S. rebaudiana* with estrogen receptor- alpha (PDB ID 3ERT).

Ligand	Dockscore	Lipophilicevdw	Hbond (Kcal/mol)	E_{Elec} (Kcal/mol)
4-Hydroxytamoxifen*	-13.98	-7.84	-1.01	-1.02
Rutin	-10.44	-4.26	-4.34	-1.3

Quercetin 3-O-Glucoside	-9.9	-3.32	-4.32	-1.83
Apigenin	-9.84	-5.33	-1.18	-0.34
Sterebin G	-9.54	-4.72	-1.92	-0.46
Quercetin	-9.35	-4.26	-2.72	-0.83
6-Austroinulin	-8.85	-5.53	-0.96	-0.1
Germacrene D	-8.57	-5.41	0	0.02
Steviol	-8.56	-4.47	-0.49	-0.27
Luteolin 7-O-Glucoside	-8.5	-4.12	-3.84	-0.31
Isosteviol	-8.48	-4.74	0	0.06
β-Cubebene	-8.44	-5.24	0	0
Apigenin 4-Glucoside	-8.44	-4.71	-2.4	-0.74
Luteolin	-8.42	-4.34	-1.89	-0.51
Sterebin N	-8.31	-3.62	-1.66	-0.74
Chlorogenic Acid	-8.26	-3.76	-2.4	-0.49
Jhanol	-8.22	-4.42	-0.7	-0.09
α-Humulene	-8.2	-5.17	0	0.02
Calamenene	-8.19	-4.98	0	-0.01
α-Cubebene	-8.1	-4.89	0	-0.01
γ-Cadinene	-8.06	-5.23	0	0.03
β-Caryophyllene	-8.05	-4.93	0	0.03
7 Austroinulin	-8.01	-4.8	-0.97	-0.18
Torreyol	-7.95	-4.73	0	-0.04

α -Copaene	-7.92	-4.76	0	0.04
3hydroxy-2-Phenyl-Chromone	-7.91	-4.86	-0.48	0.07
α -Bergamotene	-7.81	-5.16	0	0
β-Bourbonene	-7.75	-4.58	0	0.03
3-Hydroxy-2-Phenylchromone	-7.73	-5.17	0	0.11
δ-Cadinene	-7.68	-4.5	0	0.02
Kaempferol	-7.61	-4.28	-1.31	-0.48
β-Elemene	-7.51	-4.92	0	0
Dihydroisosteviol	-7.49	-4.22	0	0.06
Spathulenol	-7.49	-4.38	0	0.01
Bisabolene	-7.46	-4.72	0	-0.05
Kaempferol-3-O-Rhamnoside	-7.3	-3.8	-2.25	-0.83
Steviolbioside	-7.15	-2.78	-3.84	-0.75
γ-Elemene	-7.03	-4.29	0	-0.01
Quercetin 3-0-Rhamnoside	-7.03	-2.9	-2.93	-1.3
2-Phenyl-Chromone	-6.94	-5.23	0	-0.17
β-Sitosterol	-6.86	-4.44	-0.32	-0.14
Eugenol.	-6.86	-3.52	-1.18	-0.16
Indole 3- Acetonitrile	-6.84	-3.74	0	0

Caffeic Acid	-6.83	-2.85	-1.86	-0.42
Nerolidol	-6.78	-4.77	-0.66	-0.36
Myrtenal	-6.61	-3.38	0	-0.02
β-Pinene	-6.56	-3.67	0	0.01
Stigmasterol	-6.55	-4.48	-0.18	-0.12
1,8-Cineole	-6.54	-3.38	0	0.03
Centaureidin	-6.54	-3.67	-1.92	-0.38
β-Ionone	-6.54	-4.03	0	0.04
Carvacrol	-6.45	-3.22	-0.6	-0.11
Geraniol	-6.42	-3.15	-0.94	-0.48
Steviolmonoside	-6.36	-2.92	-2.35	-1.1
Apigenin 7-O-Glucoside	-6.34	-2.56	-1.87	-2
Pinocarvone	-6.33	-3.45	0	0.05
Terpinolene	-6.25	-3.16	0	-0.02
Trans-Verbenol	-6.24	-2.93	-0.7	-0.15
Borneol	-6.22	-3.51	0	0.02
α –Pinene	-6.22	-3.4	0	0.02
Luperol Palmitate	-6.17	-5.66	0	-0.01
P-Cymene	-6.16	-3.37	0	-0.01
Trans-Linalool Oxide	-6.15	-3.2	-0.35	-0.29
Cis-Linalool Oxide	-6.11	-3.58	-0.35	-0.25
γ-Terpinene	-5.99	-3.38	0	0

Sterebin F	-5.97	-2.58	-2.1	-0.87
Sterebin M	-5.97	-2.95	-2.25	-0.61
Sterebin I	-5.96	-3.2	-1.44	-0.5
Sterebin J	-5.91	-2.91	-1.92	-0.44
Limonene	-5.91	-3.22	0	0
Sabinene	-5.84	-3.17	0	0.03
Terpineol	-5.81	-3.14	-0.35	-0.31
Glucose	-5.76	-1.89	-3.07	-0.51
Sterebin K	-5.69	-3.47	-1.44	-0.48
Sterebin L	-5.66	-3.2	-1.92	-0.25
Pinocarveol	-5.66	-3.09	-0.4	0.01
Camphor	-5.51	-3.12	0	0.08
Sterebin C	-5.32	-3.29	-1.1	-0.23
Linalool	-5.17	-3.04	-0.66	-0.35
Trans-β-Farnesene	-5.17	-5.06	0	0.02
Sitosterol D-Glucoside	-5.07	-3.22	-1.92	-0.37
Austroinulin	-5.04	-2.86	-1.44	-0.15
Campesterol	-4.95	-3.66	0	-0.07
Terpinen-4-Ol	-4.86	-3.07	-0.26	-0.16
Sterebin B	-4.78	-2.64	-1.18	-0.35
Myrcene.	-4.58	-3.56	0	0.03
Sterebin A	-4.46	-2.28	-1.31	-0.43

Sterebin E	-4.09	-2.66	-0.96	-0.16
Citric Acid	-3.61	-1.49	-1.27	0.18
Sterebin O	-3.55	-1.66	-0.96	-0.24
Sterebin D	-3.41	-2.22	-0.35	-0.39
Succinic Acid	-2.18	-0.89	-0.83	0
Formic Acid	-1.45	-0.17	-0.89	0.05

*Pubchem ID/Name of standard estrogen receptor-alpha inhibitor used in the study

6.5.2 Androgen receptor

In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -12.97, -4.66, -4.97 and -1.17 Kcal/mol respectively. The interaction pattern is represented in Figure 6.5 in which all the amino acids interact with hydroxyl group of the ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 704 and ASN 705.

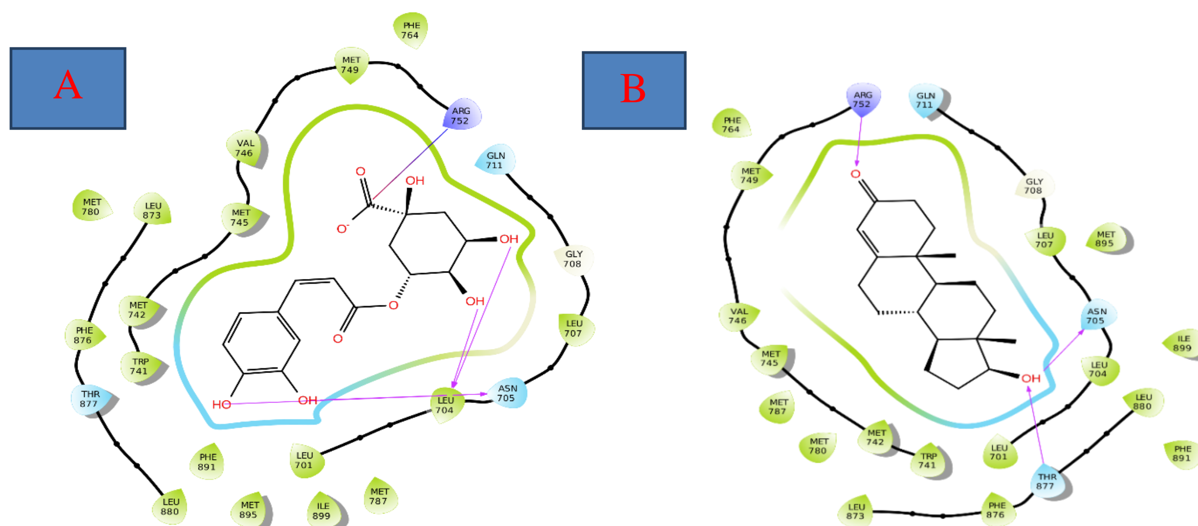

Figure 6.5: Ligand interaction diagrammed of (A) Chlorogenic and (B) Testosterone with androgen receptor (PDB ID 2AM9)

The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard androgen inhibitor testosterone was found to be -12.17, -6.64, -1.33 and -0.64Kcal/mol respectively which is comparatively higher than chlorogenic acid. The main interaction pattern included is Hydrogen bonding with carbonyl group and the hydroxyl group of the ligand which is shown in Figure 6.5. The main residues showing interaction with ligand includes ARG 752, THR 877and ASN 705. Thus on the basis of docking score phytochemical of S. rebaudiana have shown significant binding affinity towards androgen receptor (2AM9). The overall result of various compounds docked with 2AM9 is as represented in Table 6.5.

Table 6.5: Docking score of various phytochemical constituents of *S. rebaudiana* with androgen receptor (PDB ID 2AM9).

Ligand	Dockscore	Lipophilicevdw	Hbond (Kcal/mol)	E_{Elec} (Kcal/mol)
Chlorogenic Acid.	-12.97	-4.66	-4.97	-1.17
Quercetin	-12.25	-5	-4.34	-0.82
Testosterone*	-12.17	-6.64	-1.33	-0.64
Kaempferol	-12.09	-4.85	-3.5	-1.04
Luteolin	-11.98	-5.27	-3.16	-0.73
Sterebin E.	-11.24	-4.71	-2.63	-1.05
Sterebin G	-10.85	-4.49	-2.82	-0.77
Apigenin	-10.8	-5.31	-2.3	-0.51
Sterebin L	-10.69	-4.11	-2.54	-1.34
Austroinulin	-10.22	-4.85	-1.7	-0.78
Sterebin O	-10.13	-5.02	-1.11	-0.4
Sterebin F	-9.98	-4.33	-2.17	-0.64
Isosteviol	-9.89	-5.94	0	-0.01
Sterebin J	-9.88	-3.99	-2.18	-0.55

Sterebin B	-9.49	-4.62	-0.89	-0.51
Sterebin N	-9.47	-4.06	-1.65	-0.9
3-Hydroxy-2-Phenylchromone	-9.39	-5.55	-0.96	-0.24
Steviol	-9.25	-5.63	-0.16	-0.08
Sterebin A	-9.24	-4.77	-1.31	-0.29
Centaureidin	-9.07	-4.75	-2.59	-0.55
Sterebin I	-8.94	-4.05	-1.79	-0.36
Sterebin M	-8.9	-4.16	-2.14	-0.44
Jhanol	-8.86	-5.58	-0.21	-0.06
Dihydroisosteviol	-8.77	-5.29	0	-0.04
2-Phenylchromone	-8.68	-5.64	0	0.02
Torreyol	-8.67	-4.45	-0.86	-0.33
Sterebin D	-8.52	-4.81	-0.35	-0.29
Spathulenol	-8.51	-4.27	-0.7	-0.34
Germacrene D	-8.43	-5.25	0	0.02
α –Humulene	-8.39	-5.23	0	0.03
β-Caryophyllene	-8.28	-5.07	0	-0.01
β-Bourbonene	-8.27	-5.06	0	-0.01
β-Cubebene	-8.2	-4.98	0	-0.02
Calamenene	-8.18	-5	0	0.02
6-Austroinulin	-8.18	-4.62	-0.96	-0.19
α –Copaene	-8.13	-4.93	0	0
δ-Cadinene	-7.88	-4.76	0	0.03
α –Cubebene	-7.87	-4.67	0	0
7-Austroinulin	-7.85	-4.32	-1.28	-0.18
γ-Elemene	-7.7	-4.93	0	0
γ-Cadinene	-7.7	-4.66	0	-0.01
β-Elemene	-7.6	-5.01	0	-0.04
Caffeic Acid	-7.54	-2.85	-2.01	-0.54

Pinocarveol	-7.44	-3.83	-0.9	-0.35
Glucose	-7.41	-2.09	-4.55	-0.86
Bisabolene	-7.39	-5.06	0	0.03
Terpinen-4-ol	-7.28	-3.32	-1.11	-0.44
Beta-Ionone	-7.23	-4.32	0	-0.02
Trans-Linalool Oxide	-7.18	-3.47	-1.49	-0.43
Nerolidol	-7.18	-4.89	-1.33	-0.49
Cis-Linalool Oxide	-7.16	-3.49	-1.44	-0.39
Indole 3- Acetonitrile	-7.14	-3.34	-0.69	-0.26
Myrtenal	-7.08	-3.13	-0.7	-0.32
α –Bergamotene	-7.03	-4.85	0	0.02
Carvacrol	-7.03	-3.27	-1.09	-0.63
Borneol	-6.97	-3.23	-1.25	-0.49
Eugenol	-6.9	-3.31	-1.51	-0.49
P-Cymene	-6.85	-3.67	0	0.01
β-Pinene	-6.76	-3.71	0	0.01
Terpineol	-6.69	-3.37	-1.05	-0.45
Geraniol	-6.66	-3.38	-1.33	-0.81
Trans-Verbenol	-6.63	-3.44	-0.7	-0.23
Camphor	-6.44	-3.35	-0.23	-0.08
1,8-Cineole	-6.37	-3.6	0	0.05
Limonene	-6.21	-3.49	0	0.01
α –Pinene	-6.19	-3.59	0	-0.01
Pinocarvone	-6.13	-3.61	-0.06	-0.07
Trans-β-Farnesene	-6.05	-5.17	0	-0.04
Terpinolene	-5.96	-3.51	0	0
Sabinene	-5.96	-3.71	0	0.01
γ-Terpinene	-5.95	-3.6	0	0
Linalool	-5.48	-3.43	-1.33	-0.49
Myrcene	-4.64	-3.52	0	0

Citric Acid	-4.15	-1.59	-1.33	-0.08
Succinic Acid	-2.29	-0.95	-0.35	-0.27
Formic Acid	-1.54	-0.17	-0.35	-0.31

*Pubchem ID/Name of standard androgen inhibitor used in the study

6.5.3 Aromatase receptor

In Aromatase receptor (PDB ID 3EQM) Sterebin G have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -10.84, -6.17, -2.82 and -0.44 Kcal/mol respectively. The interaction pattern is represented in Figure 6.6 in which all the amino acids interact with hydroxyl group of the ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 372 and LEU 477.

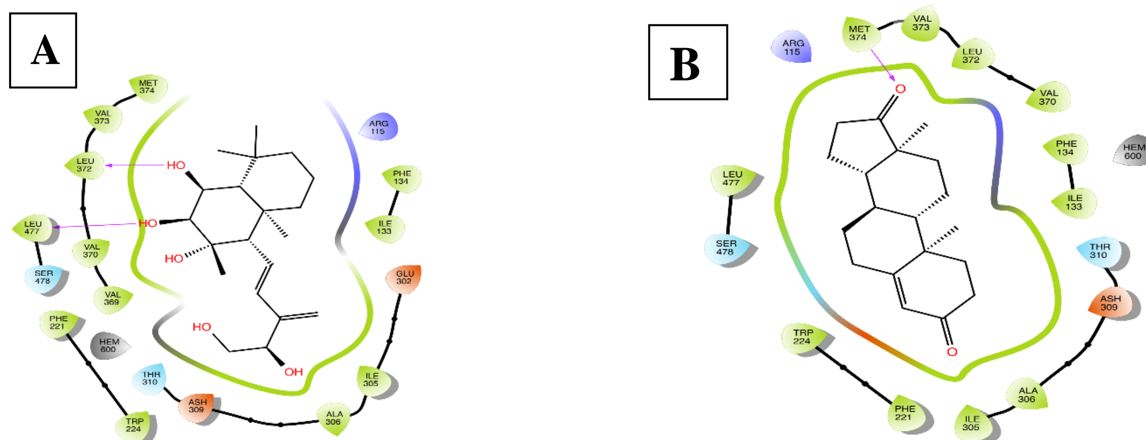

Figure 6.6: **Ligand interaction diagrammed of (A) Sterebin G and (B) 4-Androstene-3-17- dione with aromatase receptor (PDB ID 3EQM).**

The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard aromatase inhibitor 4-androstene-3-17-dione was found to be -5.2, -6.92, -0.7 and -0.25Kcal/mol respectively which is comparatively much higher than top ranked compounds as per dock score. The main interaction pattern included is Hydrogen bonding with carbonyl group of the ligand which is shown in Figure 6.6. The main residues showing interaction with ligand includes is MET 374. Based on the result of docking score phytochemicals of S. rebaudiana have shown significant binding affinity

towards aromatase receptor (3EQM). The overall result of various compounds docked with 3EQM is as represented in Table 6.6

Table 6.6: **Docking score of various phytochemical constituents of *S. rebaudiana* with Aromatase receptor (PDB ID 3EQM).**

Ligand	**Dockscore**	**Lipophilicevdw**	**Hbond (Kcal/mol)**	**E_{Elec} (Kcal/mol)**
Sterebin G	-10.84	-6.17	-2.82	-0.44
Sterebin E	-10.52	-5.91	-2.87	-0.57
Sterebin N	-10.41	-5.63	-3.01	-0.77
Sterebin M	-9.16	-5.84	-1.92	-0.4
Quercetin	-8.41	-5.16	-2.6	-0.62
Luteolin	-8.36	-5.08	-2.38	-0.68
Kaempferol	-8.03	-5.12	-1.96	-0.68
Apigenin	-7.34	-5.11	-1.48	-0.66
Indole3- Acetonitrile	-7.3	-3.4	-0.7	-0.35
Sterebin F	-7.28	-5.1	-2.4	-0.1
Chlorogenic Acid	-7.27	-4.2	-2.71	-0.05
Borneol	-6.72	-3.35	-0.7	-0.11
Torreyol	-6.66	-4.73	-0.7	-0.34
Nerolidol	-6.57	-5.28	0	-0.15
Sterebin A	-6.52	-5.19	-0.96	-0.18
Calamenene	-6.44	-4.84	0	-0.02
Sterebin D	-6.38	-5.09	0	-0.07
Sterebin O	-6.36	-3.88	-0.99	-0.24
Caffeic Acid.	-6.3	-3.29	-1.56	-0.26
Linalool Oxide	-6.13	-3.73	-0.96	-0.37

Eugenol.	-6.07	-3.92	-1.21	-0.26
Sterebin C	-6.04	-3.97	-0.62	0.02
Cis-Linalool Oxide	-6.03	-4.01	-0.7	-0.28
Austroinulin	-6.03	-4.93	-1.44	-0.24
Pinocarveol	-6.02	-3.47	-0.7	-0.26
Spathulenol	-5.82	-4.39	0	-0.03
Myrtenal	-5.79	-3.03	-0.7	-0.16
Terpineol	-5.78	-3.61	-0.52	-0.17
β-Elemene	-5.73	-5.47	0	-0.04
δ-Cadinene	-5.71	-4.87	0	-0.03
7-Austroinulin.	-5.59	-4.41	-0.96	-0.08
γ-Elemene.	-5.58	-5.06	0	-0.01
Geraniol	-5.55	-3.8	-0.75	-0.07
α –Copaene	-5.52	-4.99	0	-0.03
α –Pinene	-5.51	-3.8	0	-0.05
Carvacrol	-5.5	-3.88	-0.4	-0.28
Pinocarvone	-5.44	-2.86	-0.7	-0.11
P-Cymene	-5.37	-3.77	0	0.06
γ-Terpinene	-5.3	-3.66	0	-0.02
Terpinen-4-Ol	-5.29	-3.67	-0.35	-0.05
β-Cubebene	-5.22	-5.05	0	-0.05
4-Androstene-3-17-Dione*	-5.2	-6.92	-0.7	-0.25
Trans-Verbenol	-5.17	-3.68	0	-0.08
Linalool	-5.16	-3.37	-0.52	-0.14
Sabinene	-5.15	-3.64	0	-0.03
β-Pinene	-5.03	-3.46	0	-0.03
Camphor	-5	-3.38	0	-0.06
Sterebin J	-5	-5.13	-2.42	-0.43
Germacrene D	-4.95	-4.71	0	-0.03

β-Ionone	-4.81	-4.22	-0.26	-0.08
Terpinolene	-4.72	-3.61	0	-0.03
β-Bourbonene	-4.71	-5.09	0	-0.03
Sterebin L	-4.34	-6.11	-1.44	0.18
Dihydroisosteviol	-4.19	-6.34	-0.72	-0.11
Limonene	-4.19	-3.5	0	0.01
1,8-Cineole	-4.17	-4.17	0	0
α –Humulene	-4.14	-4.74	0	0.09
Steviol	-4.04	-5.62	-0.7	-0.34
β-Caryophyllene	-3.44	-3.82	0	0
Jhanol	-3.24	-5.22	-0.7	-0.18
Isosteviol	-3.22	-5.58	-0.28	0.11
γ-Cadinene	-3.06	-5.24	0	-0.05
2-Phenylchromone	-2.95	-5.38	0	-0.05
α –Cubebene	-2.94	-5.3	0	-0.05
Citric Acid	-2.81	-1.63	-0.83	0.16
α –Bergamotene	-2.48	-5.72	0	-0.06
3-Hydroxy-2-Phenylchromone	-2.48	-5.35	-0.48	0.08
Trans-β-Farnesene	-2.28	-4.91	0	0.01
Succinic Acid	-2.07	-1.17	-0.35	-0.05
Bisabolene	-1.85	-4.87	0	0
Formic Acid	-0.97	-0.2	-0.27	0
Myrcene	0.54	-3.83	0	-0.1

*Pubchem ID/Name of standard aromatase inhibitor used in the study

6.6 ADME PREDICTION

ADME properties of all studied compounds have been predicted using Qikprop application and are represented in Table 6.7. The phytochemicals of Stevia rebaudiana have shown good binding affinity towards aromatase receptor also the pharmacokinetic profile as well as percentage oral bioavailability of the phytochemical was found to be good. Incase of androgen receptor only two phytochemical that is chlorogenic acid and quercetin have shown better docking score compare to the standard inhibitor testosterone but the pharmacokinetic profile of these phytochemical including the percentage oral bioavailability and predicted apparent caco-2 cell permeability was found to be very poor and incase of estrogen receptor –alpha none of the phytochemical have shown the better binding affinity towards the receptor compare to the standard inhibitor 4-hydroxytamoxifen.The best docking score obtained is of standard inhibitor and after that the best dock score was shown by rutin which have poor pharmacokinetic profile. Thus from the docking study it was clear that the phytochemicals of S. rebaudiana have significant anticancer potential towards these receptors but by the ADME study results it was concluded that these compounds have poor pharmacokinetic profile and required some structural mofication for the best results. Mapping inbuilt pharmacophores and simplification of structure is a logical approach to be considered in this scenario. Moreover such compounds may be useful in cancers where absorption is not required. Incase of steviol and isosteviol predicted oral absorption is more than 90% so there is a chance of modification of these compounds to get good anticancer compounds with good ADME properties which can be more justified by the fact that isosteviol have shown multiple anticancer properties.

Table 6.7: **ADME properties of various phytochemical constituents of *S. rebaudiana* as predicted by Qikprop**

Molecule	Mol_MW	Qplogpo/w	Qppcaco	H-Bond donor	H-Bond acceptor	Qplogbb	Qplogkhsa	% oralabsorption
1,8-Cineole	154.3	2.461	9906	0	0.75	0.605	0.224	100
6-Austroinulin	350.5	4.088	1326	2	4.45	-0.616	0.664	100
7-Austroinulin	350.5	4.131	1510	2	4.45	-0.571	0.663	100
α –Bergamotene	204.4	5.895	9906	0	0	1.111	0.986	100
α -Pinene.	136.2	3.634	9906	0	0	0.871	0.349	100
α –Copaene	204.4	5.321	9906	0	0	1.069	0.933	100
α –Humulene	204.4	5.308	9906	0	0	1.076	1.013	100
α –Cubebene	204.4	5.414	9906	0	0	1.053	0.969	100
Apigenin	270.2	1.607	114.4	2	3.75	-1.447	-0.038	73.194
Apigenin 4-Glucoside	432.4	-0.342	9.229	5	12.25	-3.318	-0.696	29.261
Apigenin 7-O-Glucoside	432.4	-0.314	8.58	5	12.25	-3.405	-0.69	28.853
Austroinulin	322.5	3.518	1599	3	4.15	-0.517	0.399	100
Caffeic Acid	180.2	0.545	22.36	3	3.5	-1.546	-0.804	54.29
Bisabolene	204.4	6.156	9906	0	0	1.132	1.055	100

β –Sitosterol	414.7	7.621	3378	1	1.7	-0.353	2.076	100
β-Carotene	536.9	16.752	9906	0	0	2.22	3.887	100
β -Amyrin Acetate	468.8	7.956	4347	0	2	0.159	2.438	100
β –Pinene	136.2	3.537	9906	0	0	0.857	0.348	100
β -Cubebene.	204.4	5.518	9906	0	0	1.082	1.005	100
β –Bourbonene	204.4	5.302	9906	0	0	1.059	0.938	100
β –Caryophyllene	204.4	5.125	9906	0	0	1.039	0.962	100
β –Elemene	204.4	5.697	9906	0	0	0.995	0.948	100
β –Ionone	192.3	3.111	3430	0	2	-0.027	0.234	100
Borneol	154.3	2.062	4099	1	1.7	0.205	-0.098	100
Dulcoside A	788.9	-1.728	4.716	10	26.55	-4.053	-1.331	0
Dihydroisosteviol	320.5	3.757	282.8	2	3.7	-0.337	0.38	92.824
Citric Acid	192.1	0.115	0.292	3	5.75	-1.964	-1.332	18.053
Glucose	180.2	-2.179	34.16	4	9.5	-2.108	-1.154	41.634
Germacrene D	204.4	5.42	9906	0	0	1.044	0.998	100
δ-Cadinene	204.4	5.591	9906	0	0	1.114	0.999	100
Cis-Linalool Oxide	170.3	2.772	5547	1	1.5	0.17	0.06	100
Chlorophyll A	871.2	13.046	588.7	1	8.25	-2.833	3.579	100
Centaureidin.	360.3	2.252	255.8	2	6	-1.391	0.038	83.228
Carvacrol	150.2	3.298	3684	1	0.75	0.071	0.056	100

Camphor	152.2	1.945	3965	0	2	0.259	-0.175	100
Campesterol	400.7	7.301	3379	1	1.7	-0.291	1.978	100
Calamenene	202.3	5.417	9906	0	0	0.966	0.927	100
Chlorogenic Acid	354.3	-0.297	1.39	6	9.65	-3.414	-0.913	14.802
Chlorophyll B	885.2	11.228	94.65	2	11	-4.143	2.957	100
Dulcoside B	951	-3.977	0.21	13	35.05	-6.891	-2.332	0
Eugenol.	164.2	2.661	3043	1	1.5	-0.129	-0.108	100
2-Phenyl-Chromone	222.2	3.552	2922	0	2.5	0.079	0.135	100
2-Phenyl-4-Chromanol	238.2	2.604	1297	1	3.25	-0.324	0.083	100
Formic Acid	46.03	-0.304	86.02	1	2	-0.502	-1.138	59.792
γ-Cadinene	204.4	5.554	9906	0	0	1.062	0.993	100
γ –Elemene	204.4	5.617	9906	0	0	1.046	0.985	100
γ –Terpinene	136.2	4.097	9906	0	0	0.849	0.402	100
Geraniol	154.3	2.612	2872	1	1.7	-0.235	-0.007	100
Indole 3- Acetonitrile	156.2	2.096	1253	1	1.5	-0.315	-0.185	94.667
Luteolin	286.2	0.927	40.83	3	4.5	-1.956	-0.197	61.208
Luperol Palmitate	665.1	13.532	4313	0	2	-0.914	4.276	100
Lupeol Caffeate	588.9	8.533	520.7	2	3.5	-1.349	2.602	100
Lupeol Acetate	468.8	8.023	3946	0	2	0.042	2.434	100

Lupeol	426.7	7.111	4371	1	1.7	0.11	2.036	100
Linalool Oxide, Trans	170.3	2.742	4862	1	1.5	0.119	0.066	100
Linalool	154.3	3.14	5247	1	0.75	0.015	0.135	100
Lanosterol	426.7	7.706	4352	1	1.7	-0.111	2.172	100
Limonene	136.2	3.986	9906	0	0	0.833	0.382	100
Kaempferol	286.2	1.042	51.22	3	4.5	-1.893	-0.191	63.641
Kaempferol-3-O-Rhamnoside	432.4	0.012	15.8	5	11.3	-2.724	-0.525	35.513
Jhanol	306.5	4.581	4071	1	2.45	0.042	0.877	100
Isosteviol	318.5	3.737	307.4	1	4	-0.239	0.385	93.351
Luteolin 7-O-Glucoside	448.4	-0.947	3.615	6	13	-3.846	-0.812	5.47
Myrcene	136.2	4.601	9906	0	0	0.868	0.4	100
Myrtenal	150.2	1.805	2075	0	2	-0.049	-0.203	96.881
Nerolidol	222.4	4.831	6122	1	0.75	-0.121	0.723	100
P-Cymene	134.2	3.669	9906	0	0	0.702	0.343	100
Pinocarveol	152.2	2.14	4048	1	1.7	0.195	-0.076	100
Pinocarvone	150.2	1.977	3775	0	2	0.165	-0.213	100
Quercetin	302.2	0.368	18.2	4	5.25	-2.419	-0.343	51.655
Quercetin 3-O-Glucoside	464.4	-1.313	4.017	7	13.75	-3.386	-0.848	4.151

Quercetin 3-0-Rhamnoside	448.4	-0.41	12.04	6	12.05	-2.866	-0.636	17.967
Rebaudioside A	967	-4.634	0.243	14	36.75	-6.323	-2.461	0
Rebaudioside B	804.9	-2.16	0.238	11	28.25	-5.221	-1.799	0
Rebaudioside C	951	-4.087	0.149	13	35.05	-6.714	-2.236	0
Rebaudioside T	1247	-9.05	0.004	19	52.05	-9.915	-4.333	0
Rebaudioside S	951	-4.162	0.168	13	35.05	-6.796	-2.313	0
Rebaudioside O	1437	-10.326	0.004	22	60.55	-9.413	-4.822	0
Rebaudioside N	1275	-8.455	0.009	19	52.05	-9.365	-4.205	0
Rebaudioside M	1291	-9.268	0.003	20	53.75	-10.544	-4.534	0
Rebaudioside L	1129	-6.968	0.026	17	45.25	-9.112	-3.71	0
Rebaudioside J	1099	-6.508	0.044	16	43.55	-7.324	-3.068	0
Rebaudioside I	1129	-6.856	0.019	17	45.25	-8.585	-3.451	0
Rebaudioside H	1113	-6.157	0.085	16	43.55	-6.852	-2.988	0
Rebaudioside G	804.9	-2.297	1.707	11	28.25	-4.863	-1.559	0
Rebusoside	642.7	-0.514	7.039	8	19.75	-3.826	-0.837	0.229
Rebaudioside F	937	-4.162	0.381	13	35.05	-5.876	-2.238	0
Rebaudioside E	967	-4.781	0.18	14	36.75	-6.638	-2.53	0
Rebaudioside D	1129	-6.872	0.04	17	45.25	-8.217	-3.531	0
Rebaudioside U	1097	-5.848	0.048	16	42.3	-7.449	-2.956	0

Rebudioside R	937	-4.213	0.444	13	35.05	-5.818	-2.275	0
Rutin	610.5	-2.472	1.113	9	20.55	-4.366	-1.247	0
Sabinene	136.2	3.793	9906	0	0	0.875	0.364	100
Sitosterol D-Glucoside	576.9	5.109	281.3	4	10.2	-2.052	0.936	74.779
Spathulenol	220.4	3.919	5103	1	0.75	0.264	0.662	100
Sterebin A	310.4	2.054	531.6	3	6.15	-0.89	0.028	87.756
Sterebin B	352.5	2.798	467.2	2	6.45	-1.002	0.311	91.105
Sterebin C	352.5	2.853	534	2	6.45	-0.928	0.319	92.469
Sterebin D	294.4	2.929	983.1	2	4.45	-0.576	0.331	100
Sterebin E	338.5	2.536	434.8	4	5.85	-1.151	0.145	89.014
Sterebin F	338.5	2.439	364.1	4	5.85	-1.235	0.126	87.068
Stigmasterol	412.7	7.5	3378	1	1.7	-0.282	2.066	100
Stevioside	804.9	-2.255	2.627	11	28.25	-4.515	-1.544	0
Steviolmonoside	480.6	2.076	26.66	5	11.25	-1.763	-0.22	64.617
Steviolbioside	642.7	-0.305	1.136	8	19.75	-3.659	-0.952	0
Steviol	318.5	3.903	218.6	2	2.75	-0.445	0.505	91.673
Sterebin O	282.4	1.255	558.1	2	6.4	-0.575	-0.17	83.456
Sterebin N	338.5	2.873	574.1	4	4.9	-0.977	0.237	93.151
Sterebin K	352.5	3.39	1292	3	5.85	-0.721	0.348	100

Sterebin M	338.5	3.005	722.4	4	4.9	-0.903	0.253	95.706
Sterebin L	352.5	3.241	1253	3	5.85	-0.67	0.305	100
Sterebin J	336.5	2.28	209.1	3	6.15	-1.427	0.137	81.825
Sterebin I	336.5	2.308	226.1	3	6.15	-1.397	0.14	82.598
Sterebin G	354.5	1.655	194.8	5	7.55	-1.582	-0.166	77.615
Stigmasterol β -D-Glucoside	574.8	5.191	278.2	4	10.2	-1.999	1.002	75.172
Succinic Acid	118.1	-0.577	5.807	2	4	-1.263	-1.194	37.241
Terpinen-4-ol	154.3	2.978	5690	1	0.75	0.244	0.118	100
Terpineol	154.3	2.962	4923	1	0.75	0.189	0.123	100
Terpinolene	136.2	4.202	9906	0	0	0.889	0.433	100
Torreyol	222.4	4.08	4709	1	0.75	0.163	0.705	100
Trans-β-Farnesene	204.4	6.983	9906	0	0	1.132	1.061	100
Trans-Verbenol	152.2	2.099	3562	1	1.7	0.143	-0.077	100

CHAPTER 7.0

SUMMARY

Chapter 7

Summary

On the basis of In-vitro study results in which the anticancer potential of various extracts have been determined using MTT based in-vitro assay using T-47d cell line and have shown significant activity. In T47d cell line AD-2 that is chloroform extract and AD-4 that is aqueous methanol have shown excellent activity with IC_{50} value of 7.79μg/ml and 9.53 μg/ml respectively. Moreover AD-1 that is petroleum ether has shown IC_{50} value of 9.58 μg/ml and on the basis of preliminary test results aqueous methanol extract was selected for isolation process and Total four compounds have been isolated on the basis of single black spot visualized by spraying 5% sulphuric acid on the precoated silica gel $60F_{254}$ TLC plates. However, only two compounds (**ASP-2** and **ASP-4**) were characterized. **ASP-2** was found to be stevioside and **ASP-4** was found to be Rebaudioside A both of which are known compounds. Apart from In-vitro determination of anticancer potential, In-silico approach have also been implemented to determine the anticancer potential of reported compounds obtained from S. rebaudiana. Docking study was performed on Estrogen receptor-α (PDB ID 3ERT), Androgen receptor (PDB ID 2AM9) and Aromatase receptor (PDB ID 3EQM). In case of Estrogen receptor-alpha (PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern. Its docking score was found to be -13.98 Kcal/mol. In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score was found to be -12.97 Kcal/mol. Moreover, in case of Aromatase receptor (PDB ID 3EQM) best dock score of -10.84 Kcal/mol was obtained for Sterebin G. However its major constituents that is steviol glycosides have not shown activity in the inhibition of respected receptors and as a result of In-silico study the Antiproliferative activity was shown by other constituents including the flavonoids. from the literature it has been confirmed that the major constituents showing the Antiproliferative activity are present in the aqueous methanol extract. Thus Phytochemicals of Stevia rebaudiana have shown good binding affinity for all the three receptors and thus seem to have significant anticancer potential. But poor pharmacokinetic profile of these compounds as predicted by

QikProp is the major problem associated with them but structural modification can be tried to improve the ADME properties of these compounds.

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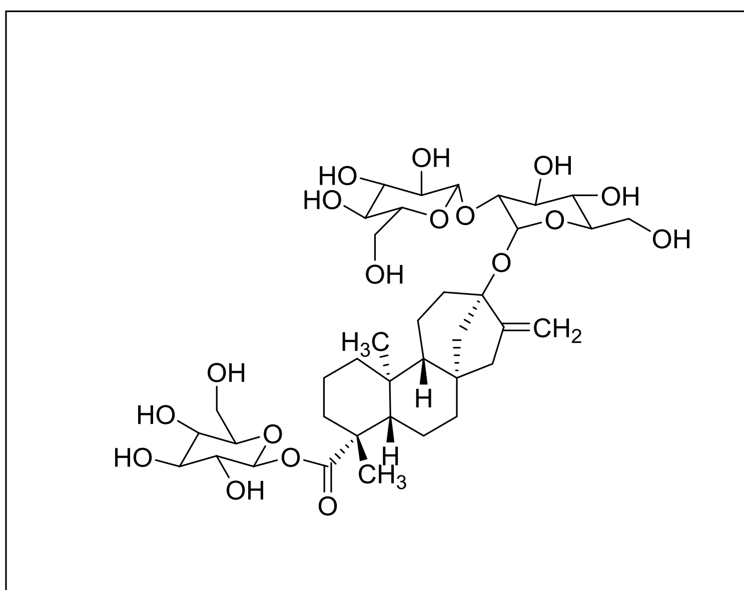
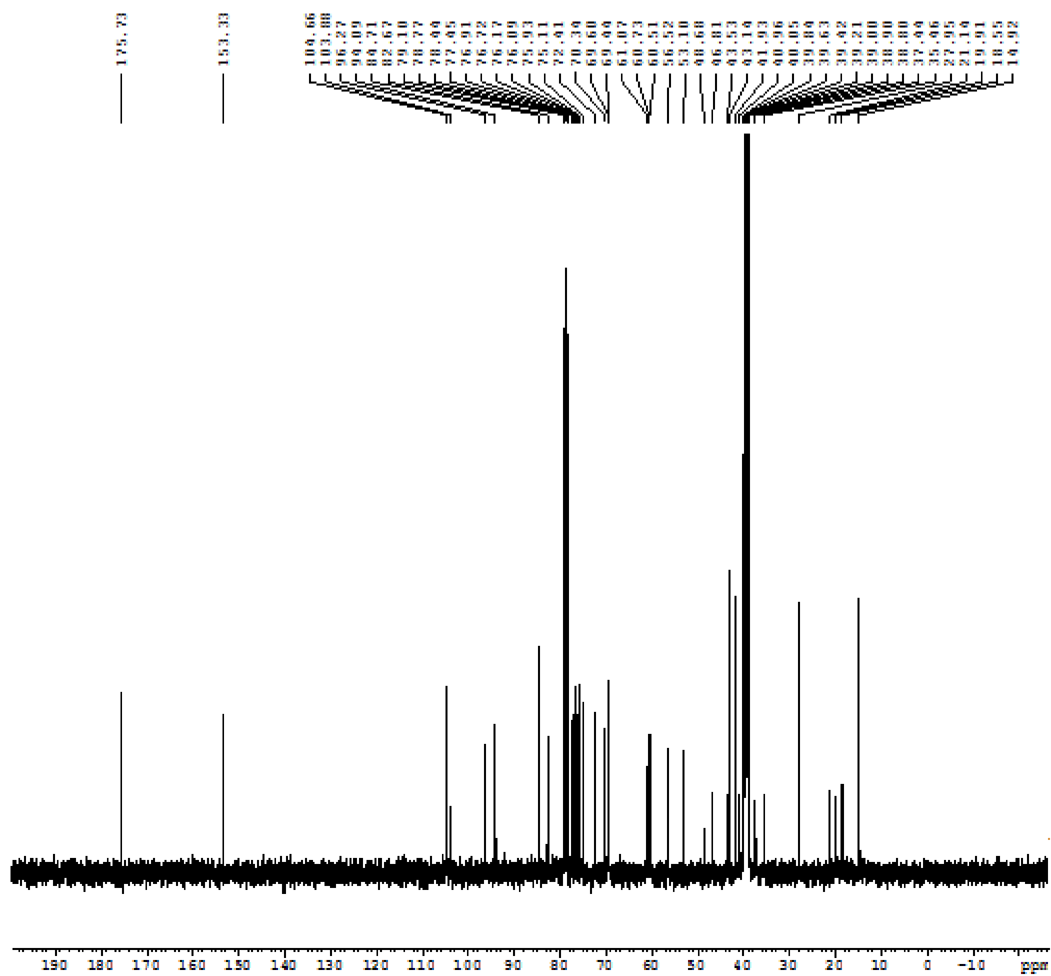
Appendix A

Spectral Data of ASP-2

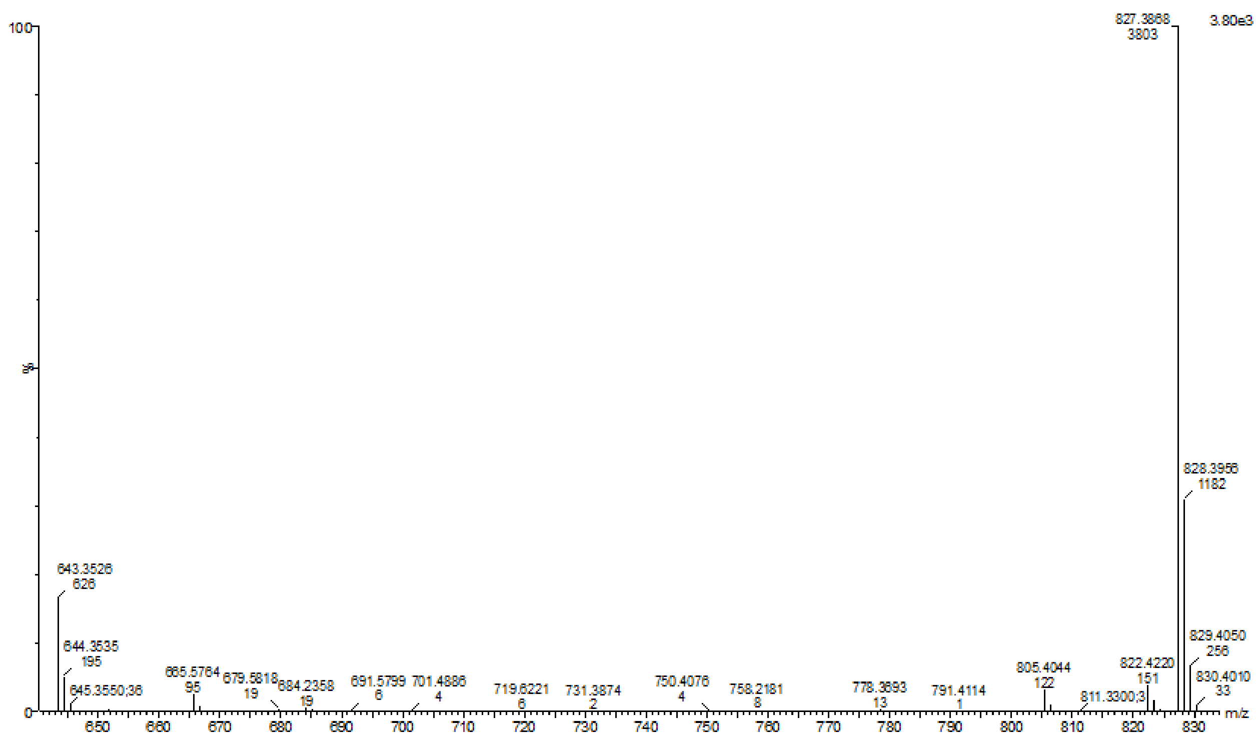


Proton NMR of ASP-2

^{13}C NMR of ASP-2

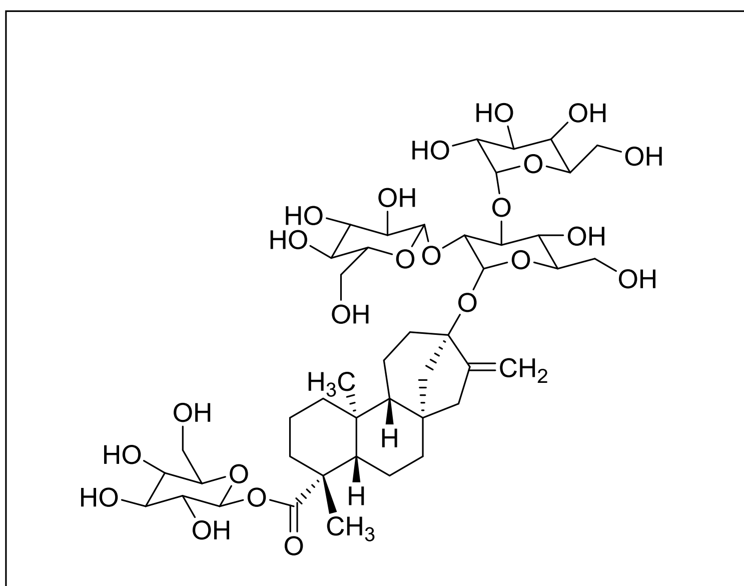


ESI-MS of ASP-2



Appendix B

Spectral Data of ASP-4



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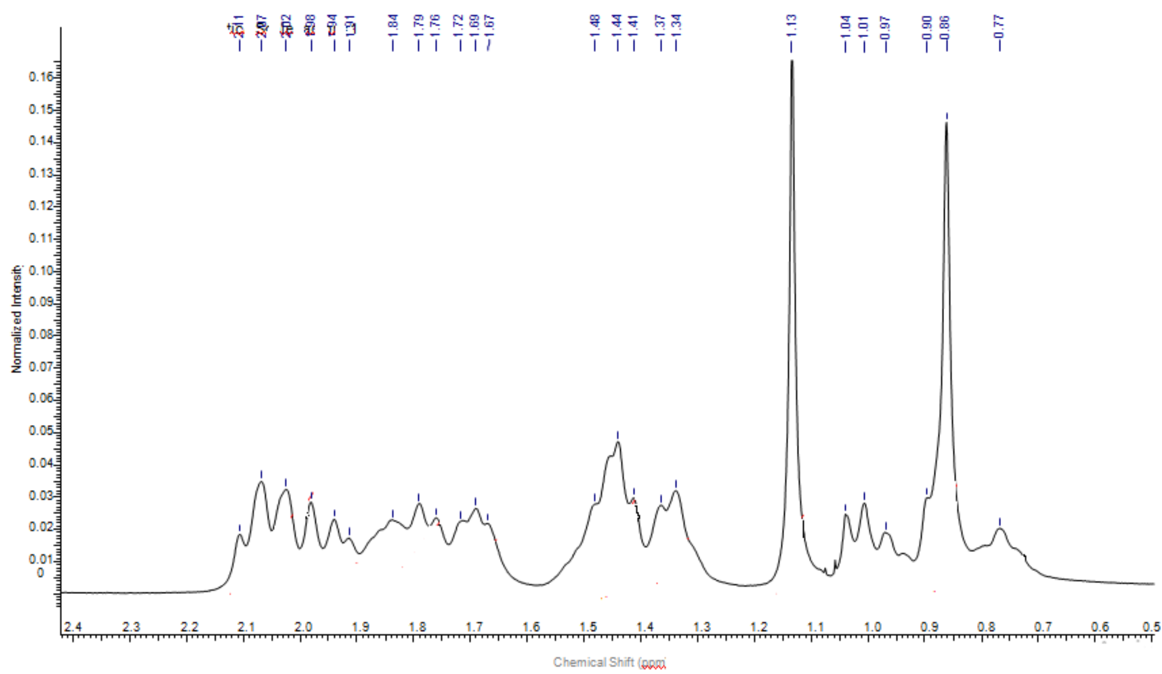

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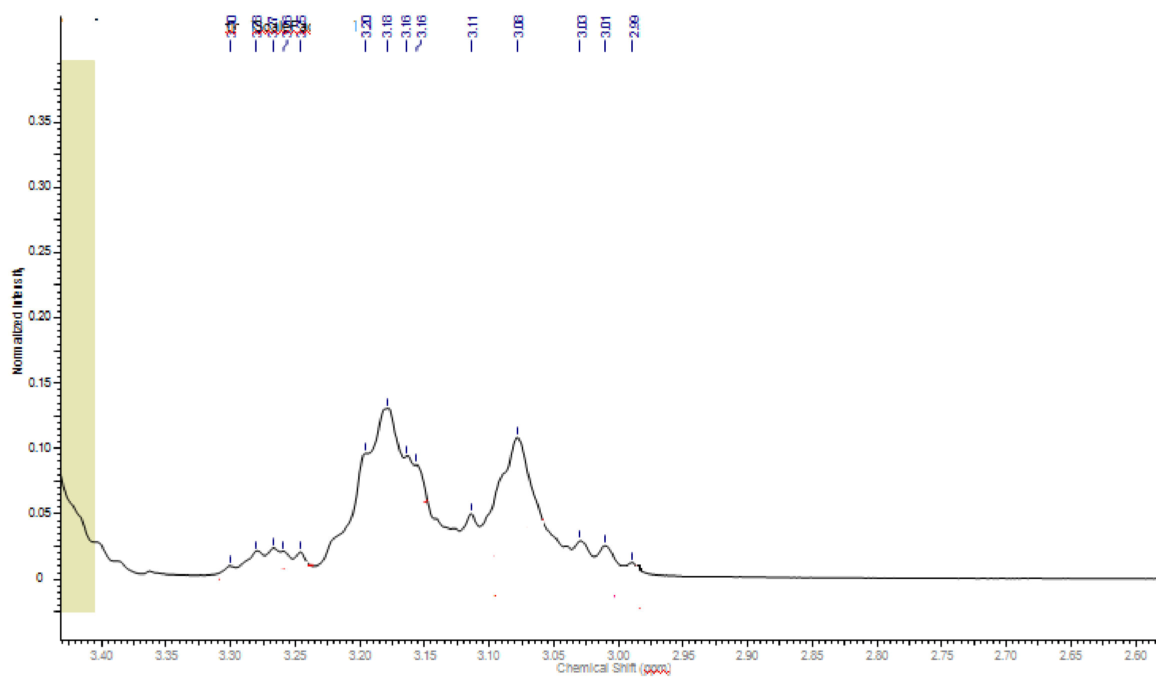

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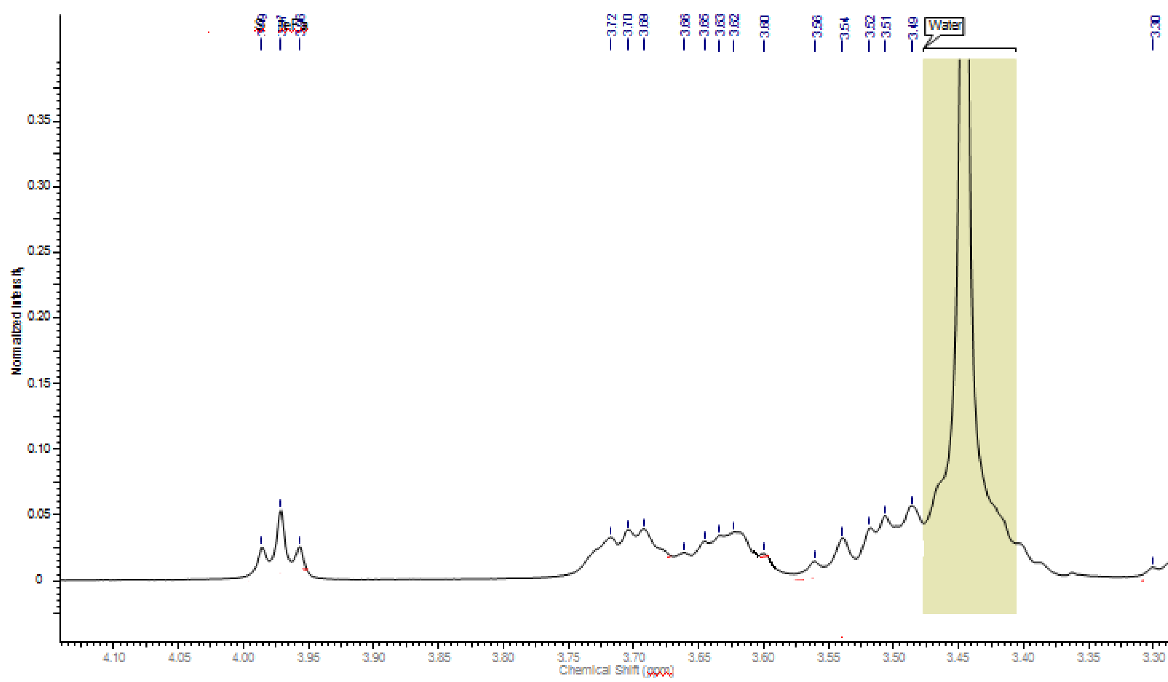

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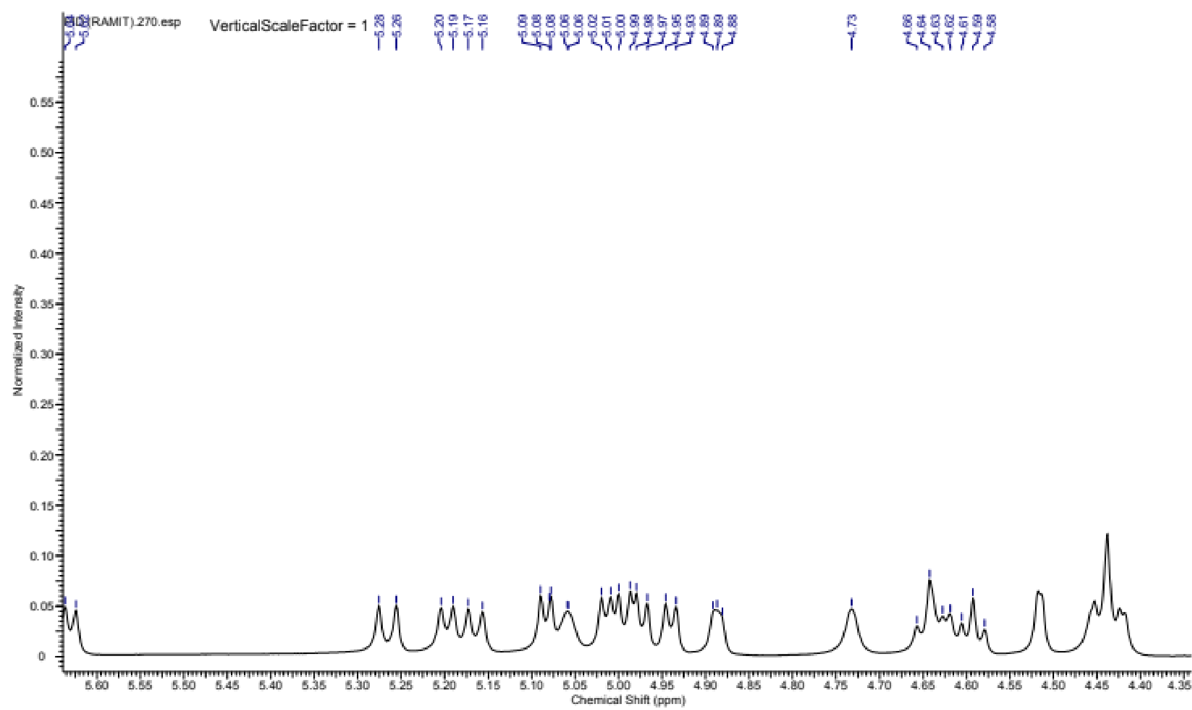
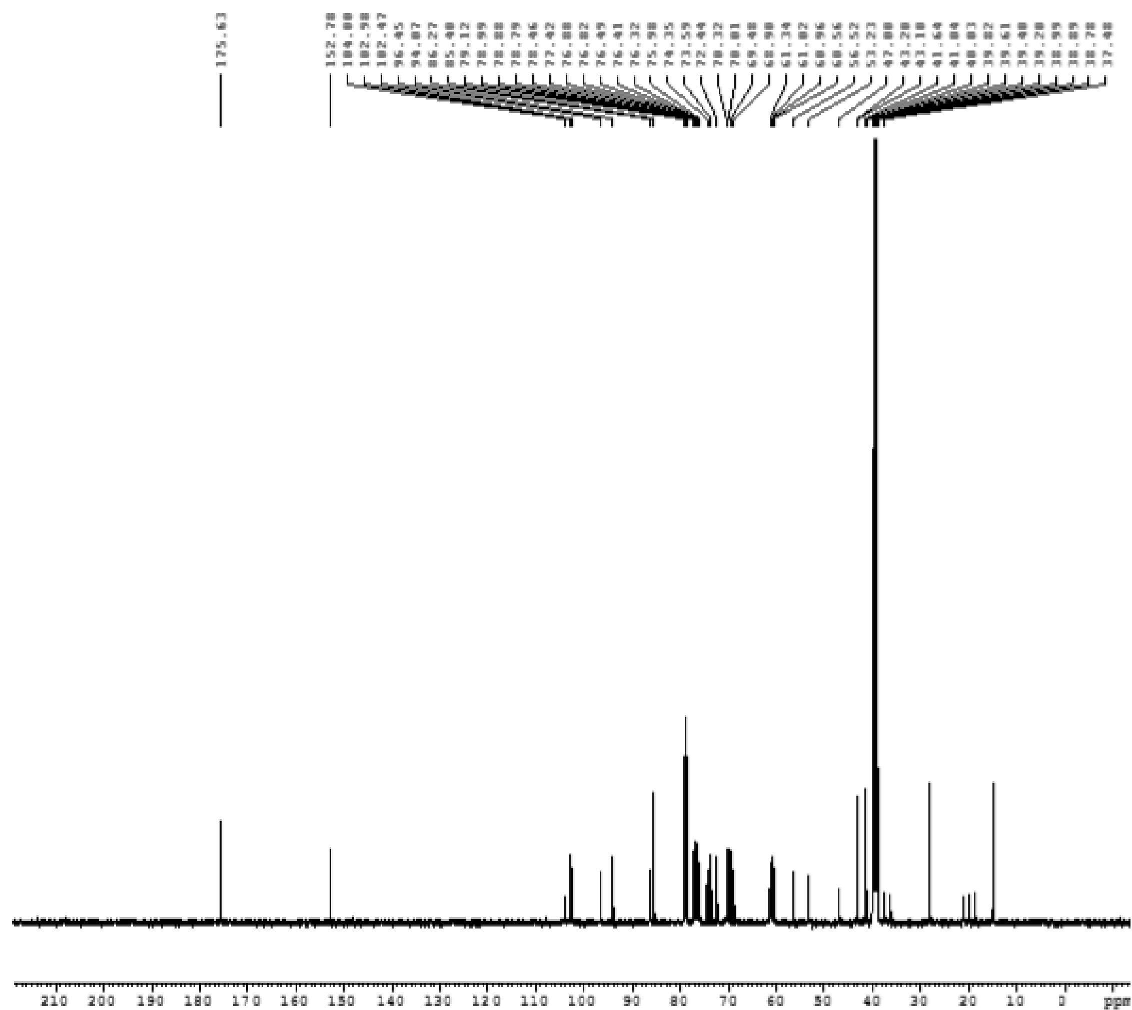


^{13}C NMR of ASP-4



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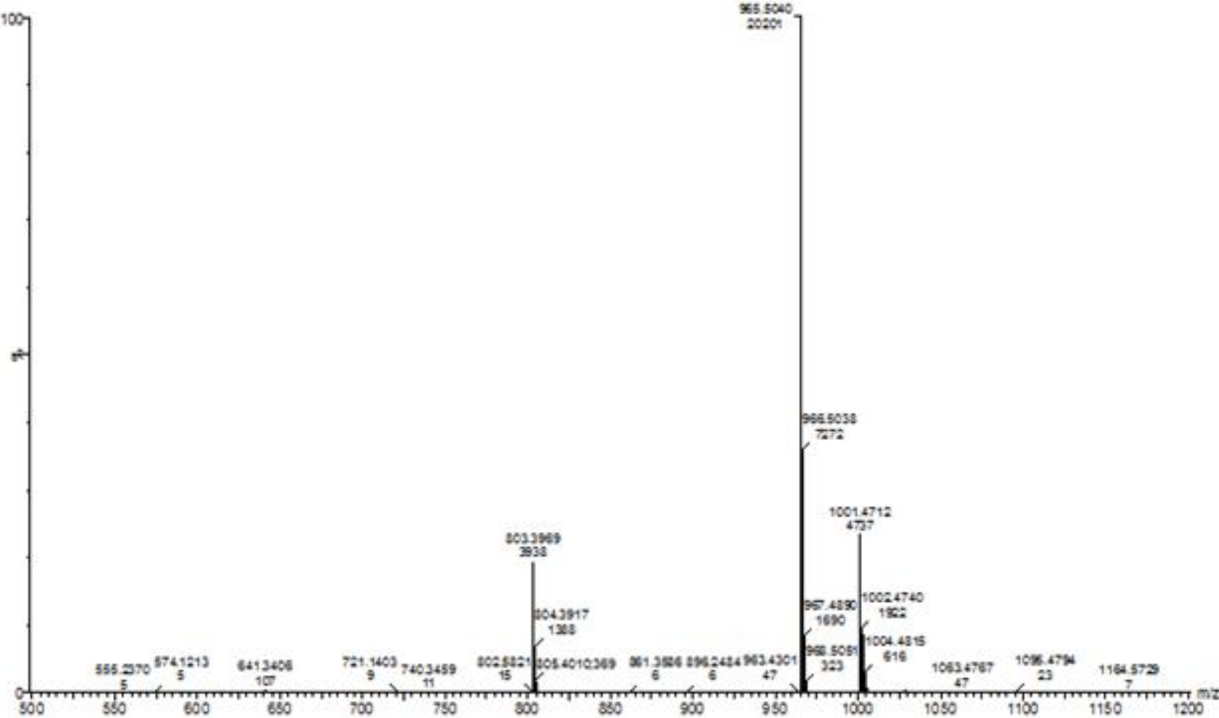

URKUND

Urkund Analysis Result

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4

CHAPTER 1.0

INTRODUCTION

1 INTRODUCTION

The word “cancer” for the first time was coined by Hippocrates, known as father of western medicine, who connected Greek words “carcinoma” and “Karakinos” to express tumor Cancer is considered as the hazardous disease in which the cells keeps on dividing without any control that can also attack to other close tissues. In normal condition the process which takes place to protect the secure condition of tissues is meiosis and apoptosis whereas the procedure which is involved in Carcinogenesis is consists of multi mechanism (figure 1) (Safarzadeh et al.,2014). Cancer is considered as the foremost public health problem in both developed and developing countries. More than 9.7 million cases are detected each year and 6.7 million people died from cancer and everyday, around 1700 Americans died of this disease and approx 20.4 million people are living with cancer in the world today and almost 1 in 3 people were diagnosed with cancer in the UK and 1 in 4 died from this deadly disease. It has been predicted by WHO that the new cases of cancer will reach 15 million until 2020 (Tavakoli et al., 2012). Cancer is the second most important cause of death in the United States after the cardiovascular disease where every one person among the four dies due to cancer (Manju et al., 2017). The substances which are responsible to cause cancer are known as carcinogens. The main reason behind the cause of cancer is mutation which make changes in DNA and loses the control over the growth of the cells (Manju et al., 2017) . Likewise some other factors which causes cancer includes external factors including radiations, smoking, tobacco, toxins in drinking water, foodstuff, atmosphere, certain metals and transmittable agents and internal factors like hereditary mutations, body immune system and hormonal issues are highly responsible for this deadly disease (Iqbal et al., 2017). Today there are more than 100 different types of cancer are known and are usually named by the organ or type of cell from where they start like a cancer that begin in the colon is called colon cancer (Zaid et al., 2017). According to National Cancer Institute (NCI) classification, different types of cancer are classified as follows (Safarzadeh et al., 2014). • Carcinoma: Cancers resulting from epithelial cells. It includes most of the regular cancers, mainly in older adults like breast, prostate, lung, pancreas, and colon cancer. • Sarcoma: Cancers resulting from connective tisssue like bone, cartilage, fat, nerve each of which develop from cells originating in mesenchymal cells exterior to the bone marrow. • Lymphoma and leukemia: these both types of cancer results from the cells tht are involved to make blood. Leukemia is the most general type of cancer mainly in children accounting for almost 30%.conversely, in case of adults both types of cancer can develop. • Germ cell tumor: these types of Cancers are resulting from pluripotent cells, mainly present in the testicle or the ovaries . • Blastoma: These types of Cancers are resulting from undeveloped "precursor" cells or developing tissue. Its mainly occur in the children compare to adults.

Among these the lung cancer is reported the top listed in male followed by breast cancer in female (Zhou et al., 2017) 1.1 BREAST

0: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3711134/ 63%

CANCER Breast cancer is the most commonly diagnosed cancer and is the leading cause of death among women

worldwide (Tabatabaei et al., 2016). It is a malignant tumor that gets started in the cells of breast. The most common Signs

0: https://www.baptisthealth.com/madisonville/pages/services/cancer-care/cancer-types/breast-cancer.aspx 68%

of breast cancer include a lump in the breast, changes in shape and size of the

breast, dimpling of the breast skin, release of liquid from the nipple without squeezing ,pain in the breast that last forever, swelling in the armpit area and at the site of collarbone and a red scaly patches in the skin. Breast cancer most commonly develops in cells lining the milk ducts and the lobules that supply the ducts with milk (Kabel et al., 2015).Similar to other cancers, there are various factors that can elevate the risk of receiving breast cancer like female sex, obesity, short of physical exercise, consumption of alcohol, hormone replacement therapy in menopause, high exposure to ionizing radiation, early age at the time of first menstruation and old age (Gøtzsche et al., 2013). High exposure of estrogen damage the DNA and causes genetic mutation which can result into the breast cancer. In addition to this few people acquire defects in the DNA and mutation in BRCA1, BRCA2 and P53 genes due to hereditary reasons as compare to other peoples. Thus the person who have the family history of ovarian or breast cancer are at increased risk of breast cancer (Kamińska et al., 2015).

1.1.1 STATISTICS In high wages countries like the United States, about 232340 ladies has been diagnosed and 39620 has been died from breast carcinoma in 2016 (Siegel et al., 2018). In 2017, an estimated 252,710 new instances of invasive breast cancer has been diagnosed among ladies and 2,470 instances has been diagnosed in men. Moreover, 63,410 cases of in situ breast carcinoma were diagnosed among ladies and Around 40,610 ladies and 460 men died from breast cancer in 2017 (DeSantis et al., 2017) and In 2018 more 1,735,350 new cancer cases and 609,640 cancer deaths are expected to occur in the United States (Siegel et al., 2018). It has been reported that in an American lady, the danger of rising breast cancer in their entire lifetime is 12.38% or 1 out of 8 (Nowsheen et al., 2017).

1.1.2

0: https://www.agingcare.com/articles/breast-cancer-signs-symptoms-treatments-136545.htm 78%

TYPES OF BREAST CANCER Breast cancer can be categorized as invasive or non-invasive.

Ductal carcinoma in situ (DCIS) is a non-invasive breast cancer moreover it is also recognized as stage 0. In the DCIS, the unusual cells are controlled in the milk ducts of the breast and do not broaden into the neighboring breast tissue. Whereas incase of Invasive breast cancer abnormal cells do multiply from their original site that can be either the milk ducts or the

lobules that is the sacs that make breast milk to the neighboring breast tissue. It might also have multiply to the lymph nodes. (Horlings et al., 2013). All the invasive breast cancers and DCIS are tested for hormone receptors. steroid hormone are mainly responsible for this deadly disease specially estradiol, which plays an significant function in the development of breast cancer, and a majority of the human breast cancers begin out as estrogen dependent and convey the estrogen receptor (ER) (Clarke et al., 2003). From the literature it has been confirmed that the elevated levels of endogenous estrogens and androgens and minor levels of sex hormone binding globulin (SHBG) are linked with higher danger of postmenopausal breast cancer (Hormone et al., 2013)These breast cancers can be treated with hormone therapy such as tamoxifen and aromatase inhibitors. Nearly all the invasive breast cancers are hormone receptor-positive (Abeshouse et al., 2015). 1.1.3 ROLE OF ESTROGEN, ANDROGEN AND AROMATASE IN BREAST CANCER The steroid hormone, estradiol, has the very significant function in the development of breast cancer, and mostly the human breast cancers found were estrogen dependent and convey the estrogen receptor (ER). Estrogen mediates its biological effect by binding to one of the structurally and functionally different ERs that is ERα and ERβ (Saha Roy et al., 2012). Over expression of estrogen caused the risk of breast cancer as much clear mechanism is not known. In normal condition where the level of estrogen is maintained the chance of getting breast cancer is very low, but mutation in the certain metabolites of estrogen that when bind to DNA causes the hydroxylation of estrogen and induce the quinone and semiquinone formation (Santen et al., 2015). The quinone form of estrogen binds with DNA and produce DNA adducts and begins the transcription of proteins which is responsible for the cause of breast cancer. Semiquinone intermediate are free radicals which can bind to oxygen and produce superoxide radicals and alter the structure of DNA (figure1.2.) (Santen et al., 2015). Aromatase receptor play an important role in breast carcinogenensis. The aromatase enzyme is the important enzyme for the biosynthesis of estrogen. This enzyme is also known as estrogen synthase. It induces aromatization of androgens to estrogens hence responsible for the overexpression of estrogen (Yamamoto et al., 2014). From the literature it has been confirmed that about 60% of breast cancer state this enzymes with elevated levels of mRNA expression and activity when compared among the non-malignant tissue. It catalyzes the final steps of estrogen biosynthesis androgens to estrogens particularly conversion of testosterone to estradiol and androstenedione (Group et al., 2015). Androgen receptor is expressed in 60-70% of breast cancer and plays a dual role with estrogen. Inhibition of Androgen receptor is mainly responsible for the down regulation of ERK signaliing therefore androgen receptor induce its effect by genomic and non genomic pathways. In the genomic pathway androgen bind and directly control the expression of gene in DNA and in case of non genomic signaling where nucleus receptor gets signals with other proteins interactions. Androgen receptor is responsible for the breast cancer mainly due to irregulation of growth hormone and cytokines. this combined signaling contribute majorly for the breast cancer (Iacopetta et al., 2012).

1.1.4 THERAPIES FOR BREAST CANCER The main treatments used for cancer are chemotherapy, radiotherapy and surgery (Solowey et al., 2014). A successful anticancer drug should destroy or injure the cancer cells devoid of causing unnecessary damage to the normal cells. But this ideal process by the anticancer drug is very complex, or maybe impossible to achieve and is the main reason for the unpleasant side effects from which the patients have to

suffer when under-going treatment (Manju et al.,2017). the main Chemotherapeutic agents includes cytostatic and cytotoxic drugs which have revealed excellent outcome either alone or in the grouping with additional cancer therapies. Some main chemotherapeutic agents includes the topoisomerase inhibitors like- Irinotecan which shows the side effects including neutropenia, sensory neuropathy, and diarrhoea and doxorubicin whose having the high risk of causing cardiotoxicity. The alkylating agents used for curing cancer includes oxaliplatin, melphalan, carboplatin, cisplatin and cyclophosphamide but shows the side effects including cardiovascular toxicity, nephrotoxicity, gastrointestinal toxicity, pulmonary and hematologictoxicity (Pegram et al., 2004). In addition to this the main drawback about these drugs is the cancer cells resistance to these drugs as they go through mutation like ABCA4 and ABCA12 are the Drug resistant genes which are over-expressed in human MCF-7 breast cancer cells correspondingly when the drug docetaxel was used for the treating the cancer (Iqbal et al., 2017)

As there are many drugs available for the treatment of cancer but it also have number of side effect thus it is very important to look for novel anticancer agents which have superior efficiency and minor side effects. In this case Natural compounds are considered as the good sources for developing new remedies for the treatment of various diseases (Aung et al., 2017).

1.1.5 USE OF MEDICINAL PLANTS TO TREAT BREAST CANCER

Medicinal plants and herb have been used from ancient times to treat human chronic diseases including cancer much before the invention of modern drugs (Manju et al., 2017). At present about 60% of drugs used for the treatment of cancer are isolated from natural products in which the plant kingdom has been the most important source (Solowey et al., 2014).

Plants have various active compounds which work synergistically for giving the therapeutic benefits and bringing down the dangers of side effects so that no other supplemental therapy is required to control the cancer debility. Thus it has made very important to uphold the use of natural Ayurvedic therapies for curing various types of cancers and imply an incorporated approach for the management of tumor and for its treatment (Shukla et al., 2015).Some important plants used for the treatment of breast cancer as well as for other cancers are mentioned in the table 1.1. (Safarzadeh et al., 2014)

The therapeutic significance of the plants are due to the occurrence of chemical substances that generate a specific physiological action in the human body as mentioned above In the table 1.1. Main other bioactive compounds of the plants used for the treatment of diseases include alkaloids, flavanoids, tannins and phenolic compounds. Also, several number of plant leaves tends to have antimicrobial principles such as tannins, essential oils and new aromatic compounds. In addition to that some preclinical studies have confirmed that the phytochemicals have great importance in the prevention of colorectal cancer and other cancers (Jayaraman et al., 2008). Thus Herbal medicines are refined natural compounds which can control the different phases of diseases at the same time by the different mechanisms (Shukla et al., 2015). Whereas the chemical medicines are the individual synthetic compounds that can be intrusive in an ideal condition by the single mechanism. Mainly the anticancer drugs used for the treatment of cancer whether it is synthetic chemicals or the natural

products, tends to cooperate with the DNA or its precursors which produces the irreversible harm to DNA and restrain the synthesis of proteins. Thus, curing the cancer cells by using the mono-target chemical agent is not an efficient method. hence, on the basis of broad research conclusion, the phytochemicals and their resulting analogues are considered as the best option for the enhanced and less lethal for the cancer treatment (Singh et al., 2016). As there are number of plants whose phytochemicals are used today for the treatment of many diseases as well as for the cancer and among them one of the very important plant is Stevia rebaudiana belonging to the Asteraceae family in which the major sweet constituent in the leaves of the Stevia rebaudiana (Bertoni) is stevioside which is 300 times sweeter as compare to sucrose and has just gained significance as a natural non-caloric sweetener (Siddique et al., 2016).Beside sweetness, stevioside along with other associated compounds including rebaudioside A, steviol, and isosteviol may also recommend therapeutic uses, such as antioxidative, antihyperglycaemic, anti-hypertensive, antitumor, antidiabetic, anti-HIV (Paul et al., 2012). It is also reported that the isosteviol inhibited DNA polymerases and DNA topoisomerase II. Which are considered as the

0: http://www.scielo.br/pdf/rbfar/v19n2a/a02v192a.pdf 88%

important cellular targets for the development of anti-cancer agents (Mizushina et al., 2005).

Moreover Stevia leaf extracts and the presense of polyphenolic constituents have revealed the inhibitory effect on tumor commencement and its promotion (Heikal et al., 2008). Thus, information concerning the structural characteristics of stevioside-based compounds may provide valuable insight for the design of new anti-cancer agents.

CHAPTER 2 REVIEW OF LITERATURE

Chapter 2 Review of literature Stevia rebaudiana (SR) is considered as the medicinal herb which has been utilized in the traditional Armenian medicine to lowering down the glucose, cholesterol and blood pressure levels and also adjust the immune function of the body. (Aghajanyan et al.,2017). It is originated from the northeast of Paraguay (Mathur et al.,2017). The initial botanical explanation of the plant was acknowledged by M. S. Bertoni In 1889. The plant was earlier recognized as Eupatorium rebaudianum Bert. in tribute of Rebaudi, the first chemist who studied the chemical distinctiveness of the substances extracted. Its name was afterward changed to the present one. The genus Stevia incorporates 230 species but only S. rebaudiana provides the sweet taste property. Some other related species include S. eupatoria, S. micrantha, S. plummerae, S. rhombifolia, S. serrata, S. salicifolia, S. viscida, S. commixta, S. origanoides, S. leptophylla, S. satureiaefilia, S. ophryphylla, S. selloi, S. nepetifolia and S. triflora (Ruiz-Ruiz et al., 2017). Stevia is about 200-300 times sweeter than the sugar; its sweetness effectiveness is alike to that of aspartame. Stevia is cultivated worldwide due to its function as a non-caloric sweetener. It has been utilized for many years in the treatment of diabetes in various countries as it have no toxic harm as well as no side effect (Yadav et al., 2011). It have great value as a economic medicinal plant since it have pharmaceutically active compounds all through the world. Stevia has been used a non-caloric natural-source having other remedial applications together with anti-cariogenic, anticancer, antioxidant and

antidiabetic properties. Biotechnological techniques present novel approaches for its industrial production, propagation as well as conservation and management of Stevia (Karimi et al., 2017).

2.1. PHARMACOGNOSTICAL CHARACTERISTICS

2.1.1 COMMON NAMES Sweet leaf, sweet herb, honey leaf, Stevia, sweet leaf of Paraguay, caa-he-éé, kaa jheéé, ca-a-jhei, ca-a-yupi, azucacaa, eira-caa, capim doce, erva doce, sweet-herb, honey yerba, yaa waan, candy leaf, sugar leaf, sweet honey leaf, Eupatorium rebaudianum (Jayaraman et al., 2008; Marković et al., 2008).

2.1.2 GEOGRAPHICAL SOURCE The genus Stevia, mainly Stevia rebaudiana is initially from Paraguay. The sweet feature of Stevia has been used by the Paraguayan Indians from several centuries. It is native to the northern regions of South America. Stevia is still found growing wild in the highlands of the Amambay and Iguacu districts which is the border area flanked by Brazil and Paraguay. It is predicted that almost 200 species of Stevia are native to South America; conversely, none of the other Stevia plants have acquired the similar intensity of sweetness as the S. rebaudiana. It is developed commercially in many parts of Paraguay, Brazil, Israel, Uruguay, Thailand, Central America and China. it is also refined on a minor scale in Canada, Mexico, USA, some European countries and also with the Israel. In india it is mainly cultivated in Karnataka, Punjab, himachal Pradesh, Haryana, uttar Pradesh, west Bengal , Madhya Pradesh and tamil nadu. In Japan Stevia have been utilized for more than 50 years as a sweetening agent in a variety of foods and beverages (Brandle et al., 1998; Mandal et al., 2013).

2.1.3 PLANT MORPHOLOGY Stevia rebaudiana is a herbaceous perennial plant belongs to the Asteraceae family. The plant reaches upto 65cm in height, having sessile, oppositely arranged lanceolate to oblanceolate, dark green colour leaves with toothed margins. The taste of leaves is sweet. The seeds of Stevia are commonly small, brown and black in colour and on maturation the seeds turn into black coloured fruits which are dispersed with the help of persistent pappus bristles as in the case of dandilon. The root system of Stevia rebaudiana is on the surface and with a little lateral root. The flower are little in size, white in colour, are bisexual and fashioned in clusters of 2 -6 florets, through sprouts that are initially tender and finally gets harden (Lester et al.,1999; Shaffert et al.,1994). 2.2. CHEMICAL COMPOSITION 2.2.1 Steviol glycosides Steviol glycosides are the most important components of Stevia rebaudiana which is responsible for its sweetener capacity. It commonly contains glucose moieties which are joined with the aglycone part termed as Steviol (1) (Puri et al., 2011) . Isosteviol (2) is the oxidized product of steviol mainly famous for its therapeutic benefits including anticancer activity (Jaitak et al., 2008). The plant consists of more than 40 steviol glycosides in varying concentrations (Oehme et al.,2017). The major steviol glycosides found are Stevioside (3) and Rebaudioside A (4), having the highest content in the plant. The percentage of stevioside found to be 6–10%, and of rebaudioside A is 2–4% and the other minor glycosides is up to 0.1-1%. Stevioside is 250-300 times sweeter than sucrose whereas rebaudioside-A is 300-400 times sweetener than sucrose (Singla et al., 2016). the other steviol glycosides found in the plant are Rebaudioside B (5), Rebaudioside C (6), Rebaudioside D (7), Rebaudioside E (8),

Rebaudioside F (9), Rebaudioside G (10), Rebaudioside H(11), Rebaudioside I(12), Rebaudioside J (13), Rebaudioside L (14), Rebaudioside M (15), Rebaudioside N (16), Rebaudioside O(17), Rebaudioside R (18), Rebaudioside S (19), Rebaudioside T (20), Rebaudioside U (21), Dulcoside A (22), Dulcoside B (23), Rebusoside (24), steviolbioside (25). Structure of various isolated steviol glycosides. 2.2.2 Non-glycosidic diterpenes Labdane-type diterpenes comes under this category of constituents of S .rebaudiana. the various compounds of this group are austroinulin (26) jhanol (27), Sterebins (28-40), (Ibrahim, El-Gengaihi, Motawe, & Riad, 2007). The chemical structure of these non –glycosidic diterpenes.

2.2.3 Flavonoids The plant contains numerous number of flavonoids including quercetin (41), apigenin 4-O-glucoside (42), apigenin (43), Kaempferol 3-O-rhamnoside (44), luteolin 7-O-glucoside (45), quercetin 3-O-rhamnoside (46), quercetin 3-O-glucoside (47), apigenin 7-O-glucoside (48), rutin (49), centaureidin (50) (Wölwer-Rieck et al., 2012). The chemical structure of these flavonoids has been shown in figure 2.3

2.3. BIOSYNTHETIC PATHWAY OF SGS Steviol glycoside molecules come under the diterpene group of compounds which tends to have huge number of biological functions in plants. The Production of these diterpene group of compounds have the common primary biosynthetic pathway. Poly –isoprene is the basic backbone of all the diterpene compounds. Isoprene is a simple organic compound having the following chemical structure (figure 2.4).

The structure of poly-isoprene is mentioned in figure 2.5. structure of Geranylgeranyl diphosphate is meintioned below (figure 2.6) it is the main precursor for numerous compounds found in the plants including the steviol glycosides. Biosynthesis of steviol glycosides consist of two stages. first stage includes the Geranylgeranyl diphosphate or GGDP synthesis from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Geranylgeranyl diphosphate is the initial point for various biologically essential diterpene compounds present in the plants. In the second stage, steviol glycosides are formed by the conversion of Geranylgeranyl diphosphate by following multiple steps. The two main precursors that is isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are produced by a very important pathway in plants known as 2-C-methyl-D-erythritol-4 phosphate pathway or MEP pathway. The MEP pathway is described below with the formation of GGDP (Brandle et al., 1998). The steps followed in this pathway can be catalyzed only in the presence of some definite enzymes. In case if these specific enzymes are absent, the biochemical reactions cannot be completed. STEP 1 In this step Pyruvate and Glyceraldehyde 3 phosphate are combined to form Deoxyxyulose 5 phosphate which further gets phosphorylated by the Cytidine triphosphate. This is the beginning of the MEP Pathway STEP 2 Further phosphorylation by Adenosine Triphosphate (ATP) followed by formation of 2-C-Methyl-D-Erythritol-2,4-Cyclodiphosphate STEP 3 In this step Isopentenyl Diphosphate and Dimethylallyl Diphosphate are formed. These are known to be the precursors for variety of isoprenoid compounds found in the plants. This is the last step of MEP pathway STEP 4 In this step Copalyl diphosphate is form by the Geranylgeranyl diphosphate formation and its cyclization. This is the beginning of the late stage Steviol glycoside synthesis (figure 2.10). STEP 5 In this step, ionization dependent cyclization takes place and Kaurene is produced formed from Copalayl Diphosphate which then gets oxidized to kaurenoic acid. The next step involves

the formation of steviol by the hydroxylation of kaurenoic acid. From this step steviol glycoside biosynthetic pathway takes a distraction from gibberellin biosynthesis pathway. STEP 6 In this step various steviol glycosides are form as the steviol gets glycosilated by the Glucosyl Transferase enzymes.

2.4 Pharmacological properties of S. rebaudiana

2.4.1 Anti-hyperglycemic effect Stevia rebaudiana extracts has been used from the ancient times for treating the diabetes as it increases the sensitivity and secreation of insulin (Chatsudthipong et al., 2009).The effect of stevioside as Anti-hyperglycemic was found to be mediated by its effect on phosphophenol pyruvate carboxy kinase (PEPCK) which is the rate-limiting enzyme intended for gluconeogenesis and consequently controls the glucose production in the liver. It was observed that stevioside repress the PEPCK gene expression and lastly reduces gluconeogenesis. In addition stevia powder is more proficient in down-regulating PEPCK in comparison to stevioside which specify that except stevioside various other chemical compounds can be present which are responsible for down-regulating PEPCK (Chatsudthipong et al., 2009). In the study it is found that the production of insulin is enhanced by the action of Rebaudioside A in the pancreatic islet cells of mouse. Also stevioside promotes the glucose-activated insulin secretion, devoid of any disturbance in case of fasting insulinemia (Mathur et al., 2017).

2.4.2 Antimicrobial activity It has been reported that Stevia inhibits growth and reproduction of bacteria which is responsible for gum disease and tooth decay thus showing anti-microbial activity.It was also further found that the major cariogenic organism, Streptococcus mutans, experiences growth repression and less acid secretion when grown on media containing stevoside in comparison to media containing the fructose, glucose or the sucrose (Mathur et al., 2017).

2.4.3 Anti-inflammatory and immunomodulatory activity Various SGs have shown important effects on pro-inflammatory cytokines. 1 mM dose of stevioside moderately amplified the creation of tumor necrosis factor (TNF-α), interlukin (IL-1β) and nitric oxide (NO), in un-stimulated human THP-1 cell by interacting through toll-like receptor-4. Therefore stevioside can be valuable to healthy individual as it is accomplished to develop the innate immunity (Boonkaewwan et al., 2013). In the study the effects of stevoside and steviol as anti-inflammatory was studied on epithelial cells of colon. Moreover Stevoside has been found to reveal inhibitory effects on the contraction of smooth muscles of intestine whose inhibition deals with the hyper-motility related diarrhea. It has been onserved that the steviol along with its analogs has antidiarrheal effect as it inhibits cAMP regulated Cl- secretion in T84cells (Mathur et al., 2017). 2.4.4 Anti-diarrheal activity The anti-diarrheal potential of SGs was reported by Pariwar and his co-workers . According to the information stevioside and its foremost metabolite, steviol, were establish to influence the ion transport in various types of tissues including kidney, pancreas and intestine. Moreover short-circuit current measurements by them in the study showed that steviol and its analogs isosteviol, dihydroisosteviol and isosteviol 16-oxime inhibits forskolin-induced chloride secretion in a dose-dependent manner and have IC50 values of 101, 100, 9.6, and 50 mM, respectively.

Parent compound stevioside was found to be free from this effect. In the same study apical current measurement indicated that dihydroisosteviol besieged the cystic fibrosis transmembrane regulator. Inhibitory action of this compound was found reversible and was not linked with changes in the intracellular CAMP level. Pariwar and co-workers further recognized that it did not affect calcium-activated chloride secretion and T84 cell viability. In-vivo studies using a mouse closed-loop model of cholera toxin-induced intestinal fluid secretion showed that intra-luminal injection of 50 mM dihydroisosteviol reduced intestinal fluid secretion by 88.2% devoid of altering fluid absorption, thus indicating that dihydroisosteviol and related compounds could be a new class of cystic fibrosis transmembrane regulator inhibitors that may be helpful for additional development as anti-diarrheal agents (Brahmachari et al., 2011).

2.4.5 Anti-hypertensive effect Stevia and stevioside extract property as a antihypertensive could be due to their effects on the plasma volume. Stevioside administration by I.V causes natriuresis, diuresis and increased renal plasma flow but glomerular filtration rate (GFR) is unaffected (Melis, et al., 1992). It is reported that the stevioside causes vasodilation therefore reduces the total peripheral resistance as it inhibits the influx of Ca2+ in the vascular smooth muscle (Chatsudthipong et al., 2009). However stevioside has no influence on vasopression induced vasoconstriction when the medium is free of Ca2+.Thus it is clear that it causes vaso-relaxation by inhibiting the influx of Ca2+ (Ruiz-Ruiz et al., 2017).

2.4.6 Anticancer activity From the literature it has been confirmed that the stevioside have the cytotoxic ability. it mediates the apoptosis in MCF-7 human breast cancer cell line by the induction of reactive oxygen species (ROS) through mitochondrial pathway. By the intracellular ROS generation it conveyed the apoptotic signal (Gupta et al., 2017).Hence it was concluded that the stevioside increases the expression of apoptotic proteins such as Bax, Bcl-2 and Caspase-9 By the induction of the ROS-mediated mitochondrial permeability transition (Gupta et al., 2017). In the study it was reported that the Stevioside have inhibited tumor promotion by the TPA in the mouse skin cancer (Karimi et al., 2017). In addition isosteviol inhibits DNA polymerase and human topoisomerase II which are prominent targets of anticancer drugs (Chatsudthipong et al., 2009). Chaiwat reported the effects of steviol and stevioside on the human colon carcinoma cell lines by the MTT method. The cell viability in Caco-2, HT29 cells and T84 decreases when the concentrations of stevioside was 2–5 mM and that of steviol was 0.2–0.8 mM (Brahmachari et al., 2011). It is also mentioned that the Stevia leaf extracts and the presense other constituents such as its polyphenolic constituents have the inhibitory effect on tumor commencement and its promotion (Heikel et al., 2008). As a result SGs have revealed major anticancer activity which can be more explored for investigating the better anticancer compounds.

CHAPTER 3 Aim and Objectives

Chapter 3 Aim and Objectives

Work has been conducted by following these objectives • Isolation and characterization of secondary metabolites from S. rebaudiana leaves • In-vitro study of different extracts of S. rebaudiana (Bertoni).

• In-silico Study of reported secondary metabolites from S. rebaudiana.

CHAPTER 4 Rational

Chapter 4 Rational

• Therapies available for treating the cancer cause maximum side effects such as bone marrow depression, neutropenia, cardiotoxicity, mouth soreness, hairfall, nerve changes and also affects the normal cells which is danger for the person suffering from it and ultimate can cause the death of the individual, so the research for drugs having minimum side effects is necessary.

• Drugs obtained from natural products are considered safe as it have minimal side effects and the chances for curing the disease is much higher and today almost 60% drugs available for curing cancer are obtained from natural products therefore plant kingdom is considered the main source.

• From literature review, we observed that Stevia rebaudiana which is a very famous plant for its sweet taste and having zero caloric value mainly used for the treatment of diabetes is also having many other therapeutic benefits including Antiproliferative activity and as mentioned in literature various cell line including HT-29, MCF-7 the Antiproliferative action of the phytochemicals from S. rebaudiana has been observed.

• Keeping in view the above mentioned facts about the medicinal value of the plant present study was conducted to further explore the phytochemical and medicinal aspect of Stevia rebaudiana using In-vitro and In-silico approach.

Thus from this plant, we can get the Phytochemicals as Antiproliferative agent for curing the aggressive disease cancer

CHAPTER 5

Material and Method

Chapter 5

Material and Method

5.1 Chemical and Instruments Solvent methanol and ethyl acetate (laboratory grade) was procured from Finar limited India, petroleum ether (laboratory grade) was procured from SDFCL (sd fine-chem limited) Mumbai India. Solvent Chloroform laboratory grade was procured from Thomas baker chemicals Pvt. Ltd India. RPMI 1640 and DMEM, antibiotic solution, Phosphate buffer and bovine serum media were used to culture cancer cell lines. Sulphuric acid (91%) was purchased from Loba Chemie Pvt Ltd. Silica gel 60\120 for column chromatography was procured from SDFCL, glassware of fine quality were used and procured from Borosil JSGW, readymade TLC plates F254 from Merck were used, Rota vapor instrument LABINDIA was used. Laboratory grade reverse osmosis water R.O system was used. T-47 (Breast Cancer) cancer cell lines were selected for biological activity. These cell lines procured

from National Cell Repository located at NCCS Pune. For visualizing the TLC plates procured UV chamber from Mac Company,the characterization of structure of isolated compound NMR (H1 and C13) 400 MHz were used. Isolation of compounds was done by flash chromatography procured from Biotage. Other instruments used for thesis work such as Incubator for incubation,oven, automatic cell counter, UV-Vis spectrophotometer, laminar airflow. For docking studies of reported phytochemicals and isolated compounds Maestro software 2015 procured from Schrodinger Company. 5.2 Procurement and Preparation of Plant material Dried aerial part of plant S. rebaudiana was procured in the month of july 2013 from Green Valley farm situated at Pojewal (Garhshanker) in Punjab (Altitude 355m; Latitude 31.191769oN, longitude 76.258774oE) (figure 5.1).

5.3 Preliminary phytochemical investigation of extracts The powdered material (10g) was subjected to successive extraction with methanol and water (80:20) in the sonicator at 55°C for 30 min. The proportion of methanol from the extract was evaporated by using the rotavapour and the remaining proportion of water was fractionated with petroleum ether, chloroform, ethyl acetate, and aqueous methanol and was dried on a rotavapor yielding 42mg, 128mg, 234mg, and 304mg respectively. Now the qualitative chemical test of all the extracts of stevia rebaudiana was subjected to detect the presence of various phytoconstituents. 5.3.1 Test for Alkaloid: The samples are added with 3ml of picric acid saturated solution. Samples giving yellow precipitate shows presence of alkaloid. 5.3.2 Test for Fixed oil: On a clean filter paper add 2 drops of each sample. If it leaves a translucent spot then it is the presence of fixed oil

5.3.3 Test for Volatile oil: All samples are added with alcoholic solution of Sudan III dye. If the samples become red then volatile oil is present.

5.3.4 Test for Tannins: Add FeCl3 to all the sample. Yellow colour give hydrolysable tannin whereas green colour give condensed tannin.

5.3.5 Test for Flavanoid: The samples are added with NaOH solution producing yellow colouration. On adding dilute acid if the mixture goes colourless then presence of flavonoid is confirmed.

5.3.6 Test for Glycoside: Part A: Add dilute H2SO4 in the samples then add 5% NaOH neutralizing it. To it add equal volume of Fehling solution 1 and solution 2. Red colour is produced.

Part B: Add DM Water to all the test tubes until it is diluted then add equal volumes of Fehling solution 1 and 2. Red colouration

Compare the redness if part A has more intensity than part B then we can say that there is presence of reducing sugar. 5.3.7 Test for Steroids and triterpenes: All samples are added with conc. Sulphuric acid. Yellow on top layer show steroid and green on bottom show triterpenes. 5.4 In vitro anticancer activity

Different Extracts of S. rebaudiana were prepared for evaluating its anticancer activity. For studying this activity MTT assay was performed using the T-47 D breast cancer cell lines. In this cell lines 7500 cells were taken in each well consisting of 96 well total in plate. All the

prepared extracts were completely dried and stored in an eppendorf tube and weighed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a stock solution The tubes were submitted to animal cell culture laboratory in central university of Punjab. The eppendorf tubes were marked as AD-01 (Petroleum ether extract), AD-2(chloroform), AD-3 (Ethyl acetate), AD-4 (Methanol + water extract). In Animal cell culture laboratory T-47D cells are taken on petridish after thawing it from -80oC to body temperature and then media is added after 24 hours adherence is formed and then splitting of adherent cells . The cells of the cell lines was treated with different extracts of the plant in triplicate of its concentration and the experiment was also repeated three times .This process continues until sufficient cells for 96 well plate can be used enough. In the 96 well plate Plant extract of different concentration is added along with serum free media and MTT. Violet colour formation is seen due to formazone ring formation with dehydrogenase enzyme. The colouration is checked under 570 nm which is directly proportional to cell viability. Results were then plotted in graphs to calculate cytotoxic potential and IC50 values. 5.5 Isolation and characterization compounds

5.5.1 Isolation

On the basis of preliminary test performed and the in-vitro study aqueous methanol extract was selected for the isolation of compounds. The extract was further subjected to successive extractions with 100% methanol, methanol: chloroform (50:50) and aqueous methanol. All the extracts were dried using rotavapour. On the basis of TLC observation 100% methanol extract was selected for isolation process. The aqueous methanol extract (23.67g) was dissolved in methanol and 14g silica was added. The mixture was dried over rotavapor and further was subjected to successive extractions with 100% methanol, methanol: chloroform (50:50) and aqueous methanol to yield 10g,6g and 4g respectively and a TLC for all the three extracts was prepared. Progress of the reaction was monitored by TLC using solvent system ethyl acetate/ ethanol/H2O (8:2:1.2, v/v/v).The developed plate was dried in oven and spots were visualized by spraying with 5% sulfuric acid. Maximum spots were obtained with 100% methanol. The 100% methanol extract was subjected to flash column chromatography using a gradient elution of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 45, 50%, 55%, 60%, 65%, 70%, 75%, 80% chloroform: methanol to give 1,792 fractions. The analysis of all the fractions was performed by TLC using the mobile phase consists of ethyl acetate/ethanol/H2O (8:2:1.2 v/v/v). Fraction 239-290 were combined and dried on the rotavapour yielding ASP-1 JMG (4mg). Fraction 423-443 were combined and dried on the rotavapour yielding ASP-2 (22mg).Fraction 529-533 were combined together on the basis of a single spot on precoated silica gel 60F24 TLC plates .The combined fractions were dried on rotavapour yielding ASP-3JMG (17mg). The Fractions 600-610 were combined together on the basis of single spot on precoated silica gel 60F24 TLC plate. The combined fractions were dried on a rotavapour to give 7mg of ASP-4JMG.

5.5.2 Characterization of ASP-2 Melting point was found to be 197-199oC.

1H NMR (400 MHz, DMSO): Showed three anomeric peaks at δHn 4.87 (1H, d, J= 4Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) respectively.

13C NMR peaks of ASP-2 in (PPM,100MHz,DMSO): 14.92, 18.55, 19.91, 21.14, 27.95, 35.46, 37.44, 40.96, 41.93, 43.14, 43.53, 46.81, 48.65, 53.10,56.52, 60.51,60.73, 61.07, 69.44, 69.60,

70.34, 72.41, 75.11, 75.93, 76.09, 76.17, 76.72, 76.91, 77.45 , 78.44, 82.67, 84.71, 94.09, 96.27, 103.88, 104.66, 153.33, 175.73.

HRMS (ESI): m/z calc. [M+Na]+ calculated for (C38H60O18Na)+ 827.3868 corresponding to molecular formula C38H60O18.

5.5.3 Characterization of ASP-4

Melting point was found to be 244-246oC

13C -NMR (100 MHz, DMSO): 14.89, 18.56, 19.79, 21.14, 28.04, 37.48, 38.70, 39.20, 40.03, 41.04, 41.64, 43.10, 43.20, 47.0, 53.23, 56.52, 60.56, 61.02, 61.34, 68.90 69.48, 70.01, 70.32, 72.44, 73.59, 74.35, 75.98, 76.49, 76.88, 76.98, 77.42, 78.46, 78.79, 78.88, 79.12, 85.40, 86.27, 94.45, 96.45, 102.47, 102.98, 104.0, 152.78, 175.63.

1H NMR (400 MHz, DMSO): Showed four anomeric peaks at δHn 5.64 (1H, d, J = 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz) respectively.

HRMS (ESI): m/z calc. for C44H70O23 [M-H]- 965.5040 corresponding to molecular formula C44H70O23.

5.6 In-silico activity The computational study of molecular recognition is an important part of structure-based drug design. The molecular docking problem is commonly cast as a problem of finding the low-energy binding modes of a small molecule, or ligand, within the active site of a macromolecule, or receptor, whose structure is known. With the help of molecular docking environment, using Maestro software 11. The mechanism behind the antiproliferative activity of phytochemical from S. rebaudiana was determined. The targets selected for the study were estrogen receptor alpha, androgen receptor.

5.6.1 Ligand preparation With the help of ChemBio Draw ultra 12.0 structures of all the phytochemicals (figure 3.1)including the structures mentioned in chemical constituents in the review section were drawn and saved in SDF format. The molecules were changed from 3D structure to the 2D by using the Lig Prep version 2.5. These ligands were imported from workspace option then were subjected to ligand preparation using ‘LigPrep’ wizard application in Maestro 9.6 The Lig Prep developed a single, low energy, 3D structure with correct chiralities for the each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Lig Prep application of the Maestro operates OPLS 2005 force field. 5.6.2 Protein Preparation: The PDB for the crystal structure of estrogen alpha (PDB Id 3ERT) has been obtained from RSCB protein data bank. It is mainly responsible for the breast cancer. crystal structure of human androgen receptor in complex with testosterone (PDB ID 2AM9) have also been downloaded from protein data bank and the crystal structure of human aromatase receptor in complex with androstenedione (PDB ID 3EQM)has obtained from protein data bank.The protein preparation wizard option was worn for preparation of protein structure with polar hydrogen. This procedure consist of two steps that is preparation and refinement. In this step, bond orders were consigned, all hydrogen atoms were added ,bonds to metals were deleted and formal charges were set on the metal and the neighboring atoms and water molecules were deleted

that were more than the 5Å specific distance. Also the reorientation of hydroxyl groups, water molecule and amino acids to the optimization of hydrogen bond network. The refinement process is the last step for protein preparation. This steps involves firstly optimization then add hydrogen only and minimization. Thus in this process the restrained impact of minimization of protein was taken and stearic clashes were revealed and made the protein ready to dock. 5.6.3 Receptor Grid generation Generation of the grid at a exacting site in the protein is an important factor to perform docking study. It is not possible to perform Ligand docking without the generation of receptor grid. In case of 3ERT, 2AM9 and 3EQM the grid was generated at the position of co-crystallized ligand which already has to bound to protein. The ligand molecule has to be selected for grid generation. The length of ligands which are selected for docking were made to 36Å. 5.6.4 Glide Docking GLIDE (Grid Based Ligand Docking with Energetics) software was used for docking procedure developed by Schrödinger. Three docking precision is available in GLIDE docking module such as HTVS (High throughout visual screening), standard precision (SP) and Extra precision (XP). HTVS is proposed for quick screening of very large numbers of ligands. It has controlled conformational sampling. SP is set as a default parameter and is used to screen thousands of compounds. Extra precision docking and scoring is more dominated and sharp procedure. Extra precision is developed to locate active compounds that binds to a particular conformation of receptors. Docking of reported phytochemicals and the inhibitor was done with extra precision. Extra precision descriptions were written, ligand was taken as flexible. Epik penalties were added to the docking score. Docking score was taken into consideration for comparing the results, more negative the docking score more potent the compound and indicates the good binding potential. Various components are present in GLIDE score like hydrogen bonds, hydrophobic contacts, Van der Waals interaction, and Columbic interaction, Polar interaction in the binding site, Metal binding and Freezing rotable bonds. 5.6.5 ADME Study For estimating the absorption, distribution, metabolism and excretion (ADME) properties of chemical compounds Qikprop module application present in Schrödinger suit 2013 is used. It predicts physically significant descriptions and evaluates the pharmacokinetics properties of ligands by retrieving the drug like properties. Using this module we have calculated ADME properties of chemical compounds of S. rebaudiana used in the study.

CHAPTER 6.0 RESULTS AND DISCUSSION

Chapter 6.0 Results and discussion

6.1 Preparation of Extracts The powdered material (4.3 kg) was subjected to successive extractions (figure 6.1) with petroleum ether, ethyl acetate, CHCl3 extract, and aqueous methanol (20:80 v/v).

6.2 Preliminary phytochemical investigation of extracts Phytochemical test of Petroleum ether extract, CHCl3 Extract, ethyl acetate and Aqueous methanol extracts of S. rebaudiana were subjected to tests detection for the presence of various phytoconstituents. General test were performed such as alkaloid test, fixed oil test, test for tannin, volatile oil test, Flavonoid Test, Glycoside test, Steroid test, Trirepenes test and test for saponin. From the phytochemical investigations of extracts of S. rebaudiana, CHCl3 Extract extract and methanol- water

exhibited that the most chemical constituents were present. So aqueous methanol extract was selected for further study and for the isolation of the compounds. 6.3 In- vitro study For estimating the anticancer activity of extract of the S. rebaudiana MTT assay was performed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a stock solution and subjected to in- vitro antiproliferative activity. MTT evaluates demonstrated results in varied range of concentration. In the MTT cell proliferation measurement of all proliferation rates and similarly, when metabolic events lead to apoptosis or necrosis, that reduction in cell viability. The MTT reagent yields get low. The T-47 D breast cancer cell lines confirmed that the chloroform extract has high activity against breast cancer cell lines after that aqueous methanol shows the high activity against the cell line and than the petroleum ether extract. Further IC50 values of aqueous methanol extract, petroleum ether extract, and chloroform extract were calculated. 6.4 Characterization of isolated compounds

6.4.1 Characterization of ASP-2 Compound ASP-2 was a crystalline white solid, produced a single black spot which was visualized by spraying with 5% sulfuric acid on a precoated silica gel 60F254 TLC plate. Its m/z ratio indicated an ion peak at m/z [M+Na]+ calculated for (C38H60O18Na)+ 827.3868 corresponding to molecular formula C38H60O18. The 1H and 13C NMR of compound ASP-2 showed three anomeric values at δHn 4.87 (1H, d, J= 3.44Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) and δ 13C 94.09, 96.27 and 104.66 respectively assigned to three glucose molecules. In 13C NMR, δc at 14.92 (C-20) and δc 27.95 (C-18) indicates the presence of two methyl groups. δc value at 175.73 and δc 103.88 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively. Above mentioned observation confirmed that the compound ASP-2 is stevioside which is represented in Figure 4.2. stevioside is a SG Containing three glucose molecules attached with steviol moiety having melting point of 197-199oC. Further confirmation of structure was done on the basis of literature (Danieli et al., 1997).

6.4.2 Characterization of ASP-4

Compound ASP-4 was a crystalline white solid, produced a single black spot on a precoated silica gel 60F254 TLC plate. Its m/z ratio indicated an ion peak at m/z calc. for C44H70O23 [M-H]- : 965.5040 corresponding to molecular formula C44H70O23. The 1H and 13C NMR of compound ASP-4 showed four anomeric values at δHn 5.64 (1H, d, J = 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz). and δ 13C 94.45, 96.45, 102.98 and 102.47 respectively assigned to four glucose molecule. In C13 NMR, δc at 14.89 (C-20) and δc 28.04 (C-18) indicates the presence of two methyl groups. δc value at 175.63 and δc 104.0 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively and additional peaks for sugar moiety at C-3" was obtained to be 102.98, 78.46, 74.35, 73.59, 70.01 and 61.02. Which confirms the presence of one extra glucose moiety compare to stevioside. Above mentioned observation confirmed that the compound ASP-4 is Rebaudioside A which is represented in Figure 4.3. Rebaudioside A is a SG Containing four glucose molecules attached with steviol moiety having melting point of 244-246oC. Further confirmation of structure was done on the basis of literature (Steinmetz et al., 2009). 6.5 In silico study

The preliminary screening for anticancer property of S. rebaudiana extracts by MTT assays using T47D cell line demonstrated that the extracts of S. rebaudiana retains antiproferative activity specially showing the activity against the breast cancer, due to presence of phytochemical constituents. From the literature it is also confirmed that the constituents showing excellent dock score are obtained from aqueous methanol extract (Muanda et al., 2011).Estrogen receptors like estrogen alpha play an important role in regulation of estrogen hormone in women. Irregulaties in these receptors causes to induce the breast cancer. Docking study experiment shown that phytochemical constituents S. rebaudiana regulate the estrogen receptors. Phytochemical constituents prevent the binding of tamoxifen with its receptor (Estrogen α). Androgen receptor also playing key role in the induce of breast cancer. Testosterone used as a standard inhibitor for androgen receptor. Aromatase receptor also initiated the breast cancer by play a key role in biosynthesis of estrogen. Aromatase convert the testosterone into estrone by aromatization. 4- Androstene-3-17- dione used as a standard inhibitor for the aromatase receptor. These inhibitor makes down regulation of the signaling pathway and these phytochemical constituents also acts as inhibitor of enzymes involved in the biosynthesis of estrogen hormones. The targets selected for the docking study are estrogen receptor alpha , androgen receptor and aromatase receptor. All these targets having a vibrant role in the breast cancer proliferation. anticancer drug candidates were identified by glide docking of compounds of S.rebaudiana on all the three receptors and the best compounds were selected on the basis of lowest docking score. Reported 123 compounds of S.rebaudiana were taken for the docking study and the topmost compound showing the best docking result for antiproliferation is mentioned in detail below with compare to the standard inhibitor taken and other compounds result are mentioned in the table. 6.5.1 Estrogen receptor alpha In Estrogen receptor alpha (PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -13.98, -7.84, -1.01 and -1.02 Kcal/mol respectively. The interaction pattern is represented in figure 6.4 in which majority of amino acid residues interact with hydroxyl group and with the amino group of ligand by forming the hydrogen bond. The main residues showing interaction with ligand include GLU 353, ARG 394 and ASP351.

Next in the rank order according to docking score was Rutin which showed dock score, Lipophilicity, H bond and electrostatic energy of -10.44, -4.26, -4.34 and -1.3Kcal/mol respectively. The main interaction pattern included stacking interaction and Hydrogen bonding which is shown in figure 6.4. TRP 383 showed π-π stacking interaction with ring A of ligand. ALA 350, ASP 351, MET 528 and LEU 525 amino acid residues showed hydrogen bonding of various hydroxyl groups of ligand. Thus on the basis of docking score top two compound was mentioned in case of estrogen receptor alpha which shows significant binding affinity for the receptor. The dock score, Lipophilicity, H bond and electrostatic energies of all the compounds includes in the study are shown in table 6.4.

6.5.2 Androgen receptor In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -12.97, -4.66, -4.97 and -1.17 Kcal/mol respectively. The interaction pattern is represented in figure 6.5 in which all the amino acids interact with hydroxyl group of the

ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 704 and ASN 705. The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard androgen inhibitor testosterone was found to be -12.17, -6.64, -1.33 and -0.64Kcal/mol respectively which is comparatively higher than chlorogenic acid. The main interaction pattern included is Hydrogen bonding with carbonyl group and the hydroxyl group of the ligand which is shown in figure 6.5. The main residues showing interaction with ligand includes ARG 752, THR 877and ASN 705. Thus on the basis of docking score phytochemical of S.rebaudiana have shown significant binding affinity towards androgen receptor (2AM9). The overall result of various compounds docked with 2AM9 is as represented in table 6.5.

6.5.3 Aromatase receptor In Aromatase receptor (PDB ID 3EQM) sterebin G have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -10.84, -6.17, -2.82 and -0.44 Kcal/mol respectively. The interaction pattern is represented in figure 6.6 in which all the amino acids interact with hydroxyl group of the ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 372 and LEU 477. The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard aromatase inhibitor 4-androstene-3-17-dione was found to be -5.2, -6.92, -0.7 and -0.25Kcal/mol respectively which is comparatively much higher than top ranked compounds as per dock score. The main interaction pattern included is Hydrogen bonding with carbonyl group of the ligand which is shown in figure 6.6. The main residues showing interaction with ligand includes is MET 374. Based on the result of docking score phytochemicals of S. rebaudiana have shown significant binding affinity towards aromatase receptor (3EQM). The overall result of various compounds docked with 3EQM is as represented in table 6.6.

6.6 ADME PREDICTION ADME properties of all studied compounds have been predicted using Qikprop application and are represented in Table 6.7. The phytochemicals of Stevia rebaudiana have shown good binding affinity towards aromatase receptor also the pharmacokinetic profile as well as percentage oral bioavailability of the phytochemical was found to be good. Incase of androgen receptor only two phytochemical that is chlorogenic acid and quercetin have shown better docking score compare to the standard inhibitor testosterone but the pharmacokinetic profile of these phytochemical including the percentage oral bioavailability and predicted apparent caco-2 cell permeability was found to be very poor and incase of estrogen receptor –alpha none of the phytochemical have shown the better binding affinity towards the receptor compare to the standard inhibitor 4-hydroxytamoxifen.The best docking score obtained is of standard inhibitor and after that the best dock score was shown by rutin which have poor pharmacokinetic profile. Thus from the docking and ADME data it was clear that the phytochemicals of S. rebaudiana have significant anticancer potential towards aromatase and androgen receptor but pharmacokinetic is the major problem specially for the androgen receptor incase of aromatase it is somewhat good but required some structural mofication for the best results. Mapping inbuilt pharmacophores and simplification of structure is a logical approach to be considered in this scenario. Moreover such compounds may be useful in cancers where absorption is not required. Incase of steviol and isosteviol predicted oral absorption is more than 90% so there is a chance of modification of these compounds to get good anticancer compounds with good ADME

properties which can be more justified by the fact that isosteviol have shown multiple anticancer properties.

CHAPTER 7.0 SUMMARY

Chapter 7 Summary Dried aerial part of plants powder was extracted thrice by solvents with increasing order of polarity using petroleum ether, chloroform, ethyl acetate, and aqueous methanol respectively. All the extracts were dried using rotavapor. On the basis of In-vitro study results and than with the TLC observation aqueous methanol extract was selected for isolation process. Total four molecules have been isolated on the basis of single black spot visualized by spraying 5% sulphuric acid on the precoated silica gel 60F254 TLC plates. However, only two compounds (ASP-2 and ASP-4) were characterized. ASP-2 was found to be stevioside and ASP-4 was found to be Rebaudioside A both of which are known compounds. The anticancer potential of various extracts have been determined using MTT based in-vitro assay using T-47d cell line and have shown significant potential. In T47d cell line AD-2 that is chloroform extract and AD-4 that is aqueous methanol have shown excellent activity with IC50 value of 7.79μg/ml and 9.53 μg/ml respectively. Moreover AD-1 that is petroleum ether has shown IC50 value of 9.58 μg/ml. Apart from in-vitro determination of anticancer potential, in silico approach have also been implemented to determine the anticancer potential of reported compounds obtained from S. rebaudiana. Docking study was performed on Estrogen receptor-alpha (PDB ID 3ERT),Androgen receptor (PDB ID 2AM9) and Aromatase receptor (PDB ID 3EQM). In case of Estrogen receptor-alpha(PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern.Its docking score was found to be -13.98 Kcal/mol. In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score was found to be -12.97 Kcal/mol. Moreover, in case of Aromatase receptor (PDB ID 3EQM) best dock score of -10.84 Kcal/mol was obtained for Sterebin G. from the literature it has been confirmed that the major constituents showing the Antiproliferative activity are present in the aqueous methanol extract. Thus Phytochemicals of Stevia .rebaudiana have shown good binding affinity for all the three receptors and thus seem to have significant anticancer potential. But poor pharmacokinetic profile of these compounds as predicted by QikProp is the major problem associated with them but structural modification can be tried to improve the ADME properties of these compounds. Moreover these compounds may be used directly to target cancers where absorption is not required.

2

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TYPES OF BREAST CANCER Breast cancer can be categorized as invasive or non-invasive.	types of breast cancer. Breast cancer can be invasive or non-invasive.

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of breast cancer include a lump in the breast, changes in shape and size of the

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of breast cancer include:

• A lump in the breast • A rash around the nipple • Appearance, shape and size of the

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important cellular targets for the development of anti-cancer agents (Mizushina et al., 2005).	important cellular targets for chemical intervention in the development of anti-cancer agents (Mizushina et al., 2000).

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CANCER Breast cancer is the most commonly diagnosed cancer and is the leading cause of death among women	cancer in 2008 [1]. Breast cancer is now the most frequently diagnosed cancer and the leading global cause of cancer death in women,

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CHAPTER 1.0

INTRODUCTION

1 INTRODUCTION

The word “cancer” for the first time was coined by Hippocrates, known as father of western medicine, who connected Greek words “carcinoma” and “Karakinos” to express tumor Cancer is considered as the hazardous disease in which the cells keeps on dividing without any control that can also attack to other close tissues. In normal condition the process which takes place to protect the secure condition of tissues is meiosis and apoptosis whereas the procedure which is involved in Carcinogenesis is consists of multi mechanism (figure 1) (Safarzadeh et al.,2014). Cancer is considered as the foremost public health problem in both developed and developing countries. More than 9.7 million cases are detected each year and 6.7 million people died from cancer and everyday, around 1700 Americans died of this disease and approx 20.4 million people are living with cancer in the world today and almost 1 in 3 people were diagnosed with cancer in the UK and 1 in 4 died from this deadly disease. It has been predicted by WHO that the new cases of cancer will reach 15 million until 2020 (Tavakoli et al., 2012). Cancer is the second most important cause of death in the United States after the cardiovascular disease where every one person among the four dies due to cancer (Manju et al., 2017). The substances which are responsible to cause cancer are known as carcinogens. The main reason behind the cause of cancer is mutation which make changes in DNA and loses the control over the growth of the cells (Manju et al., 2017) . Likewise some other factors which causes cancer includes external factors including radiations, smoking, tobacco, toxins in drinking water, foodstuff, atmosphere, certain metals and transmittable agents and internal factors like hereditary mutations, body immune system and hormonal issues are highly responsible for this deadly disease (Iqbal et al., 2017). Today there are more than 100 different types of cancer are known and are usually named by the organ or type of cell from where they start like a cancer that begin in the colon is called colon cancer (Zaid et al., 2017). According to National Cancer Institute (NCI) classification, different types of cancer are classified as follows (Safarzadeh et al., 2014). • Carcinoma: Cancers resulting from epithelial cells. It includes most of the regular cancers, mainly in older adults like breast, prostate, lung, pancreas, and colon cancer. • Sarcoma: Cancers resulting from connective tisssue like bone, cartilage, fat, nerve each of which develop from cells originating in mesenchymal cells exterior to the bone marrow. • Lymphoma and leukemia: these both types of cancer results from the cells tht are involved to make blood. Leukemia is the most general type of cancer mainly in children accounting for almost 30%.conversely, in case of adults both types of cancer can develop. • Germ cell tumor: these types of Cancers are resulting from pluripotent cells, mainly present in the testicle or the ovaries . • Blastoma: These types of Cancers are resulting from undeveloped "precursor" cells or developing tissue. Its mainly occur in the children compare to adults.

Among these the lung cancer is reported the top listed in male followed by breast cancer in female (Zhou et al., 2017) 1.1 BREAST

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CANCER Breast cancer is the most commonly diagnosed cancer and is the leading cause of death among women

worldwide (Tabatabaei et al., 2016). It is a malignant tumor that gets started in the cells of breast. The most common Signs

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of breast cancer include a lump in the breast, changes in shape and size of the

breast, dimpling of the breast skin, release of liquid from the nipple without squeezing ,pain in the breast that last forever, swelling in the armpit area and at the site of collarbone and a red scaly patches in the skin. Breast cancer most commonly develops in cells lining the milk ducts and the lobules that supply the ducts with milk (Kabel et al., 2015).Similar to other cancers, there are various factors that can elevate the risk of receiving breast cancer like female sex, obesity, short of physical exercise, consumption of alcohol, hormone replacement therapy in menopause, high exposure to ionizing radiation, early age at the time of first menstruation and old age (Gøtzsche et al., 2013). High exposure of estrogen damage the DNA and causes genetic mutation which can result into the breast cancer. In addition to this few people acquire defects in the DNA and mutation in BRCA1, BRCA2 and P53 genes due to hereditary reasons as compare to other peoples. Thus the person who have the family history of ovarian or breast cancer are at increased risk of breast cancer (Kamińska et al., 2015).

1.1.1 STATISTICS In high wages countries like the United States, about 232340 ladies has been diagnosed and 39620 has been died from breast carcinoma in 2016 (Siegel et al., 2018). In 2017, an estimated 252,710 new instances of invasive breast cancer has been diagnosed among ladies and 2,470 instances has been diagnosed in men. Moreover, 63,410 cases of in situ breast carcinoma were diagnosed among ladies and Around 40,610 ladies and 460 men died from breast cancer in 2017 (DeSantis et al., 2017) and In 2018 more 1,735,350 new cancer cases and 609,640 cancer deaths are expected to occur in the United States (Siegel et al., 2018). It has been reported that in an American lady, the danger of rising breast cancer in their entire lifetime is 12.38% or 1 out of 8 (Nowsheen et al., 2017).

1.1.2

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TYPES OF BREAST CANCER Breast cancer can be categorized as invasive or non-invasive.

Ductal carcinoma in situ (DCIS) is a non-invasive breast cancer moreover it is also recognized as stage 0. In the DCIS, the unusual cells are controlled in the milk ducts of the breast and do not broaden into the neighboring breast tissue. Whereas incase of Invasive breast cancer abnormal cells do multiply from their original site that can be either the milk ducts or the

lobules that is the sacs that make breast milk to the neighboring breast tissue. It might also have multiply to the lymph nodes. (Horlings et al., 2013). All the invasive breast cancers and DCIS are tested for hormone receptors. steroid hormone are mainly responsible for this deadly disease specially estradiol, which plays an significant function in the development of breast cancer, and a majority of the human breast cancers begin out as estrogen dependent and convey the estrogen receptor (ER) (Clarke et al., 2003). From the literature it has been confirmed that the elevated levels of endogenous estrogens and androgens and minor levels of sex hormone binding globulin (SHBG) are linked with higher danger of postmenopausal breast cancer (Hormone et al., 2013)These breast cancers can be treated with hormone therapy such as tamoxifen and aromatase inhibitors. Nearly all the invasive breast cancers are hormone receptor-positive (Abeshouse et al., 2015). 1.1.3 ROLE OF ESTROGEN, ANDROGEN AND AROMATASE IN BREAST CANCER The steroid hormone, estradiol, has the very significant function in the development of breast cancer, and mostly the human breast cancers found were estrogen dependent and convey the estrogen receptor (ER). Estrogen mediates its biological effect by binding to one of the structurally and functionally different ERs that is ERα and ERβ (Saha Roy et al., 2012). Over expression of estrogen caused the risk of breast cancer as much clear mechanism is not known. In normal condition where the level of estrogen is maintained the chance of getting breast cancer is very low, but mutation in the certain metabolites of estrogen that when bind to DNA causes the hydroxylation of estrogen and induce the quinone and semiquinone formation (Santen et al., 2015). The quinone form of estrogen binds with DNA and produce DNA adducts and begins the transcription of proteins which is responsible for the cause of breast cancer. Semiquinone intermediate are free radicals which can bind to oxygen and produce superoxide radicals and alter the structure of DNA (figure1.2.) (Santen et al., 2015). Aromatase receptor play an important role in breast carcinogenensis. The aromatase enzyme is the important enzyme for the biosynthesis of estrogen. This enzyme is also known as estrogen synthase. It induces aromatization of androgens to estrogens hence responsible for the overexpression of estrogen (Yamamoto et al., 2014). From the literature it has been confirmed that about 60% of breast cancer state this enzymes with elevated levels of mRNA expression and activity when compared among the non-malignant tissue. It catalyzes the final steps of estrogen biosynthesis androgens to estrogens particularly conversion of testosterone to estradiol and androstenedione (Group et al., 2015). Androgen receptor is expressed in 60-70% of breast cancer and plays a dual role with estrogen. Inhibition of Androgen receptor is mainly responsible for the down regulation of ERK signaliing therefore androgen receptor induce its effect by genomic and non genomic pathways. In the genomic pathway androgen bind and directly control the expression of gene in DNA and in case of non genomic signaling where nucleus receptor gets signals with other proteins interactions. Androgen receptor is responsible for the breast cancer mainly due to irregulation of growth hormone and cytokines. this combined signaling contribute majorly for the breast cancer (Iacopetta et al., 2012).

1.1.4 THERAPIES FOR BREAST CANCER The main treatments used for cancer are chemotherapy, radiotherapy and surgery (Solowey et al., 2014). A successful anticancer drug should destroy or injure the cancer cells devoid of causing unnecessary damage to the normal cells. But this ideal process by the anticancer drug is very complex, or maybe impossible to achieve and is the main reason for the unpleasant side effects from which the patients have to

suffer when under-going treatment (Manju et al.,2017). the main Chemotherapeutic agents includes cytostatic and cytotoxic drugs which have revealed excellent outcome either alone or in the grouping with additional cancer therapies. Some main chemotherapeutic agents includes the topoisomerase inhibitors like- Irinotecan which shows the side effects including neutropenia, sensory neuropathy, and diarrhoea and doxorubicin whose having the high risk of causing cardiotoxicity. The alkylating agents used for curing cancer includes oxaliplatin, melphalan, carboplatin, cisplatin and cyclophosphamide but shows the side effects including cardiovascular toxicity, nephrotoxicity, gastrointestinal toxicity, pulmonary and hematologictoxicity (Pegram et al., 2004). In addition to this the main drawback about these drugs is the cancer cells resistance to these drugs as they go through mutation like ABCA4 and ABCA12 are the Drug resistant genes which are over-expressed in human MCF-7 breast cancer cells correspondingly when the drug docetaxel was used for the treating the cancer (Iqbal et al., 2017)

As there are many drugs available for the treatment of cancer but it also have number of side effect thus it is very important to look for novel anticancer agents which have superior efficiency and minor side effects. In this case Natural compounds are considered as the good sources for developing new remedies for the treatment of various diseases (Aung et al., 2017).

1.1.5 USE OF MEDICINAL PLANTS TO TREAT BREAST CANCER

Medicinal plants and herb have been used from ancient times to treat human chronic diseases including cancer much before the invention of modern drugs (Manju et al., 2017). At present about 60% of drugs used for the treatment of cancer are isolated from natural products in which the plant kingdom has been the most important source (Solowey et al., 2014).

Plants have various active compounds which work synergistically for giving the therapeutic benefits and bringing down the dangers of side effects so that no other supplemental therapy is required to control the cancer debility. Thus it has made very important to uphold the use of natural Ayurvedic therapies for curing various types of cancers and imply an incorporated approach for the management of tumor and for its treatment (Shukla et al., 2015).Some important plants used for the treatment of breast cancer as well as for other cancers are mentioned in the table 1.1. (Safarzadeh et al., 2014)

The therapeutic significance of the plants are due to the occurrence of chemical substances that generate a specific physiological action in the human body as mentioned above In the table 1.1. Main other bioactive compounds of the plants used for the treatment of diseases include alkaloids, flavanoids, tannins and phenolic compounds. Also, several number of plant leaves tends to have antimicrobial principles such as tannins, essential oils and new aromatic compounds. In addition to that some preclinical studies have confirmed that the phytochemicals have great importance in the prevention of colorectal cancer and other cancers (Jayaraman et al., 2008). Thus Herbal medicines are refined natural compounds which can control the different phases of diseases at the same time by the different mechanisms (Shukla et al., 2015). Whereas the chemical medicines are the individual synthetic compounds that can be intrusive in an ideal condition by the single mechanism. Mainly the anticancer drugs used for the treatment of cancer whether it is synthetic chemicals or the natural

products, tends to cooperate with the DNA or its precursors which produces the irreversible harm to DNA and restrain the synthesis of proteins. Thus, curing the cancer cells by using the mono-target chemical agent is not an efficient method. hence, on the basis of broad research conclusion, the phytochemicals and their resulting analogues are considered as the best option for the enhanced and less lethal for the cancer treatment (Singh et al., 2016). As there are number of plants whose phytochemicals are used today for the treatment of many diseases as well as for the cancer and among them one of the very important plant is Stevia rebaudiana belonging to the Asteraceae family in which the major sweet constituent in the leaves of the Stevia rebaudiana (Bertoni) is stevioside which is 300 times sweeter as compare to sucrose and has just gained significance as a natural non-caloric sweetener (Siddique et al., 2016).Beside sweetness, stevioside along with other associated compounds including rebaudioside A, steviol, and isosteviol may also recommend therapeutic uses, such as antioxidative, antihyperglycaemic, anti-hypertensive, antitumor, antidiabetic, anti-HIV (Paul et al., 2012). It is also reported that the isosteviol inhibited DNA polymerases and DNA topoisomerase II. Which are considered as the

0: http://www.scielo.br/pdf/rbfar/v19n2a/a02v192a.pdf 88%

important cellular targets for the development of anti-cancer agents (Mizushina et al., 2005).

Moreover Stevia leaf extracts and the presense of polyphenolic constituents have revealed the inhibitory effect on tumor commencement and its promotion (Heikal et al., 2008). Thus, information concerning the structural characteristics of stevioside-based compounds may provide valuable insight for the design of new anti-cancer agents.

CHAPTER 2 REVIEW OF LITERATURE

Chapter 2 Review of literature Stevia rebaudiana (SR) is considered as the medicinal herb which has been utilized in the traditional Armenian medicine to lowering down the glucose, cholesterol and blood pressure levels and also adjust the immune function of the body. (Aghajanyan et al.,2017). It is originated from the northeast of Paraguay (Mathur et al.,2017). The initial botanical explanation of the plant was acknowledged by M. S. Bertoni In 1889. The plant was earlier recognized as Eupatorium rebaudianum Bert. in tribute of Rebaudi, the first chemist who studied the chemical distinctiveness of the substances extracted. Its name was afterward changed to the present one. The genus Stevia incorporates 230 species but only S. rebaudiana provides the sweet taste property. Some other related species include S. eupatoria, S. micrantha, S. plummerae, S. rhombifolia, S. serrata, S. salicifolia, S. viscida, S. commixta, S. origanoides, S. leptophylla, S. satureiaefilia, S. ophryphylla, S. selloi, S. nepetifolia and S. triflora (Ruiz-Ruiz et al., 2017). Stevia is about 200-300 times sweeter than the sugar; its sweetness effectiveness is alike to that of aspartame. Stevia is cultivated worldwide due to its function as a non-caloric sweetener. It has been utilized for many years in the treatment of diabetes in various countries as it have no toxic harm as well as no side effect (Yadav et al., 2011). It have great value as a economic medicinal plant since it have pharmaceutically active compounds all through the world. Stevia has been used a non-caloric natural-source having other remedial applications together with anti-cariogenic, anticancer, antioxidant and

antidiabetic properties. Biotechnological techniques present novel approaches for its industrial production, propagation as well as conservation and management of Stevia (Karimi et al., 2017).

2.1. PHARMACOGNOSTICAL CHARACTERISTICS

2.1.1 COMMON NAMES Sweet leaf, sweet herb, honey leaf, Stevia, sweet leaf of Paraguay, caa-he-éé, kaa jheéé, ca-a-jhei, ca-a-yupi, azucacaa, eira-caa, capim doce, erva doce, sweet-herb, honey yerba, yaa waan, candy leaf, sugar leaf, sweet honey leaf, Eupatorium rebaudianum (Jayaraman et al., 2008; Marković et al., 2008).

2.1.2 GEOGRAPHICAL SOURCE The genus Stevia, mainly Stevia rebaudiana is initially from Paraguay. The sweet feature of Stevia has been used by the Paraguayan Indians from several centuries. It is native to the northern regions of South America. Stevia is still found growing wild in the highlands of the Amambay and Iguacu districts which is the border area flanked by Brazil and Paraguay. It is predicted that almost 200 species of Stevia are native to South America; conversely, none of the other Stevia plants have acquired the similar intensity of sweetness as the S. rebaudiana. It is developed commercially in many parts of Paraguay, Brazil, Israel, Uruguay, Thailand, Central America and China. it is also refined on a minor scale in Canada, Mexico, USA, some European countries and also with the Israel. In india it is mainly cultivated in Karnataka, Punjab, himachal Pradesh, Haryana, uttar Pradesh, west Bengal , Madhya Pradesh and tamil nadu. In Japan Stevia have been utilized for more than 50 years as a sweetening agent in a variety of foods and beverages (Brandle et al., 1998; Mandal et al., 2013).

2.1.3 PLANT MORPHOLOGY Stevia rebaudiana is a herbaceous perennial plant belongs to the Asteraceae family. The plant reaches upto 65cm in height, having sessile, oppositely arranged lanceolate to oblanceolate, dark green colour leaves with toothed margins. The taste of leaves is sweet. The seeds of Stevia are commonly small, brown and black in colour and on maturation the seeds turn into black coloured fruits which are dispersed with the help of persistent pappus bristles as in the case of dandilon. The root system of Stevia rebaudiana is on the surface and with a little lateral root. The flower are little in size, white in colour, are bisexual and fashioned in clusters of 2 -6 florets, through sprouts that are initially tender and finally gets harden (Lester et al.,1999; Shaffert et al.,1994). 2.2. CHEMICAL COMPOSITION 2.2.1 Steviol glycosides Steviol glycosides are the most important components of Stevia rebaudiana which is responsible for its sweetener capacity. It commonly contains glucose moieties which are joined with the aglycone part termed as Steviol (1) (Puri et al., 2011) . Isosteviol (2) is the oxidized product of steviol mainly famous for its therapeutic benefits including anticancer activity (Jaitak et al., 2008). The plant consists of more than 40 steviol glycosides in varying concentrations (Oehme et al.,2017). The major steviol glycosides found are Stevioside (3) and Rebaudioside A (4), having the highest content in the plant. The percentage of stevioside found to be 6–10%, and of rebaudioside A is 2–4% and the other minor glycosides is up to 0.1-1%. Stevioside is 250-300 times sweeter than sucrose whereas rebaudioside-A is 300-400 times sweetener than sucrose (Singla et al., 2016). the other steviol glycosides found in the plant are Rebaudioside B (5), Rebaudioside C (6), Rebaudioside D (7), Rebaudioside E (8),

Rebaudioside F (9), Rebaudioside G (10), Rebaudioside H(11), Rebaudioside I(12), Rebaudioside J (13), Rebaudioside L (14), Rebaudioside M (15), Rebaudioside N (16), Rebaudioside O(17), Rebaudioside R (18), Rebaudioside S (19), Rebaudioside T (20), Rebaudioside U (21), Dulcoside A (22), Dulcoside B (23), Rebusoside (24), steviolbioside (25). Structure of various isolated steviol glycosides. 2.2.2 Non-glycosidic diterpenes Labdane-type diterpenes comes under this category of constituents of S .rebaudiana. the various compounds of this group are austroinulin (26) jhanol (27), Sterebins (28-40), (Ibrahim, El-Gengaihi, Motawe, & Riad, 2007). The chemical structure of these non –glycosidic diterpenes.

2.2.3 Flavonoids The plant contains numerous number of flavonoids including quercetin (41), apigenin 4-O-glucoside (42), apigenin (43), Kaempferol 3-O-rhamnoside (44), luteolin 7-O-glucoside (45), quercetin 3-O-rhamnoside (46), quercetin 3-O-glucoside (47), apigenin 7-O-glucoside (48), rutin (49), centaureidin (50) (Wölwer-Rieck et al., 2012). The chemical structure of these flavonoids has been shown in figure 2.3

2.3. BIOSYNTHETIC PATHWAY OF SGS Steviol glycoside molecules come under the diterpene group of compounds which tends to have huge number of biological functions in plants. The Production of these diterpene group of compounds have the common primary biosynthetic pathway. Poly –isoprene is the basic backbone of all the diterpene compounds. Isoprene is a simple organic compound having the following chemical structure (figure 2.4).

The structure of poly-isoprene is mentioned in figure 2.5. structure of Geranylgeranyl diphosphate is meintioned below (figure 2.6) it is the main precursor for numerous compounds found in the plants including the steviol glycosides. Biosynthesis of steviol glycosides consist of two stages. first stage includes the Geranylgeranyl diphosphate or GGDP synthesis from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Geranylgeranyl diphosphate is the initial point for various biologically essential diterpene compounds present in the plants. In the second stage, steviol glycosides are formed by the conversion of Geranylgeranyl diphosphate by following multiple steps. The two main precursors that is isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are produced by a very important pathway in plants known as 2-C-methyl-D-erythritol-4 phosphate pathway or MEP pathway. The MEP pathway is described below with the formation of GGDP (Brandle et al., 1998). The steps followed in this pathway can be catalyzed only in the presence of some definite enzymes. In case if these specific enzymes are absent, the biochemical reactions cannot be completed. STEP 1 In this step Pyruvate and Glyceraldehyde 3 phosphate are combined to form Deoxyxyulose 5 phosphate which further gets phosphorylated by the Cytidine triphosphate. This is the beginning of the MEP Pathway STEP 2 Further phosphorylation by Adenosine Triphosphate (ATP) followed by formation of 2-C-Methyl-D-Erythritol-2,4-Cyclodiphosphate STEP 3 In this step Isopentenyl Diphosphate and Dimethylallyl Diphosphate are formed. These are known to be the precursors for variety of isoprenoid compounds found in the plants. This is the last step of MEP pathway STEP 4 In this step Copalyl diphosphate is form by the Geranylgeranyl diphosphate formation and its cyclization. This is the beginning of the late stage Steviol glycoside synthesis (figure 2.10). STEP 5 In this step, ionization dependent cyclization takes place and Kaurene is produced formed from Copalayl Diphosphate which then gets oxidized to kaurenoic acid. The next step involves

the formation of steviol by the hydroxylation of kaurenoic acid. From this step steviol glycoside biosynthetic pathway takes a distraction from gibberellin biosynthesis pathway. STEP 6 In this step various steviol glycosides are form as the steviol gets glycosilated by the Glucosyl Transferase enzymes.

2.4 Pharmacological properties of S. rebaudiana

2.4.1 Anti-hyperglycemic effect Stevia rebaudiana extracts has been used from the ancient times for treating the diabetes as it increases the sensitivity and secreation of insulin (Chatsudthipong et al., 2009).The effect of stevioside as Anti-hyperglycemic was found to be mediated by its effect on phosphophenol pyruvate carboxy kinase (PEPCK) which is the rate-limiting enzyme intended for gluconeogenesis and consequently controls the glucose production in the liver. It was observed that stevioside repress the PEPCK gene expression and lastly reduces gluconeogenesis. In addition stevia powder is more proficient in down-regulating PEPCK in comparison to stevioside which specify that except stevioside various other chemical compounds can be present which are responsible for down-regulating PEPCK (Chatsudthipong et al., 2009). In the study it is found that the production of insulin is enhanced by the action of Rebaudioside A in the pancreatic islet cells of mouse. Also stevioside promotes the glucose-activated insulin secretion, devoid of any disturbance in case of fasting insulinemia (Mathur et al., 2017).

2.4.2 Antimicrobial activity It has been reported that Stevia inhibits growth and reproduction of bacteria which is responsible for gum disease and tooth decay thus showing anti-microbial activity.It was also further found that the major cariogenic organism, Streptococcus mutans, experiences growth repression and less acid secretion when grown on media containing stevoside in comparison to media containing the fructose, glucose or the sucrose (Mathur et al., 2017).

2.4.3 Anti-inflammatory and immunomodulatory activity Various SGs have shown important effects on pro-inflammatory cytokines. 1 mM dose of stevioside moderately amplified the creation of tumor necrosis factor (TNF-α), interlukin (IL-1β) and nitric oxide (NO), in un-stimulated human THP-1 cell by interacting through toll-like receptor-4. Therefore stevioside can be valuable to healthy individual as it is accomplished to develop the innate immunity (Boonkaewwan et al., 2013). In the study the effects of stevoside and steviol as anti-inflammatory was studied on epithelial cells of colon. Moreover Stevoside has been found to reveal inhibitory effects on the contraction of smooth muscles of intestine whose inhibition deals with the hyper-motility related diarrhea. It has been onserved that the steviol along with its analogs has antidiarrheal effect as it inhibits cAMP regulated Cl- secretion in T84cells (Mathur et al., 2017). 2.4.4 Anti-diarrheal activity The anti-diarrheal potential of SGs was reported by Pariwar and his co-workers . According to the information stevioside and its foremost metabolite, steviol, were establish to influence the ion transport in various types of tissues including kidney, pancreas and intestine. Moreover short-circuit current measurements by them in the study showed that steviol and its analogs isosteviol, dihydroisosteviol and isosteviol 16-oxime inhibits forskolin-induced chloride secretion in a dose-dependent manner and have IC50 values of 101, 100, 9.6, and 50 mM, respectively.

Parent compound stevioside was found to be free from this effect. In the same study apical current measurement indicated that dihydroisosteviol besieged the cystic fibrosis transmembrane regulator. Inhibitory action of this compound was found reversible and was not linked with changes in the intracellular CAMP level. Pariwar and co-workers further recognized that it did not affect calcium-activated chloride secretion and T84 cell viability. In-vivo studies using a mouse closed-loop model of cholera toxin-induced intestinal fluid secretion showed that intra-luminal injection of 50 mM dihydroisosteviol reduced intestinal fluid secretion by 88.2% devoid of altering fluid absorption, thus indicating that dihydroisosteviol and related compounds could be a new class of cystic fibrosis transmembrane regulator inhibitors that may be helpful for additional development as anti-diarrheal agents (Brahmachari et al., 2011).

2.4.5 Anti-hypertensive effect Stevia and stevioside extract property as a antihypertensive could be due to their effects on the plasma volume. Stevioside administration by I.V causes natriuresis, diuresis and increased renal plasma flow but glomerular filtration rate (GFR) is unaffected (Melis, et al., 1992). It is reported that the stevioside causes vasodilation therefore reduces the total peripheral resistance as it inhibits the influx of Ca2+ in the vascular smooth muscle (Chatsudthipong et al., 2009). However stevioside has no influence on vasopression induced vasoconstriction when the medium is free of Ca2+.Thus it is clear that it causes vaso-relaxation by inhibiting the influx of Ca2+ (Ruiz-Ruiz et al., 2017).

2.4.6 Anticancer activity From the literature it has been confirmed that the stevioside have the cytotoxic ability. it mediates the apoptosis in MCF-7 human breast cancer cell line by the induction of reactive oxygen species (ROS) through mitochondrial pathway. By the intracellular ROS generation it conveyed the apoptotic signal (Gupta et al., 2017).Hence it was concluded that the stevioside increases the expression of apoptotic proteins such as Bax, Bcl-2 and Caspase-9 By the induction of the ROS-mediated mitochondrial permeability transition (Gupta et al., 2017). In the study it was reported that the Stevioside have inhibited tumor promotion by the TPA in the mouse skin cancer (Karimi et al., 2017). In addition isosteviol inhibits DNA polymerase and human topoisomerase II which are prominent targets of anticancer drugs (Chatsudthipong et al., 2009). Chaiwat reported the effects of steviol and stevioside on the human colon carcinoma cell lines by the MTT method. The cell viability in Caco-2, HT29 cells and T84 decreases when the concentrations of stevioside was 2–5 mM and that of steviol was 0.2–0.8 mM (Brahmachari et al., 2011). It is also mentioned that the Stevia leaf extracts and the presense other constituents such as its polyphenolic constituents have the inhibitory effect on tumor commencement and its promotion (Heikel et al., 2008). As a result SGs have revealed major anticancer activity which can be more explored for investigating the better anticancer compounds.

CHAPTER 3 Aim and Objectives

Chapter 3 Aim and Objectives

Work has been conducted by following these objectives • Isolation and characterization of secondary metabolites from S. rebaudiana leaves • In-vitro study of different extracts of S. rebaudiana (Bertoni).

• In-silico Study of reported secondary metabolites from S. rebaudiana.

CHAPTER 4 Rational

Chapter 4 Rational

• Therapies available for treating the cancer cause maximum side effects such as bone marrow depression, neutropenia, cardiotoxicity, mouth soreness, hairfall, nerve changes and also affects the normal cells which is danger for the person suffering from it and ultimate can cause the death of the individual, so the research for drugs having minimum side effects is necessary.

• Drugs obtained from natural products are considered safe as it have minimal side effects and the chances for curing the disease is much higher and today almost 60% drugs available for curing cancer are obtained from natural products therefore plant kingdom is considered the main source.

• From literature review, we observed that Stevia rebaudiana which is a very famous plant for its sweet taste and having zero caloric value mainly used for the treatment of diabetes is also having many other therapeutic benefits including Antiproliferative activity and as mentioned in literature various cell line including HT-29, MCF-7 the Antiproliferative action of the phytochemicals from S. rebaudiana has been observed.

• Keeping in view the above mentioned facts about the medicinal value of the plant present study was conducted to further explore the phytochemical and medicinal aspect of Stevia rebaudiana using In-vitro and In-silico approach.

Thus from this plant, we can get the Phytochemicals as Antiproliferative agent for curing the aggressive disease cancer

CHAPTER 5

Material and Method

Chapter 5

Material and Method

5.1 Chemical and Instruments Solvent methanol and ethyl acetate (laboratory grade) was procured from Finar limited India, petroleum ether (laboratory grade) was procured from SDFCL (sd fine-chem limited) Mumbai India. Solvent Chloroform laboratory grade was procured from Thomas baker chemicals Pvt. Ltd India. RPMI 1640 and DMEM, antibiotic solution, Phosphate buffer and bovine serum media were used to culture cancer cell lines. Sulphuric acid (91%) was purchased from Loba Chemie Pvt Ltd. Silica gel 60\120 for column chromatography was procured from SDFCL, glassware of fine quality were used and procured from Borosil JSGW, readymade TLC plates F254 from Merck were used, Rota vapor instrument LABINDIA was used. Laboratory grade reverse osmosis water R.O system was used. T-47 (Breast Cancer) cancer cell lines were selected for biological activity. These cell lines procured

from National Cell Repository located at NCCS Pune. For visualizing the TLC plates procured UV chamber from Mac Company,the characterization of structure of isolated compound NMR (H1 and C13) 400 MHz were used. Isolation of compounds was done by flash chromatography procured from Biotage. Other instruments used for thesis work such as Incubator for incubation,oven, automatic cell counter, UV-Vis spectrophotometer, laminar airflow. For docking studies of reported phytochemicals and isolated compounds Maestro software 2015 procured from Schrodinger Company. 5.2 Procurement and Preparation of Plant material Dried aerial part of plant S. rebaudiana was procured in the month of july 2013 from Green Valley farm situated at Pojewal (Garhshanker) in Punjab (Altitude 355m; Latitude 31.191769oN, longitude 76.258774oE) (figure 5.1).

5.3 Preliminary phytochemical investigation of extracts The powdered material (10g) was subjected to successive extraction with methanol and water (80:20) in the sonicator at 55°C for 30 min. The proportion of methanol from the extract was evaporated by using the rotavapour and the remaining proportion of water was fractionated with petroleum ether, chloroform, ethyl acetate, and aqueous methanol and was dried on a rotavapor yielding 42mg, 128mg, 234mg, and 304mg respectively. Now the qualitative chemical test of all the extracts of stevia rebaudiana was subjected to detect the presence of various phytoconstituents. 5.3.1 Test for Alkaloid: The samples are added with 3ml of picric acid saturated solution. Samples giving yellow precipitate shows presence of alkaloid. 5.3.2 Test for Fixed oil: On a clean filter paper add 2 drops of each sample. If it leaves a translucent spot then it is the presence of fixed oil

5.3.3 Test for Volatile oil: All samples are added with alcoholic solution of Sudan III dye. If the samples become red then volatile oil is present.

5.3.4 Test for Tannins: Add FeCl3 to all the sample. Yellow colour give hydrolysable tannin whereas green colour give condensed tannin.

5.3.5 Test for Flavanoid: The samples are added with NaOH solution producing yellow colouration. On adding dilute acid if the mixture goes colourless then presence of flavonoid is confirmed.

5.3.6 Test for Glycoside: Part A: Add dilute H2SO4 in the samples then add 5% NaOH neutralizing it. To it add equal volume of Fehling solution 1 and solution 2. Red colour is produced.

Part B: Add DM Water to all the test tubes until it is diluted then add equal volumes of Fehling solution 1 and 2. Red colouration

Compare the redness if part A has more intensity than part B then we can say that there is presence of reducing sugar. 5.3.7 Test for Steroids and triterpenes: All samples are added with conc. Sulphuric acid. Yellow on top layer show steroid and green on bottom show triterpenes. 5.4 In vitro anticancer activity

Different Extracts of S. rebaudiana were prepared for evaluating its anticancer activity. For studying this activity MTT assay was performed using the T-47 D breast cancer cell lines. In this cell lines 7500 cells were taken in each well consisting of 96 well total in plate. All the

prepared extracts were completely dried and stored in an eppendorf tube and weighed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a stock solution The tubes were submitted to animal cell culture laboratory in central university of Punjab. The eppendorf tubes were marked as AD-01 (Petroleum ether extract), AD-2(chloroform), AD-3 (Ethyl acetate), AD-4 (Methanol + water extract). In Animal cell culture laboratory T-47D cells are taken on petridish after thawing it from -80oC to body temperature and then media is added after 24 hours adherence is formed and then splitting of adherent cells . The cells of the cell lines was treated with different extracts of the plant in triplicate of its concentration and the experiment was also repeated three times .This process continues until sufficient cells for 96 well plate can be used enough. In the 96 well plate Plant extract of different concentration is added along with serum free media and MTT. Violet colour formation is seen due to formazone ring formation with dehydrogenase enzyme. The colouration is checked under 570 nm which is directly proportional to cell viability. Results were then plotted in graphs to calculate cytotoxic potential and IC50 values. 5.5 Isolation and characterization compounds

5.5.1 Isolation

On the basis of preliminary test performed and the in-vitro study aqueous methanol extract was selected for the isolation of compounds. The extract was further subjected to successive extractions with 100% methanol, methanol: chloroform (50:50) and aqueous methanol. All the extracts were dried using rotavapour. On the basis of TLC observation 100% methanol extract was selected for isolation process. The aqueous methanol extract (23.67g) was dissolved in methanol and 14g silica was added. The mixture was dried over rotavapor and further was subjected to successive extractions with 100% methanol, methanol: chloroform (50:50) and aqueous methanol to yield 10g,6g and 4g respectively and a TLC for all the three extracts was prepared. Progress of the reaction was monitored by TLC using solvent system ethyl acetate/ ethanol/H2O (8:2:1.2, v/v/v).The developed plate was dried in oven and spots were visualized by spraying with 5% sulfuric acid. Maximum spots were obtained with 100% methanol. The 100% methanol extract was subjected to flash column chromatography using a gradient elution of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 45, 50%, 55%, 60%, 65%, 70%, 75%, 80% chloroform: methanol to give 1,792 fractions. The analysis of all the fractions was performed by TLC using the mobile phase consists of ethyl acetate/ethanol/H2O (8:2:1.2 v/v/v). Fraction 239-290 were combined and dried on the rotavapour yielding ASP-1 JMG (4mg). Fraction 423-443 were combined and dried on the rotavapour yielding ASP-2 (22mg).Fraction 529-533 were combined together on the basis of a single spot on precoated silica gel 60F24 TLC plates .The combined fractions were dried on rotavapour yielding ASP-3JMG (17mg). The Fractions 600-610 were combined together on the basis of single spot on precoated silica gel 60F24 TLC plate. The combined fractions were dried on a rotavapour to give 7mg of ASP-4JMG.

5.5.2 Characterization of ASP-2 Melting point was found to be 197-199oC.

1H NMR (400 MHz, DMSO): Showed three anomeric peaks at δHn 4.87 (1H, d, J= 4Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) respectively.

13C NMR peaks of ASP-2 in (PPM,100MHz,DMSO): 14.92, 18.55, 19.91, 21.14, 27.95, 35.46, 37.44, 40.96, 41.93, 43.14, 43.53, 46.81, 48.65, 53.10,56.52, 60.51,60.73, 61.07, 69.44, 69.60,

70.34, 72.41, 75.11, 75.93, 76.09, 76.17, 76.72, 76.91, 77.45 , 78.44, 82.67, 84.71, 94.09, 96.27, 103.88, 104.66, 153.33, 175.73.

HRMS (ESI): m/z calc. [M+Na]+ calculated for (C38H60O18Na)+ 827.3868 corresponding to molecular formula C38H60O18.

5.5.3 Characterization of ASP-4

Melting point was found to be 244-246oC

13C -NMR (100 MHz, DMSO): 14.89, 18.56, 19.79, 21.14, 28.04, 37.48, 38.70, 39.20, 40.03, 41.04, 41.64, 43.10, 43.20, 47.0, 53.23, 56.52, 60.56, 61.02, 61.34, 68.90 69.48, 70.01, 70.32, 72.44, 73.59, 74.35, 75.98, 76.49, 76.88, 76.98, 77.42, 78.46, 78.79, 78.88, 79.12, 85.40, 86.27, 94.45, 96.45, 102.47, 102.98, 104.0, 152.78, 175.63.

1H NMR (400 MHz, DMSO): Showed four anomeric peaks at δHn 5.64 (1H, d, J = 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz) respectively.

HRMS (ESI): m/z calc. for C44H70O23 [M-H]- 965.5040 corresponding to molecular formula C44H70O23.

5.6 In-silico activity The computational study of molecular recognition is an important part of structure-based drug design. The molecular docking problem is commonly cast as a problem of finding the low-energy binding modes of a small molecule, or ligand, within the active site of a macromolecule, or receptor, whose structure is known. With the help of molecular docking environment, using Maestro software 11. The mechanism behind the antiproliferative activity of phytochemical from S. rebaudiana was determined. The targets selected for the study were estrogen receptor alpha, androgen receptor.

5.6.1 Ligand preparation With the help of ChemBio Draw ultra 12.0 structures of all the phytochemicals (figure 3.1)including the structures mentioned in chemical constituents in the review section were drawn and saved in SDF format. The molecules were changed from 3D structure to the 2D by using the Lig Prep version 2.5. These ligands were imported from workspace option then were subjected to ligand preparation using 'LigPrep' wizard application in Maestro 9.6 The Lig Prep developed a single, low energy, 3D structure with correct chiralities for the each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Lig Prep application of the Maestro operates OPLS 2005 force field. 5.6.2 Protein Preparation: The PDB for the crystal structure of estrogen alpha (PDB Id 3ERT) has been obtained from RSCB protein data bank. It is mainly responsible for the breast cancer. crystal structure of human androgen receptor in complex with testosterone (PDB ID 2AM9) have also been downloaded from protein data bank and the crystal structure of human aromatase receptor in complex with androstenedione (PDB ID 3EQM)has obtained from protein data bank.The protein preparation wizard option was worn for preparation of protein structure with polar hydrogen. This procedure consist of two steps that is preparation and refinement. In this step, bond orders were consigned, all hydrogen atoms were added ,bonds to metals were deleted and formal charges were set on the metal and the neighboring atoms and water molecules were deleted

that were more than the 5Å specific distance. Also the reorientation of hydroxyl groups, water molecule and amino acids to the optimization of hydrogen bond network. The refinement process is the last step for protein preparation. This steps involves firstly optimization then add hydrogen only and minimization. Thus in this process the restrained impact of minimization of protein was taken and stearic clashes were revealed and made the protein ready to dock. 5.6.3 Receptor Grid generation Generation of the grid at a exacting site in the protein is an important factor to perform docking study. It is not possible to perform Ligand docking without the generation of receptor grid. In case of 3ERT, 2AM9 and 3EQM the grid was generated at the position of co-crystallized ligand which already has to bound to protein. The ligand molecule has to be selected for grid generation. The length of ligands which are selected for docking were made to 36Å. 5.6.4 Glide Docking GLIDE (Grid Based Ligand Docking with Energetics) software was used for docking procedure developed by Schrödinger. Three docking precision is available in GLIDE docking module such as HTVS (High throughout visual screening), standard precision (SP) and Extra precision (XP). HTVS is proposed for quick screening of very large numbers of ligands. It has controlled conformational sampling. SP is set as a default parameter and is used to screen thousands of compounds. Extra precision docking and scoring is more dominated and sharp procedure. Extra precision is developed to locate active compounds that binds to a particular conformation of receptors. Docking of reported phytochemicals and the inhibitor was done with extra precision. Extra precision descriptions were written, ligand was taken as flexible. Epik penalties were added to the docking score. Docking score was taken into consideration for comparing the results, more negative the docking score more potent the compound and indicates the good binding potential. Various components are present in GLIDE score like hydrogen bonds, hydrophobic contacts, Van der Waals interaction, and Columbic interaction, Polar interaction in the binding site, Metal binding and Freezing rotable bonds. 5.6.5 ADME Study For estimating the absorption, distribution, metabolism and excretion (ADME) properties of chemical compounds Qikprop module application present in Schrödinger suit 2013 is used. It predicts physically significant descriptions and evaluates the pharmacokinetics properties of ligands by retrieving the drug like properties. Using this module we have calculated ADME properties of chemical compounds of S. rebaudiana used in the study.

CHAPTER 6.0 RESULTS AND DISCUSSION

Chapter 6.0 Results and discussion

6.1 Preparation of Extracts The powdered material (4.3 kg) was subjected to successive extractions (figure 6.1) with petroleum ether, ethyl acetate, CHCl3 extract, and aqueous methanol (20:80 v/v).

6.2 Preliminary phytochemical investigation of extracts Phytochemical test of Petroleum ether extract, CHCl3 Extract, ethyl acetate and Aqueous methanol extracts of S. rebaudiana were subjected to tests detection for the presence of various phytoconstituents. General test were performed such as alkaloid test, fixed oil test, test for tannin, volatile oil test, Flavonoid Test, Glycoside test, Steroid test, Trirepenes test and test for saponin. From the phytochemical investigations of extracts of S. rebaudiana, CHCl3 Extract extract and methanol- water

exhibited that the most chemical constituents were present. So aqueous methanol extract was selected for further study and for the isolation of the compounds. 6.3 In- vitro study For estimating the anticancer activity of extract of the S. rebaudiana MTT assay was performed. All extracts of S. rebaudiana was dissolved in DMSO (25mg/ml) to form a stock solution and subjected to in- vitro antiproliferative activity. MTT evaluates demonstrated results in varied range of concentration. In the MTT cell proliferation measurement of all proliferation rates and similarly, when metabolic events lead to apoptosis or necrosis, that reduction in cell viability. The MTT reagent yields get low. The T-47 D breast cancer cell lines confirmed that the chloroform extract has high activity against breast cancer cell lines after that aqueous methanol shows the high activity against the cell line and than the petroleum ether extract. Further IC50 values of aqueous methanol extract, petroleum ether extract, and chloroform extract were calculated. 6.4 Characterization of isolated compounds

6.4.1 Characterization of ASP-2 Compound ASP-2 was a crystalline white solid, produced a single black spot which was visualized by spraying with 5% sulfuric acid on a precoated silica gel 60F254 TLC plate. Its m/z ratio indicated an ion peak at m/z [M+Na]+ calculated for (C38H60O18Na)+ 827.3868 corresponding to molecular formula C38H60O18. The 1H and 13C NMR of compound ASP-2 showed three anomeric values at δHn 4.87 (1H, d, J= 3.44Hz), 4.46 (1H, d, J=5.07Hz) and 4.36 (1H, d, J=7.56) and δ 13C 94.09, 96.27 and 104.66 respectively assigned to three glucose molecules. In 13C NMR, δc at 14.92 (C-20) and δc 27.95 (C-18) indicates the presence of two methyl groups. δc value at 175.73 and δc 103.88 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively. Above mentioned observation confirmed that the compound ASP-2 is stevioside which is represented in Figure 4.2. stevioside is a SG Containing three glucose molecules attached with steviol moiety having melting point of 197-199oC. Further confirmation of structure was done on the basis of literature (Danieli et al., 1997).

6.4.2 Characterization of ASP-4

Compound ASP-4 was a crystalline white solid, produced a single black spot on a precoated silica gel 60F254 TLC plate. Its m/z ratio indicated an ion peak at m/z calc. for C44H70O23 [M-H]- : 965.5040 corresponding to molecular formula C44H70O23. The 1H and 13C NMR of compound ASP-4 showed four anomeric values at δHn 5.64 (1H, d, J = 8 Hz), 5.28 (1H, d, J = 8 Hz), 5.02 (1H, d, J = 8 Hz) and 4.95 (1H, d, J = 8 Hz). and δ 13C 94.45, 96.45, 102.98 and 102.47 respectively assigned to four glucose molecule. In C13 NMR, δc at 14.89 (C-20) and δc 28.04 (C-18) indicates the presence of two methyl groups. δc value at 175.63 and δc 104.0 indicates the presence of carbonyl carbon (C-19) and the exocyclic C=C (C-17) group respectively and additional peaks for sugar moiety at C-3" was obtained to be 102.98, 78.46, 74.35, 73.59, 70.01 and 61.02. Which confirms the presence of one extra glucose moiety compare to stevioside. Above mentioned observation confirmed that the compound ASP-4 is Rebaudioside A which is represented in Figure 4.3. Rebaudioside A is a SG Containing four glucose molecules attached with steviol moiety having melting point of 244-246oC. Further confirmation of structure was done on the basis of literature (Steinmetz et al., 2009). 6.5 In silico study

The preliminary screening for anticancer property of S. rebaudiana extracts by MTT assays using T47D cell line demonstrated that the extracts of S. rebaudiana retains antiproferative activity specially showing the activity against the breast cancer, due to presence of phytochemical constituents. From the literature it is also confirmed that the constituents showing excellent dock score are obtained from aqueous methanol extract (Muanda et al., 2011).Estrogen receptors like estrogen alpha play an important role in regulation of estrogen hormone in women. Irregulaties in these receptors causes to induce the breast cancer. Docking study experiment shown that phytochemical constituents S. rebaudiana regulate the estrogen receptors. Phytochemical constituents prevent the binding of tamoxifen with its receptor (Estrogen α). Androgen receptor also playing key role in the induce of breast cancer. Testosterone used as a standard inhibitor for androgen receptor. Aromatase receptor also initiated the breast cancer by play a key role in biosynthesis of estrogen. Aromatase convert the testosterone into estrone by aromatization. 4- Androstene-3-17- dione used as a standard inhibitor for the aromatase receptor. These inhibitor makes down regulation of the signaling pathway and these phytochemical constituents also acts as inhibitor of enzymes involved in the biosynthesis of estrogen hormones. The targets selected for the docking study are estrogen receptor alpha , androgen receptor and aromatase receptor. All these targets having a vibrant role in the breast cancer proliferation. anticancer drug candidates were identified by glide docking of compounds of S.rebaudiana on all the three receptors and the best compounds were selected on the basis of lowest docking score. Reported 123 compounds of S.rebaudiana were taken for the docking study and the topmost compound showing the best docking result for antiproliferation is mentioned in detail below with compare to the standard inhibitor taken and other compounds result are mentioned in the table. 6.5.1 Estrogen receptor alpha In Estrogen receptor alpha (PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -13.98, -7.84, -1.01 and -1.02 Kcal/mol respectively. The interaction pattern is represented in figure 6.4 in which majority of amino acid residues interact with hydroxyl group and with the amino group of ligand by forming the hydrogen bond. The main residues showing interaction with ligand include GLU 353, ARG 394 and ASP351.

Next in the rank order according to docking score was Rutin which showed dock score, Lipophilicity, H bond and electrostatic energy of -10.44, -4.26, -4.34 and -1.3Kcal/mol respectively. The main interaction pattern included stacking interaction and Hydrogen bonding which is shown in figure 6.4. TRP 383 showed π-π stacking interaction with ring A of ligand. ALA 350, ASP 351, MET 528 and LEU 525 amino acid residues showed hydrogen bonding of various hydroxyl groups of ligand. Thus on the basis of docking score top two compound was mentioned in case of estrogen receptor alpha which shows significant binding affinity for the receptor. The dock score, Lipophilicity, H bond and electrostatic energies of all the compounds includes in the study are shown in table 6.4.

6.5.2 Androgen receptor In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -12.97, -4.66, -4.97 and -1.17 Kcal/mol respectively. The interaction pattern is represented in figure 6.5 in which all the amino acids interact with hydroxyl group of the

ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 704 and ASN 705. The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard androgen inhibitor testosterone was found to be -12.17, -6.64, -1.33 and -0.64Kcal/mol respectively which is comparatively higher than chlorogenic acid. The main interaction pattern included is Hydrogen bonding with carbonyl group and the hydroxyl group of the ligand which is shown in figure 6.5. The main residues showing interaction with ligand includes ARG 752, THR 877and ASN 705. Thus on the basis of docking score phytochemical of S.rebaudiana have shown significant binding affinity towards androgen receptor (2AM9). The overall result of various compounds docked with 2AM9 is as represented in table 6.5.

6.5.3 Aromatase receptor In Aromatase receptor (PDB ID 3EQM) sterebin G have shown best interaction pattern. Its docking score, Lipophilicity, H bond and electrostatic energies were found to be -10.84, -6.17, -2.82 and -0.44 Kcal/mol respectively. The interaction pattern is represented in figure 6.6 in which all the amino acids interact with hydroxyl group of the ligand by forming the hydrogen bond. The main residues showing interaction with ligand include LEU 372 and LEU 477. The dock score, Lipophilicity, H bond and electrostatic energy of top ranked standard aromatase inhibitor 4-androstene-3-17-dione was found to be -5.2, -6.92, -0.7 and -0.25Kcal/mol respectively which is comparatively much higher than top ranked compounds as per dock score. The main interaction pattern included is Hydrogen bonding with carbonyl group of the ligand which is shown in figure 6.6. The main residues showing interaction with ligand includes is MET 374. Based on the result of docking score phytochemicals of S. rebaudiana have shown significant binding affinity towards aromatase receptor (3EQM). The overall result of various compounds docked with 3EQM is as represented in table 6.6.

6.6 ADME PREDICTION ADME properties of all studied compounds have been predicted using Qikprop application and are represented in Table 6.7. The phytochemicals of Stevia rebaudiana have shown good binding affinity towards aromatase receptor also the pharmacokinetic profile as well as percentage oral bioavailability of the phytochemical was found to be good. Incase of androgen receptor only two phytochemical that is chlorogenic acid and quercetin have shown better docking score compare to the standard inhibitor testosterone but the pharmacokinetic profile of these phytochemical including the percentage oral bioavailability and predicted apparent caco-2 cell permeability was found to be very poor and incase of estrogen receptor –alpha none of the phytochemical have shown the better binding affinity towards the receptor compare to the standard inhibitor 4-hydroxytamoxifen.The best docking score obtained is of standard inhibitor and after that the best dock score was shown by rutin which have poor pharmacokinetic profile. Thus from the docking and ADME data it was clear that the phytochemicals of S. rebaudiana have significant anticancer potential towards aromatase and androgen receptor but pharmacokinetic is the major problem specially for the androgen receptor incase of aromatase it is somewhat good but required some structural mofication for the best results. Mapping inbuilt pharmacophores and simplification of structure is a logical approach to be considered in this scenario. Moreover such compounds may be useful in cancers where absorption is not required. Incase of steviol and isosteviol predicted oral absorption is more than 90% so there is a chance of modification of these compounds to get good anticancer compounds with good ADME

properties which can be more justified by the fact that isosteviol have shown multiple anticancer properties.

CHAPTER 7.0 SUMMARY

Chapter 7 Summary Dried aerial part of plants powder was extracted thrice by solvents with increasing order of polarity using petroleum ether, chloroform, ethyl acetate, and aqueous methanol respectively. All the extracts were dried using rotavapor. On the basis of In-vitro study results and than with the TLC observation aqueous methanol extract was selected for isolation process. Total four molecules have been isolated on the basis of single black spot visualized by spraying 5% sulphuric acid on the precoated silica gel 60F254 TLC plates. However, only two compounds (ASP-2 and ASP-4) were characterized. ASP-2 was found to be stevioside and ASP-4 was found to be Rebaudioside A both of which are known compounds. The anticancer potential of various extracts have been determined using MTT based in-vitro assay using T-47d cell line and have shown significant potential. In T47d cell line AD-2 that is chloroform extract and AD-4 that is aqueous methanol have shown excellent activity with IC50 value of 7.79µg/ml and 9.53 µg/ml respectively. Moreover AD-1 that is petroleum ether has shown IC50 value of 9.58 µg/ml. Apart from in-vitro determination of anticancer potential, in silico approach have also been implemented to determine the anticancer potential of reported compounds obtained from S. rebaudiana. Docking study was performed on Estrogen receptor-alpha (PDB ID 3ERT),Androgen receptor (PDB ID 2AM9) and Aromatase receptor (PDB ID 3EQM). In case of Estrogen receptor-alpha(PDB ID 3ERT) the standard inhibitor that is 4-hydroxytamoxifen have shown best interaction pattern.Its docking score was found to be -13.98 Kcal/mol. In Androgen receptor (PDB ID 2AM9) Chlorogenic acid have shown best interaction pattern. Its docking score was found to be -12.97 Kcal/mol. Moreover, in case of Aromatase receptor (PDB ID 3EQM) best dock score of -10.84 Kcal/mol was obtained for Sterebin G. from the literature it has been confirmed that the major constituents showing the Antiproliferative activity are present in the aqueous methanol extract. Thus Phytochemicals of Stevia .rebaudiana have shown good binding affinity for all the three receptors and thus seem to have significant anticancer potential. But poor pharmacokinetic profile of these compounds as predicted by QikProp is the major problem associated with them but structural modification can be tried to improve the ADME properties of these compounds. Moreover these compounds may be used directly to target cancers where absorption is not required.

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cancer in 2008 [1]. Breast cancer is now the most frequently diagnosed cancer and the leading global cause of cancer death in women,