Phytochemical Investigation of Natural Sweetener from *Stevia rebaudiana* (Bartoni)

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Medicinal Chemistry

By

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DECLARATION

I declare that the dissertation entitled "**Phytochemical Investigation of Natural Sweetener from *Stevia rebaudiana* (Bartoni)**" has been prepared by me under the guidance of research Supervisor Dr. Vikas Jaitak, Assistant Professor, Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Mayank has prepared his dissertation entitled **"Phytochemical Investigation of Natural Sweetener from *Stevia rebaudiana* (Bartoni)"** for the award of M.Pharmacy degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

"Phytochemical Investigation of Natural Sweetener from *Stevia rebaudiana* (Bartoni)"

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ABSTRACT

S. rebaudiana is an important plant because of the high concentration of steviol glycosides (SGs). Sugars and artificial sweeteners, which are used in general as well as in pharmaceutical field have shown multiple toxic effects. On the other hand SGs have shown sweetness profile many fold compared to sucrose along with many health benefits.The substitution of SGs as sweeteners seems to be a reasonable solution towards the safety issues. The antidiabetic activity of *S. rebaudiana* extract as well as SGs is well documented in literature. The association of diabetes with cancer is also well known factor. Taking in consideration the above mentioned factors we have investigated the anticancer potential of extracts of *S. rebaudiana.* Extracts was prepared using petrolieum ether, ethyl acetate, methanol, aqueous methanol and water. Three cell lines (A-549, H-460 and MCF-7) have been used to evaluate the anticancer potential using MTT assay. In case of A-549, MVE-5 showing IC_{50} value of 10 µg/ml. Moreover, IC_{50} values of MVE-2 was also comparatively better and found to be less than 50 µg/ml and MVE-4 had shown IC_{50} value of 90 µg/ml. In case of H-460 reasonably better IC_{50} have been observed for MVE-4 and MVE-5 which is 88 µg/ml and 92 µg/ml, respectively. In H-460 reasonably better IC_{50} have been observed for MVE-4 and MVE-5 which is 88 µg/ml and 92 µg/ml, respectively. But in case of MCF-7 breast cancer cell line MVE-1 and MVE-2 have shown IC_{50} value of 90 µg/ml and 53 µg/ml, respectively. Thus, various extracts have shown good antiproliferative activity and *S. rebaudiana* can be further investigated for its anticancer potential. Furthermore docking study on EGFR,

PI3K and mTOR receptor revealed that SGs have good binding affinity towards all the three mentioned receptors and can be suitably modified to explore its anticancer potential. Moreover unfavourable 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 also been done successfully and total seven compounds have been isolated. Two compounds MVR-1 and MVR-5 have been successfully characterized and found to be quercetin and stevioside respectively which are already known compounds. Many reported SGs have shown poorer taste quality. Moreover taste quality of all SGs are different from one another. Thus docking studies were performed on SGs by constructing homology models of T1R2 and T1R3 subunits of human sweet taste receptors to explore the sweetness mechanism. Ramachandran plot, PROCHECK results and ERRAT overall quality factor indicated the acceptable quality of models. The binding pattern indicated that Asn 44, Ans 52, Ala 345, Pro 343, Ile 352, Gly 346, Gly 47, Ala 354, Ser 336, Thr 326 and Ser 329 are the main interacting amino acids residues of T1R2 and Arg 56, Glu105, Asp 215, Asp 216, Glu 148, Asp 258, Lys 255, Ser 104,Glu 217, Leu 51, Arg 52 of T1R3 respectively. Amino acids interact with SGs mainly by forming hydrogen bonds with hydroxyl group of glucose moieties. Maximum binding affinity has been obtained with SGs having total four glucose molecules attached with it and increase or decrease in glucose molecules reduced the binding afinityThere is significant variation in docked poses of all SGs. Taking in consideration the diverse binding patterns of various SGs as well as their structural features, we have proposed the mechanism of sweetness in the form of multiple point stimulation model. The present study will be helpful to know the proper mechanism of sweetness as well as binding patterns of SGs to sweet taste receptor. It will further helpful in understanding the difference in taste quality and will be used in improving the taste of SGs using semisynthetic approaches.

(Mayank) **(Dr. Vikas Jaitak)**

DEDICATED

TO

MY LOVING

MUMMY AND PAPA

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(Mayank)

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LIST OF ABBREVIATIONS

Sr no	Full form	Abbreviation
1.	Insulin dependent diabetes mellitus	IDDM
2.	Non-insulin dependent diabetes mellitus	NIDDM
3.	Human sweet taste receptor	STR
4.	G protein coupled receptor	GPCR
5.	Amino-terminal domain	ATD
6.	Cysteine rich domain	CRD
7.	Transmembrane helical domain	TMD
8.	Gibberellic acid	GA
9.	Steviol glycosides	SGs
10.	High performance liquid chromatography	HPLC
11.	Phosphophenol pyrovate carboxy-kinase	PEPCK
12.	Mean arterial blood pressure	mABP
13.	Inter venous	I.V
14.	Glomerular filteration rate	GFR
15.	12-O-tetradecanoylphorbol-13-acetate	TPA
16.	Fetal bovine serum	FBS
17.	Lipopolysaccride	LPS
18.	Phosphate buffer saline	PBS
19.	Tumor necrosis factor	TNF
20.	Interlukin	IL
21.	Nitric oxide	NO
22.	7, 12-dimethyl benz(a)anthracene	DMBA
23.	Reactive oxygen species	ROS
24.	Di-methyl sulfoxide	DMSO
25.	Nuclear magnetic resonance	NMR
26.	Mass spectroscopy	MS
27.	Ultri Violet	UV
28.	Thin layer chromatography	TLC
29.	Epidermal growth factor receptor	EGFR
30.	Protein data bank	PDB
31.	High throughput virtual screening	HTVC
32.	Standard precision	SP
33.	Extra precision	XP
34.	Absorption, distribution, metabolism and excretion	ADME
35.	Glide score	G. Score

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Natural products which include plants, animals and marine products are the richest sources of various metabolites with diverse pharmacological activities (Dahanukar *et al.*, 2000). Nature has provided a special kind of defense mechanism in the form of secondary metabolites with specific activities (Bennett and Wallsgrove, 1994). Such type of pharmacologically active secondary metabolites have been utilized in ancient time against various diseases and disorders in the form of crude extract. With the advancement of technology different secondary metabolites have been isolated and characterized and many of them are in clinical practice against various diseases. The famous examples of clinically used natural products include vinca alkaloids, taxanes, quinine, artemisinin, metformin and many more which contributes the most in economy of pharmaceutical industries. So investigation of natural resources is an important aspect in pharmaceutical field, but about 90 % of natural resources in the world are still not explored properly (Harvey, 2000). Thus, further chemical investigation of compounds from natural resources may not only improve the economy of the pharmaceutical industry, but also provide full proof treatment of various chronic diseases. Among various diseases, diabetes mellitus (DM) is the subject of major concern throughout the World. The incidence of DM is rising globally because of urbanization, physical inactivity, obesity, stressful lifestyle and many other reasons. International Diabetes Federation (IDF) estimated that the total number of people in India with diabetes were around 50.8 million in 2010 and will be around 87.0 million by 2030 and thus India will hold top rank among countries with maximum prevalence of disease (Ramachandran *et al.*, 2010). This could be a bigger problem regarding the nation's health and economy, especially for developing countries like India. DM is majorly classified as insulin dependent diabetes mellitus (IDDM) which account for only 5-10% of total diabetic cases and non insulin dependent diabetes mellitus (NIDDM) which is the major form of diabetes with about 90-95% of those with diabetes (Association, 2013). Major factors for DM include genetic factors, lifestyle, environmental factors and dietary factors (Åkerblom and Knip, 1998). Among dietary factors, use of sucrose is immediately

associated with DM. It promotes positive caloric balance, excessive weight gain and thus obesity which is immediately associated with diabetes (Jurdak and Kanarek, 2009; Leong and Wilding, 1999; Persson *et al.*, 1992). Moreover, dietary intake of sucrose and related sweeteners are responsible for many other health problems such as dental caries, candidiasis, inflammatory bowel disease and cancer (Thomas and Glade, 2010). Multiple reports indicated that there is a higher chance of the development of cancer in people with diabetes as hyperglycemia and hyperinsulinemia play a major role in the development of cancer. (Larsson *et al.*, 2006; Larsson *et al.*, 2005). Thus, regular sugar consumption is immediately related to diabetes and directly or indirectly linked with malignant neoplastic disease. Hence there is a need of alternative sweetener which is free from such health problems and must have compatibility with diabetic conditions. Several synthetic sweeteners such as aspartame, saccharin, sucralose and acesulfame potassium are currently in use, but side effects of these sweeteners have been the subject of debate over many years and is yet to be resolved. Major side effects associated with artificial sweeteners is represented in Table 1.

Table 1. Toxic effects of artificial sweeteners

Sweetener	ADI(mg/kg/d)	Acute Toxicity	Chronic toxicity	Reference
Acesulfame-K	15	Headache	Clastogenic, genotoxic at high thyroid tumors in rats	(Whitehouse *et al.*, 2008)
Aspartame	50	Headache, dry mouth, dizziness, mood change, nausea, vomiting, reduced seizure threshold, thrombocytopenia	Lymphomas, leukemias in rats	
Cyclamate	1	--	Bladder cancer in mice, testicular atrophy in mice	
Neotame	2	Headache, hepatotoxic at high dose	Lower birth rate, weight loss	
Saccharin	5	Nausea, vomiting, diarrhea	Cancer in offspring of breast-fed animals, low birth weight, bladder cancer, hepatotoxicity	
Sucralose	5	Diarrhea	Thymus shrinkage and cecal enlargements in rats	

In modern society where the challenge of maintaining a healthy calorie balance

is overwhelming to over half of the population, non-calorie natural sweetener offer hope to those who wish to avoid the debilitating disease associated with excessive sugar consumption (Ogden *et al.*, 2006). Various plants provide one or more sweet principle components and among them *S. rebaudiana* is most promising herb which consists of multiple sweet steviol glycosides (SGs) which also have many health benefits (Tanaka, 1982). SGs interact with human sweet taste receptor (STR) and produce many fold sweetness compared to sucrose (Puri *et al.*, 2011). The main drawback of SGs is taste quality. Multiple semi-synthetic approaches have been applied to improve its taste quality but we are still away from the desired outcome (de Oliveira *et al.*, 2007; Jaitak *et al.*, 2009). In this perspective binding pattern of SGs to STR is an important investigation aspect for exploring key binding interactions responsible for sweetness quality so that groups necessary for sweetness can be identified and structure as a whole can be modified to improve sweetness quality of SGs. STR is a heterodimer composed of two subunits namely T1R2 and TIR3 and belongs to class C G-protein coupled receptor (GPCR) family (Li *et al.*, 2002). Each subunit of STR consists of a large amino-terminal domain (ATD) which is joined to seven transmembrane helical domain (TMD) by extracellular cysteine rich domain (CRD) (Brauner-Osborne *et al.*, 2007; Pin *et al.*, 2003). ATD of Class C GPCR is the biggest domain containing about 500-600 residues and hold active site for endogenous ligands (Pin*, et al.*, 2003). In STR, ATD of each subunit holds the active site for sucrose, glucose, sucralose and related sugars thus these sweeteners can be considered as orthostearic agonists of STR (Nie *et al.*, 2005; Xu *et al.*, 2004). Quality and magnitude of sweetness of various SGs with little variation in their glucose molecules differ significantly, which indicate that associated glucose molecules plays an importance role in the sweetness profile of SGs by interacting with STR. SGs with three to four monosaccride subunits at C-13 position and one to two monosaccride subunits at C-19 show maximum sweetness property which further indicate the importance of associated glucose moieties in sweetness of SGs (Obtaini and Yamasaki, 2001). Thus, sweetness properties of SGs might be due to interaction of its glucose molecules with ATD domain of STR which holds active site for glucose .Thus, docking study of SGs can be done on ATD of both the subunits of STR to explore key binding interaction.

Anticancer activity of *S. rebaudiana* is yet another important aspect which can be explored further properly. Various extracts of S*. rebaudiana* was found to reduce blood glucose level by multiple known and unknown mechanism of actions and thus have inbuilt antidiabetic activity (Chatsudthipong and Muanprasat, 2009; Misra *et al.*, 2011; Shivanna *et al.*, 2013). Furthermore multiple literatures revealed that there is direct link between diabetes and cancer (Giovannucci *et al.*, 2010). The anticancer potential of biguanide and glitazone class of antidiabetic agents is well documented in literature which further indicates the direct association of diabetes and cancer. Thus it is quite reasonable to explore the anticancer properties of *S. rebaudiana.* Literature survey further revealed that stevioside which is the most abundant SG has shown significant anti-carcinogenic potential as well as apoptosis inducing ability (Brahmachari *et al.*, 2011; Paul *et al.*, 2012). Moreover isosteviol was found to inhibit DNA polymerase and human topoisomerase II, both of them are well known targets of anticancer drugs. Thus, exploring anticancer activity of *S. rebaudiana* may provide a good lead molecule which can be further modified to get useful anticancer compounds.

Keeping in view the above mentioned facts of natural products, sweetness as well as medicinal properties of SGs, present study was conducted to further explore the phytochemical and medicinal aspect of *S. rebaudiana.* Work has been conducted by considering the following two parts of objectives

Part 1

- To extract and prepare different extracts of aerial part of *S. rebaudiana.*
- *In-vitro* study of extracts of *S. rebaudiana* for its anticancer potential.
- *In-silico* study for anticancer potential of already isolated compounds from *S. rebaudiana.*
- Isolation and characterization of natural sweeteners from extracts of *S. rebaudiana*.

Part 2

- Homology modeling of human sweet taste receptor and docking study of SGs.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

S. rebaudiana (Bartoni) family *asteraceae* (Table 2) is an herbaceous perennial plant indigenous to Paraguay and Brazil where its leaves are used by local Guarani Indians as natural sweetener for hundreds of years (Jaitak *et al.*, 2008). 150 species of stevia are known, but among them only *S. rebaudiana* has sweet tasting properties (Mousumi, 2008). This plant is of worldwide importance today because its leaves are used as non-nutritive high potency natural sweetener in Japan, Korea China and in South America. It is now cultivated in some parts of Asia, Canada, China, Brazil and Paraguay. In India it is cultivated in Himachal Pradesh, Punjab, Haryana, Utter Pradesh, Madhya Pradesh, West Bengal, Karnataka and Tamilnadu (Vikas *et al.*, 2011). Overall consumption of stevia extract in Japan and Korea is around 200 and 115 tons/year respectively. Its water extract has beneficial effects on human health, having hypoglycemic, and hypotensive effects and as a source of natural antioxidant. Leaves of *S. rebaudiana* are useful in diabetes as natural substitute for sugar, used in baking, inhibits the formation of cavities and plaque in teeth, non-toxic, cardiotonic and is effective against microbes *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. Its leaves contain multiple entkaurene diterpene glycosides with steviol skeleton and characteristic organoleptic properties. Collectively all of them are known as SGs. Unlike synthetic sweeteners like aspartame, saccharine, cyclamate and sucralose, SGs are safe without any side effects (Vikas*, et al.*, 2011).

Table 2. Taxonomical classification of *S. rebaudiana.*

Kingdom	***Plantae* – Plants**
Subkingdom	*Tracheobionta*
Superdivision	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Asteridae*
Order	*Asterales*
Family	*Asteraceae*
Genus	*Stevia* Cav.
Species	*Stevia rebaudiana* (Bertoni)

2.1 Biosynthetic pathway of SGs

Biosynthetic pathway of SGs is well documented in literature (Brandle and Telmir, 2007). SGs are closely related to the gibberellins as both of them share a part of biosynthetic pathway. Kaurenoic acid is an intermediate in the biosynthesis of both gibberellic acid (GA) and SGs. In the biosynthesis of SGs the kaurenoic acid is converted into the tetracyclic diterpene steviol, which then proceeds through a multi-step glycosylation pathway to form the various SGs. Like all diterpenes, both GA and steviol are synthesized from the precursor geranylgeranyldiphosphate (Brandle and Telmir, 2007). The biosynthetic pathway showing synthesis of various SGs is as shown in Figure 1.

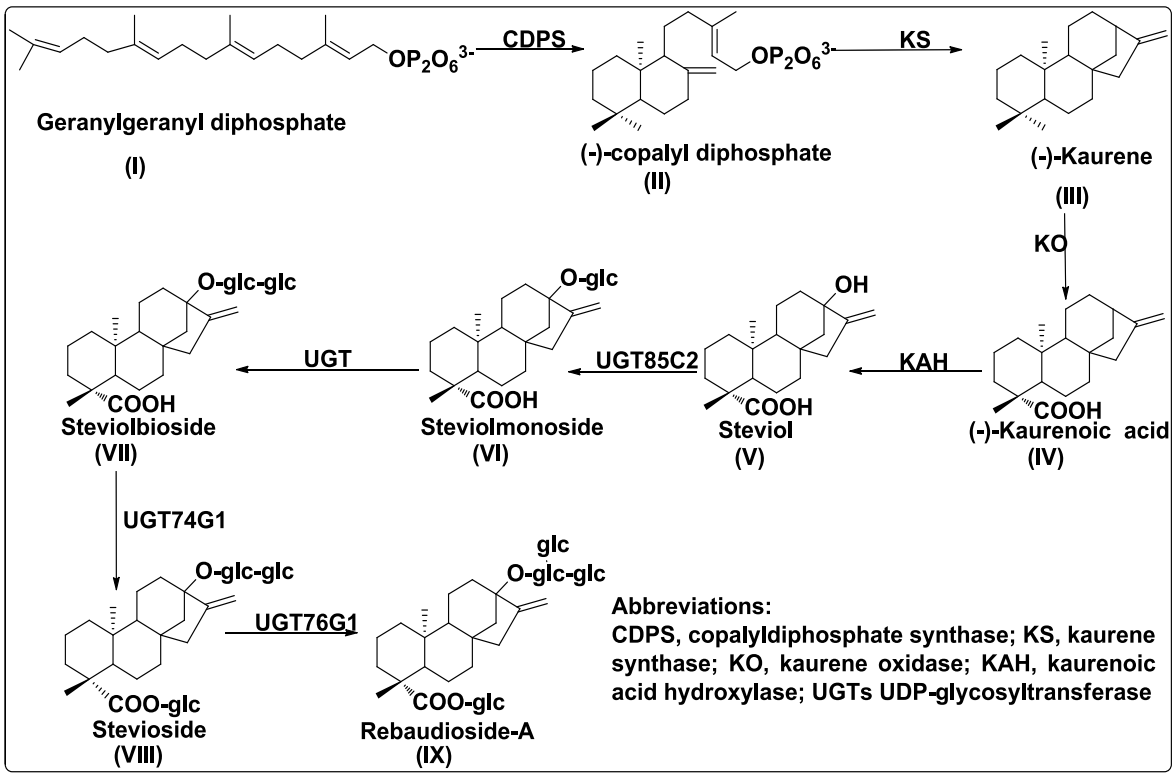

Figure 1. Biosynthetic pathway of SGs

2.2 Chemicals profile

2.2.1 Steviol glycosides

S. rebaudiana is well-known for its high concentration of SGs. SGs generally contains glucose moieties which are joined with aglycone part termed as Steviol **(1)** (Puri*, et al.*, 2011)**.** Isosteviol **(2)** which is oxidized product of steviol is well

known for many biological activities including anticancer activity. Leaves of *S. rebaudiana* generally contain 6-10% stevioside **(3)**, 2-4% rebaudioside A **(4)** and up to 1-2% other minor glycosides (Jaitak*, et al.*, 2008). The well known minor SGs include rebaudoside B **(5),** rebaudioside D **(6)**, rebaudioside E **(7)**, rebaudioside F **(8)**, dulcoside A **(9)** and steviolbioside **(10)**, (Brahmachari*, et al.*, 2011). Sweetness profile of all the above mentioned SGs except rebaudioside F is well established. Stevioside is approximately 250-300 times sweeter than that of sucrose. Similarly rebaudioside A is 350-450 times, rebaudioside B is 300-350 times, rebaudioside D is 200-300 times, rebaudioside E is 250-300 times, steviolbioside is 100-125 times and dulcoside is 50-120 times more sweeter than that of sucrose (Puri*, et al.*, 2011).

Taking in consideration the industrial importance of this plant several efforts were made to further isolate new SGs and a group of researchers have isolated three noble SGs which include 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-Dglucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid **(11)**, 13-methyl-16-oxo-17-nor-*ent*-kauran-19-oic acid-β-D-glucopyranosyl ester **(12)** and 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-16β-hydroxy-*ent*-kauran-19-oic acid **(13)** (Prakash Chaturvedula *et al.*, 2011). Yet another group of researchers have isolated two new and unique minor diterpene SGs having α -glucosyl linkage. The structure of these compounds were found to be 13-[(2-O-(3-α-O-D-glucopyranosyl)-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D glucopyranosyl) oxy] ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester **(14)** and 13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester **(15)** (Chaturvedula, Upreti*, et al.*, 2011)**.** Yet another attempt was made to isolate minor SGs and three new and one already known SGs have been isolated. The isolated new SGs were found to be 13-[(2-O-β-D-glucopyranosyl-β-Dglucopyranosyl)oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester **(16)**, 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-D-glucopyranosyl ester **(17)** and 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid β-D-glucopyranosyl ester **(18)**. 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-15-en-18-oic

acid β-D-glucopyranosyl ester **(19)** is the already known SG isolated during this process (Chaturvedula, Clos*, et al.*, 2011). Structure of various isolated SGs in represented in Figure 2.

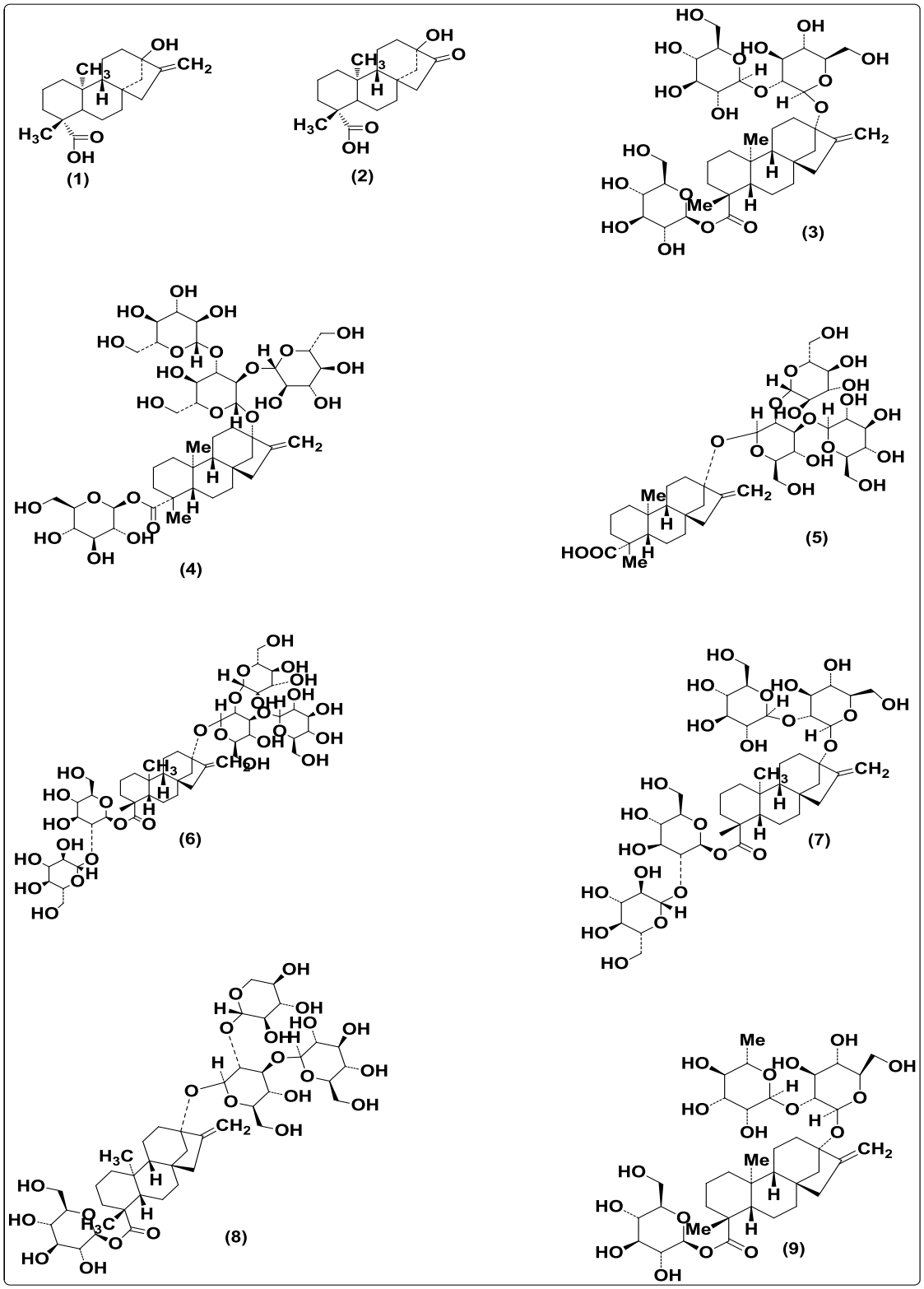

Figure 2. Chemical structure of SGs

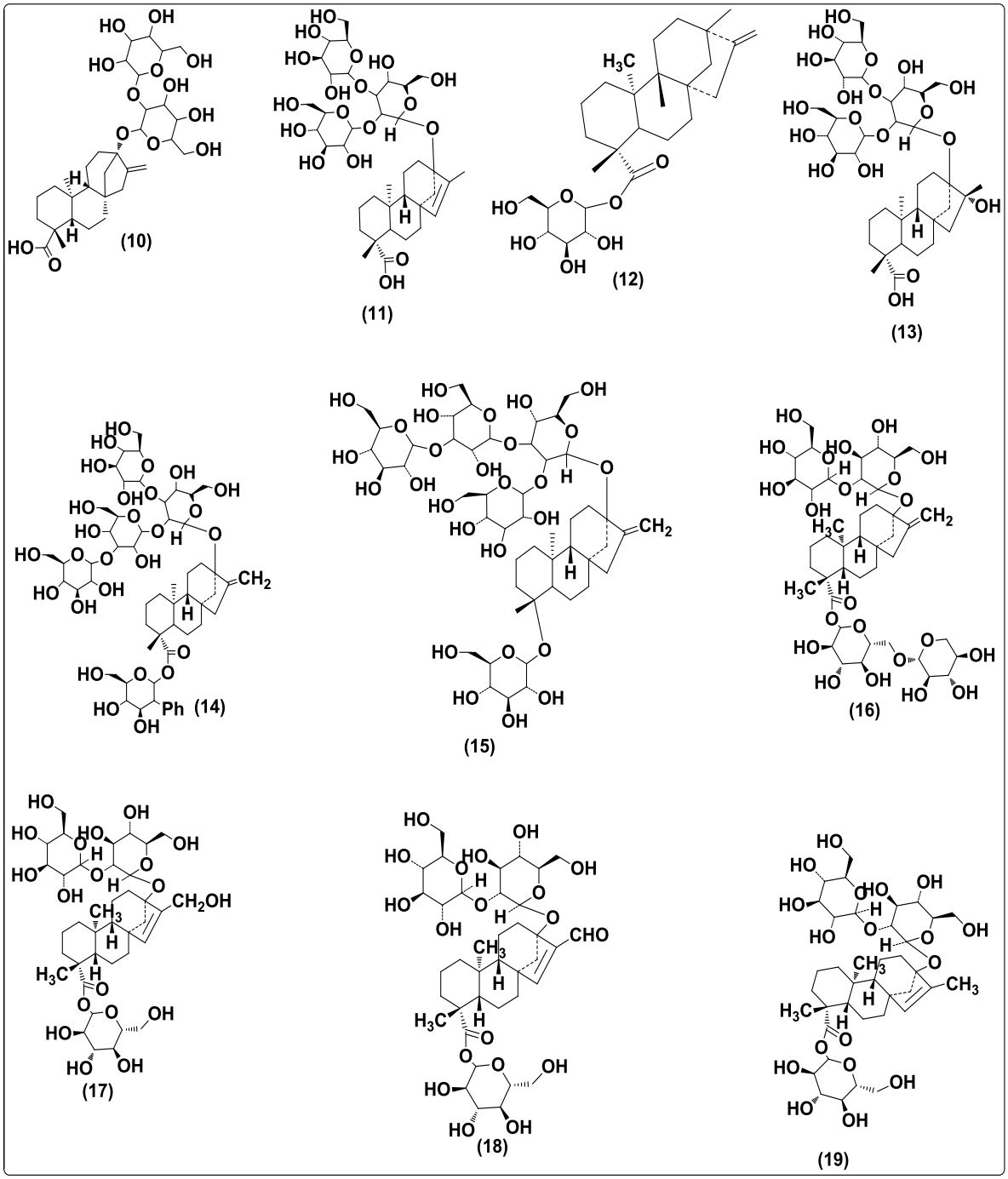

Figure 2. Continue

2.2.2 Non-glycosidic diterpenes

Labdane-type diterpenes have also been isolated from *S. rebaudiana.* The various compounds of this category isolated from *S. rebaudiana* include austroinulin **(20)** (Figure 3)**,** 6-O-Acetylaustroinulin **(21),** jhanol **(22)**, Sterebins A-N **(23-36),** (De *et al.*, 2013; Wölwer-Rieck, 2012).

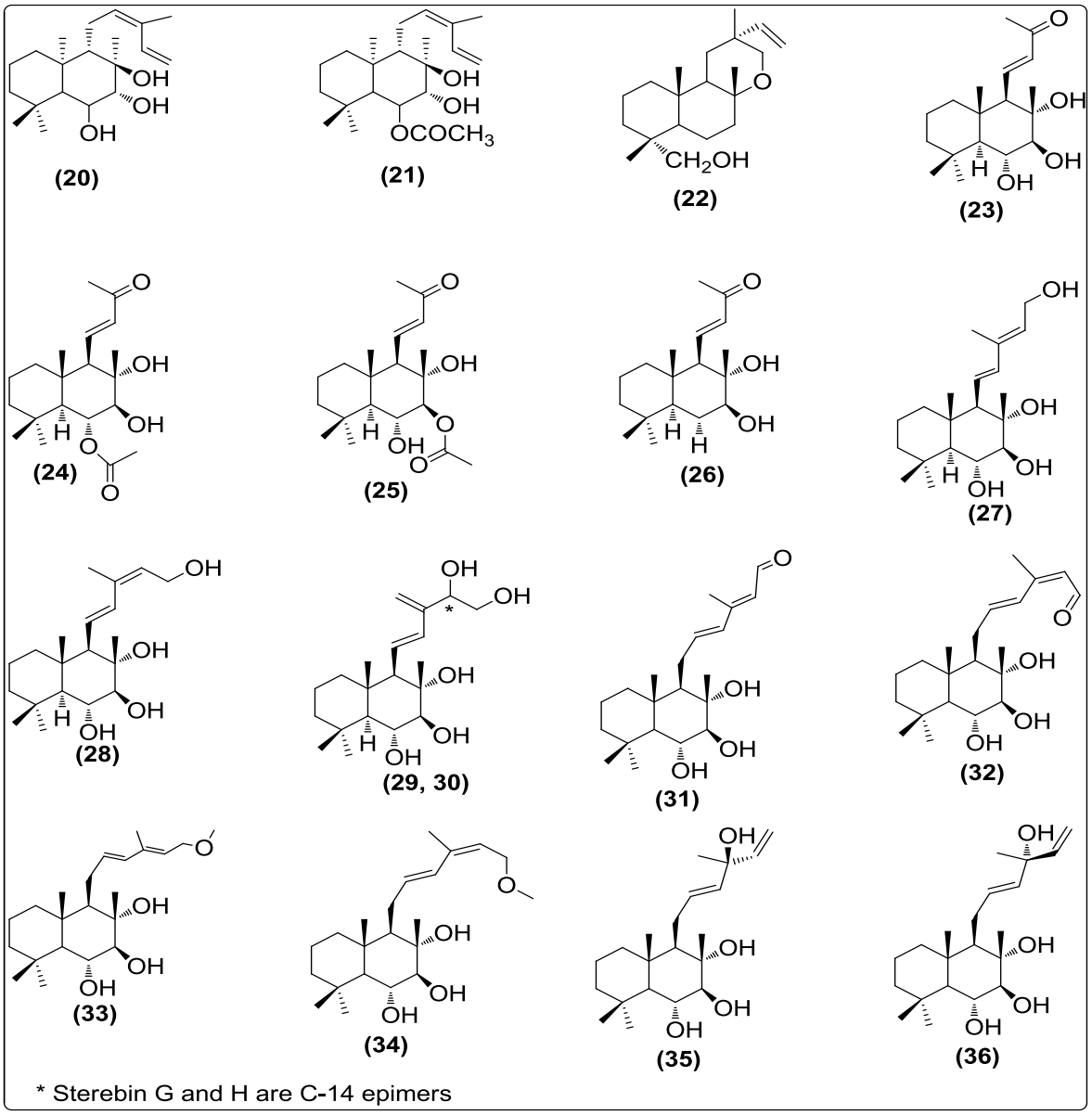

Figure 3. Chemical structure of labedene type of glycosides isolated from *S. rebaudiana*

2.2.3 Flavonoids and alkaloids

S. rebaudiana is also a rich source of flavonoids. The various flavonoids present in it include quercetin **(37)**, quercetin-3-O-β-D-arabinoside **(38)**, quercetin-3-O-β-D-rhamnoside **(39),** quercetin-3-O-glucoside **(40),** quercetin-3-O-rutinoside **(41),** kaempferol-3-O-rhamnoside **(42),** apigenin **(43),** apigenin-4′-O-β-D-glycoside **(44),** apigenin-7-O-β-D-glycoside **(45),** luteolin-7-O-β-D-glycoside **(45)** (Wölwer-Rieck, 2012)**.** An alkaloid named steviamine **(47)** has also been

isolated from the *S. rebaudiana* (Wölwer-Rieck, 2012). The chemical structures of all these flavonoids and steviamine has been shown in Figure 4.

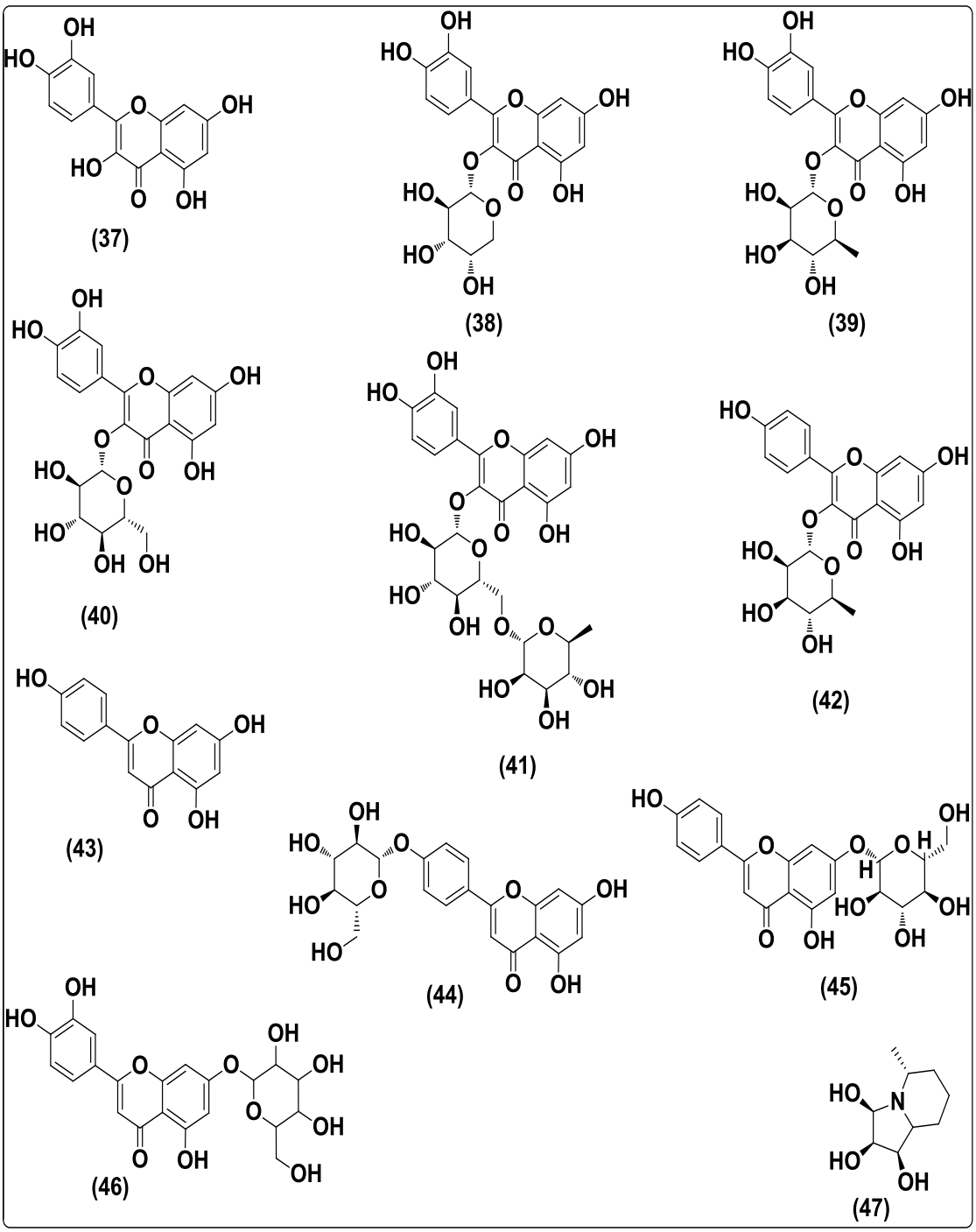

Figure 4. Chemical structures of flavonoids and alkaloid from *S. rebaudiana*

2.3 Pharmacokinetics profile of SGs

Pharmacokinetics of SGs particularly of stevioside is well established. Stevioside, being hydrophilic diterpenoid glycoside and also having relatively

high molecular weight is not absorbed through intestine. The digestive enzymes of animals as well as humans are also unable to degrade stevioside ((Chatsudthipong and Muanprasat, 2009; Koyama *et al.*, 2003; Wingard *et al.*, 1980). Intestinal bacterial flora of various animals including humans are able to break stevioside and generate free aglycone steviol (Chatsudthipong and Muanprasat 2009; Gardana *et al.*, 2003). Thus after oral administration of stevioside, it is the steviol that is taken up by the blood from the intestine (Chatsudthipong and Muanprasat, 2009). The mechanism of steviol absorption involves both passive diffusion as well as carrier mediated transport through monocarboxylic transporter (Chatsudthipong and Muanprasat, 2009). In an experiment after intravenous injection of ^{131}I stevioside into Wister male rat, radioactivity was found to be very less in case of heart, testis and muscles during the entire 24 hour experiment (Cardoso *et al.*, 1996), however significant radioactivity was detected in liver, kidney and intestine. The accumulation was higher in liver after 10 min of injection. Significant radioactivity was present in bile after 2 hours. Its presence was maximal in small and large intestine after 2 and 4 hours following stevioside injection. HPLC analysis of bile showed steviol as the major metabolite. Urine analysis indicated the presence of stevioside as well as some unknown metabolite which was also found in the bile (Cardoso*, et al.*, 1996; Chatsudthipong*, et al.*, 2009). These results show that conversion of stevioside to steviol in rats occurs in liver. There are two routes of stevioside excretion i.e. bile and urine. In addition steviol may undergo enterohepatic circulation where steviol is excreted into bile and reabsorbed back to circulation but the presence of steviol into portal system was not documented in the study (Cardoso*, et al.*, 1996; Chatsudthipong and Muanprasat, 2009). Steviol which is the major metabolite of stevioside appears in blood after oral administration passes through phase I and phases II metabolism (Chatsudthipong and Muanprasat, 2009). Incubating steviol with hepatic microsomes from rat and human monohydroxy and dihydroxy metabolites of steviol are formed and this process requires NADPH-generating system, suggesting phase I metabolism of steviol by cytochrome P450 (Chatsudthipong and Muanprasat, 2009; Koyama*, et al.*, 2003). Phase II metabolism of steviol occurs through glucuronide conjugation in liver (Figure 5) (Brahmachari*, et al.*, 2011).

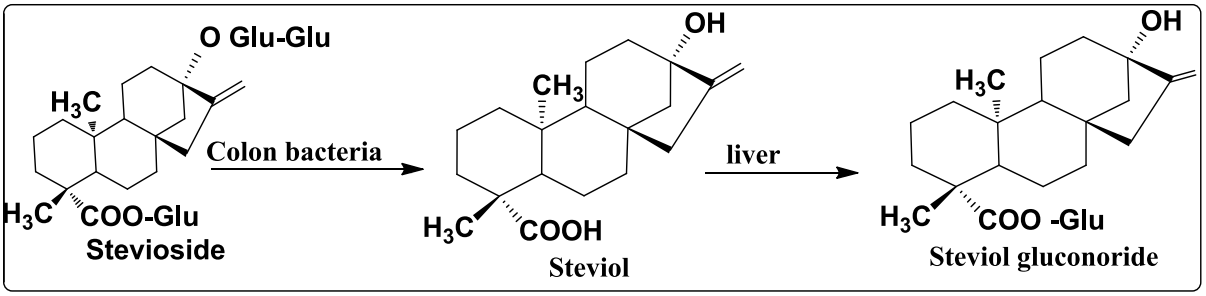

Figure 5. Phase II metabolism of steviol glucosides

Steviol glucuronide the major metabolite is mainly excreted through biliary and urinary tracts. In case of rats the principle route of excretion of steviol glucuronide is feces (Nakayama, Kasahara, & Yamamoto, 1986; Roberts & Renwick, 2008; Wingard *et al*., 1980) but in human the urinary excretion plays a major role (Chatsudthipong & Muanprasat, 2009; Geuns et al., 2006; Wheeler et al., 2008). It has been observed that after 72 h of oral administration of stevioside in humans steviol glucuronide excreted in urine is about 62% of total administered dose and free steviol excreted in feces accounts only 5.2% of total administered dose (Wheeler et al., 2008). This difference in humans and rats is thought to be due to different molecular weight threshold for humans and rat biliary excretion of organic anions like glucuronide. Organic anions having molecular mass more than 600 Da in humans and of 325 Da in rats are excreted in bile instead of through glomerular filtration process in kidney and steviol glucuronide is having molecular weight of 512.9 Da (Chatsudthipong & Muanprasat, 2009). Thus, in humans steviol glucuronide is the major metabolite of stevioside which is mainly excreted through urine. The excretion process probably involves renal organic anion transporters (Srimaroeng, Chatsudthipong, Aslamkhan, & Pritchard, 2005).

2.4 Pharmacological properties of *S. rebaudiana*

2.4.1 Anti-hyperglycemic effect

SGs help to regulate the plasma glucose level by multiple ways (Figure 6). The most important effect of SGs is its impact and usefulness in NIDDM which is a metabolic disorder caused by the defects in insulin secretion and insulin action. It has been observed that in NIDDM there exist abnormalities in pancreatic

alpha cells which cause hyper-glucagon secretion (Unger, 1997). Thus postprandial hyperglycemia in NIDDM is due to an increase in entry of glucose in plasma by basal hepatic glucose production and decrease in exit of glucose from plasma by peripheral glucose disposal mechanisms. Correction of this imbalance at either entry or exit step of plasma glucose thus seems to be useful strategy to correct various pathological condition of diabetes. Stevioside and related glycosides are free from calorie and only small quantity is needed to produce require sweetness as these are many fold much sweeter then sucrose. Thus it seems to be a good alternative to sugar for diabetic patients. It also provides various beneficial pharmacological effects in NIDDM. Extract of *S. rebaudiana* has long been used for the treatment of diabetes in South America (Kinghorn and Soejarto, 2002). SGs and related compounds affects plasma glucose level by influencing glucose absorption, glucose synthesis, insulin secretion and increasing insulin sensitivity (Chatsudthipong and Muanprasat, 2009). The effect of steviol on glucose absorption has been well investigated by various researchers (Toskulkac *et al.*, 1997). Steviol at the dose of 1mM inhibits intestinal glucose absorption by about 40% (Toskulkao *et al.*, 1995). Stevioside at the dose of 1 mM and 5 mM, does not inhibit intestinal glucose absorption in hamster jejunum but 1 mM dose of steviol inhibits glucose absorption by about 30% without affecting the activity of intestinal Na^+-K^+-ATPase (Toskulkao, *et al.*, 1995). It was further reported that there was reduction in intestinal ATP content which caused the reduction of glucose absorption. The decrease in ATP content was due to reduction in intestinal mitochondrial enzyme activity at the level of phosphorylation (Chatsudthipong and Muanprasat, 2009). Stevioside affects the glucose synthesis in both type 1 as well as type 2 diabetic animal models (Chatsudthipong and Muanprasat, 2009). The hypoglycemic effect of stevioside was found to be mediated via its effect on phosphophenol pyruvate carboxy kinase (PEPCK) which is a rate-limiting enzyme for gluconeogenesis and thus controlling glucose production in liver (Chen *et al.*, 2005). It seems that stevioside suppress the PEPCK gene expression and finally reduces gluconeogenesis (Chatsudthipong and Muanprasat, 2009). Furthermore stevia powder is more efficient in down-regulating PEPCK as compared to stevioside which indicate that except stevioside some other chemical compounds might be present which are responsible for down-regulating PEPCK (Chatsudthipong and

Muanprasat, 2009). Stevioside and steviol also effect insulin secretion and insulin sensitivity. *In-vitro* experiments revealed that stevioside and steviol have insulinotropic/hypoglycemic effect. Further anti-hyperglycemic, insulinotropic and glucagonostatic effect of stevioside is established through *in-vivo* experiments using diabetic rate animal model (Jeppesen *et al.*, 2002; Jeppesen *et al.*, 2000). Stevioside has also been found to sensitize peripheral tissue towards insulin in many experiments and thus helps to overcome insulin resistance in NIDDM (Chatsudthipong and Muanprasat, 2009). Insulin independent direct effect of stevioside on glucose transport activity in skeletal muscle in animal models have also been observed which suggested that there may be some potential site of action in skeletal muscle glucose transport system where stevioside act to produced such outcomes (Chatsudthipong and Muanprasat,2009). Furthermore several studies on animal models indicated that insulinotropic/hypoglycemic effect of stevioside was only associated with diabetic condition and no hypoglycemia occurs in normal conditions (Jeppesen, *et al.*, 2002; Jeppesen, *et al.*, 2000). Stevioside was also reported to stimulate the insulin release in isolated mouse islet only at high glucose concentration (Jeppesen, *et al.*, 2000). The reduction of glucagon has also been found to be associated with stevioside which decreases glycolysis and helps to decrease plasma glucose level.

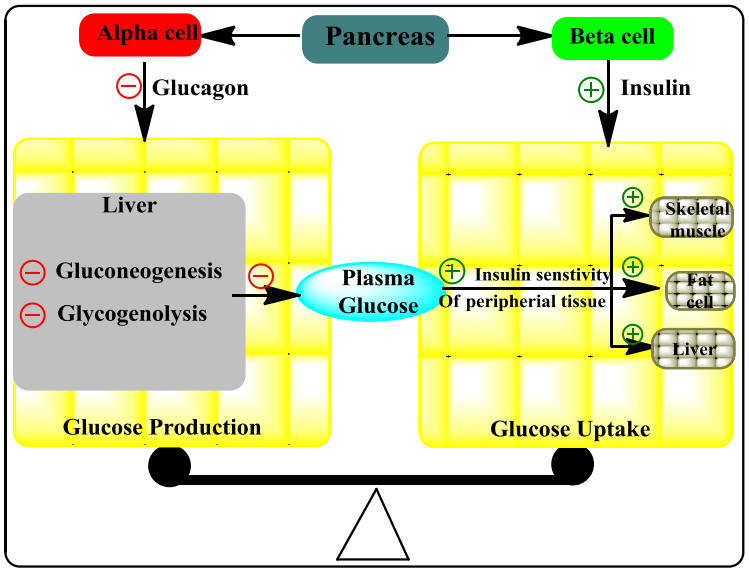

Figure 6. The possible anti-hyperglycemic actions of stevioside and related compounds.(Adopted from Chatsudthipong & Muanprasat, 2009)

This effect may be due to enhancing mRNA expression of cernitine palmitoyltransferase peroxisome proliferator-activated receptor gamma (PPARγ) and stearoyl-CoA desturase (Chatsudthipong and Muanprasat, 2009). Rebaudiana A also stimulates insulin secretion from isolated mouse islets in glucose and Ca^{2+} dependent manner (Chatsudthipong and Muanprasat, 2009). It seems that rebaudioside A cause the release of insulin by inactivating K_{ATP} channels which requires high glucose concentration and also by activating calcium channels (Chatsudthipong and Muanprasat, 2009).

2.4.2 Anti-hypertensive effect

Studies on animals models and humans provided much evidences that stevia extract as well as stevioside decrease mean arterial blood pressure (mABP) by inducing vasodilation, diuresis and natriuresis (Chatsudthipong and Muanprasat, 2009). Anti-hypertensive effect requires long time to onset following oral administration of *S. rebaudiana* extract to normal and hypertensive rat as no significant changes in BP occurred for the first 20 days. The hypotensive effect of the extract comes in observation after 40 to 60 days following administration (Chatsudthipong and Muanprasat, 2009). But I.V infusion of stevioside on the other hand reduced blood pressure in general without any delay in animal models (Melis, 1992). I.V administration of stevioside in conscious hypertensive rats produced reduction in both systolic as well as diastolic blood pressure in dose dependent manner (Chatsudthipong and Muanprasat, 2009). Thus, sharp hypotensive effect only occurred if stevioside was given directly in systemic circulation. Hypotensive effect of stevioside has also been observed in many human studies. It has been observed that it produced bradicardia and hypotension when implemented to human subjects (Chatsudthipong and Muanprasat, 2009). The mechanism behind anti-hypertensive activity of SG is still unclear but ability of indomethacin to abolish the hypotensive action of stevioside indicates that its mechanism may involve prostaglandin activity (Melis and Sainati, 1991). It was further observed that stevioside induces hypotensive effect without changing serum dopamine, norepinephrine and epinephrine level which indicate the non-involvement of sympathetic tone (Chan *et al.*, 1998). The hypotensive effect of stevia may be partially due to effect on plasma volume. I.V stevioside administration causes

natriuresis, dieresis and increased renal plasma flow but glomerular filtration rate (GFR) is unaffected (Chatsudthipong and Muanprasat, 2009). As these phenomena are reduced by indomethacin which suggested that SGs cause vasodilatation of both afferent and efferent arterioles leading to increased renal plasma flow without changing the GFR (Melies and Sainati,1991). The diuresis may be due to decrease fluid and sodium re-absorption in proximal tubule (Chatsudthipong and Muanprasat, 2009). Thus stevioside reduces mABP by reducing plasma volume as well as vascular resistance.

2.4.3 Antimicrobial activity

S. rebaudiana have been deeply studied for its antimicrobial potential. In a study among multiple extracts water extract showed activity against *B. subtilis* and *S. aureus.* Methanol extract have shown good activity against *P. aeruginosa.* Moreover *B. megaterium* and yeast were extensively susceptible towards ethyl acetate and hexane extracts, respectively (Tadhani and Subhash, 2007). In another study the antiviral activity of *S. rebaudiana* was explored. Hot water extract of *S. rebaudiana* was found to inhibit the replication of all four serotypes of human rotavirus *in-vitro*. Further investigation suggested that hot water extract bind to 37 kD, VP7 and interfere with the binding of VP7 to the cellular receptors by steric hindrance, which results in the blockade of the virus attachment to cells (Takahashi *et al.*, 2001). Thus *S. rebaudiana* have shown wide spectrum of antimicrobial activity

2.4.4 Anti-inflammatory and immunomodulatory activity

SGs have shown significant anti-inflammatory and immunomodulatory activity in multiple studies. In a report, stevioside, rebaudiosides A, C and dulcoside A have been studied for its anti-inflammatory activity. These compounds showed strong inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced inflammation in mice. Fifty percent inhibitory dose of these compounds for TPA induced inflammation ranges from 54.1-291.6 mg/ear (Yasukawa*, et al.*, 2002). Various SGs have shown significant effects on pro-inflammatory cytokines. 1 mM dose of stevioside moderately increased the production of tumor necrosis factor (TNF-α), interlukin (IL-1β) and nitric oxide (NO), in un-stimulated human THP-1 cell by interacting with toll-like receptor-4. Thus

stevioside can be useful to healthy individual as it is capable to enhance the innate immunity. On the other hand in LPS stimulated THP1 cells, the same concentration of stevioside suppress the release of TNF-α, IL-1β, and NO by interfering with the signaling pathway of NF-κB, a transcription factor that controls the expression of inflammatory cytokines in the immune cells. Thus, in several circumstances, stevioside may be useful due to its ability to prevent inflammatory response, and in healthy condition, it may offer an immune-related benefit by boosting immunity system (Boonkaewwan *et al.*, 2006).

2.4.5 Anti-diarrheal activity

Pariwar and co-workers reported anti-diarrheal potential of SGs. According to the report stevioside and its major metabolite, steviol, were found to influence ion transport in many types of tissues including kidney, pancreas and intestine. Further 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 such type effect. In the same study apical current measurement indicated that dihydroisosteviol targeted the cystic fibrosis transmembrane regulator. Inhibitory action of this compound was found reversible and was not associated with changes in the intracellular C_{AMP} level. Pariwar and co-workers further demonstrated 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% without altering fluid absorption, thereby indicating that dihydroisosteviol and similar compounds could be a new class of cystic fibrosis transmembrane regulator inhibitors that may be useful for further development as anti-diarrheal agents (Pariwat *et al.*, 2008)

2.4.6 Anticancer activity

It is well established that hyperinsulinemic condition is also associated with high risk of cancers (Figure 7) (Erbach *et al.*, 2012). SGs thus seem to indirectly reduce the risk of cancer by reducing hyperglycemic condition. Moreover, many

studies indicated that SGs have direct anticancer activities (Wasuntarawat *et al.*, 1998).

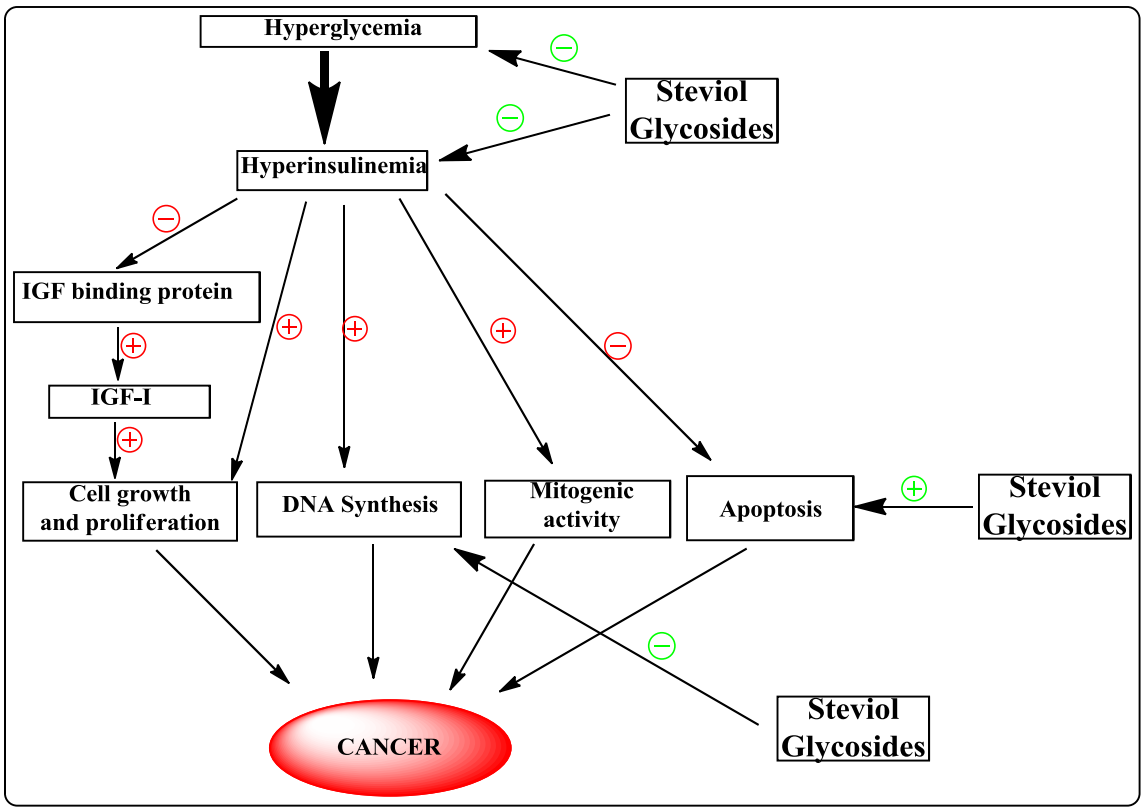

Figure 7. Anticancer activity of SGs

Oral administration of 96.6% pure stevioside at a dose of 2.5% and 5% prevents significantly the induction of carcinogenicity in fischer 344 rats animal models (Wasuntarawat*, et al.*, 1998). It was also reported that stevioside have inhibitory effect on tumor promotion by TPA in two- stages carcinogenesis in mouse skin (Brahmachari*, et al.*, 2011). Furthermore Yasukawa and his coworkers studied the time course of skin tumor formation as well as average number of tumors/mouse treated with 7, 12-dimethyl benz(a)anthracene (DMBA) plus TPA, with or without stevioside mixture. The group of animals treated with DMBA plus TPA produced 8.1 tumors/ mouse at 20 weeks, whereas the groups treated with DMBA plus TPA and a mixture of steviosides at doses of 0.1 and 1.0 mg had produced 2.2 and 0.3 tumors/mouse, respectively (Yasukawa *et al.*, 2002). Hence, SGs at the dose of 0.1 mg and 1.0 mg caused 73% and 96% reductions respectively, in the average number of tumors/mouse at the fixed time (Yasukawa*, et al.*, 2002). In another study, it has

been established that the stevioside also have cytotoxic ability. In the study, stevioside was found to induced reactive oxygen species (ROS) mediated apoptosis through mitochondrial pathway in MCF-7 human breast cancer cell line (Paul*, et al.*, 2012). It conveyed the apoptotic signal via intracellular ROS generation, inducing chances in mitochondrial trans membrane potential and thus inducing mitochondrial mediated apoptotic pathway. Results indicated that stevioside induces ROS-mediated mitochondrial permeability transition which results in increased expression of apoptotic proteins such as Bax, Bcl-2 and Caspase-9 (Paul*, et al.*, 2012). Furthermore isosteviol inhibits DNA polymerase and human topoisomerase II which are well known targets of anticancer drugs (Chatsudthipong and Muanprasat, 2009). Thus SGs have shown significant anticancer activity which can be further explored for the search of better anticancer leads

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CHAPTER 3

MATERIAL AND METHODS

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3.1 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 355 m; Latitude 31.191769^{o} N, longitude 76.258774^{o} E)

3.2 Chemicals

Petroleum ether, ethyl acetate, chloroform and methanol used in this study were purchased from SD fine-Chem limited Mumbai. Water used for the experiment was free from ions and is double distilled. RPMI 1640 and DMEM, Penicillin/ Streptomycin antibiotic solution, phosphate buffer saline and fetal bovine serum media were used to culture cancer cell lines. DMSO, extra pure AR was purchased from SRL. Sulphuric acid (91%) was purchased from LobaChemi Pvt. Ltd.

3.3 Cancer cell lines

Three cancer cell lines used in this study include H-460, A-549 and MCF-7. H-460 and A-549 are large cell lung cancer cell lines while MCF-7 is the breast cancer line. All the cell lines were procured from national cell repository located at NCCS, Pune.

3.4 Instruments

Various instruments used in the study are included in table 3.

Table 3. List of instruments used in the study

Instruments used	**Company**	**Purpose**
Aspirator	JSWG	Extraction
Rotavapor	Ilmvac	Drying extracts/fractions
^{1}H and ^{13}C NMR (400 MHz)	Bruker Avance II	Structure characterization

Automatic cell Counter	Invitrogen	For counting of cells
Incubator	GIalaxy, New Brunswick	Incubation
Centrifuge 5430 R	Eppendorf, Germany	Centrifugation
Laminar air flow	Macro Scientific Works	For aseptic condit Ion
UV-VIS Spectrophotometer	Shimadzu	Absorption studies
Inverted microscope	Magnus, Olympus	Visualization of the cancer cells

3.5 Computer and software

HP-2800 workstation with configuration of Intel (R) Xenon (R) X5660 @ 2.80 GHz 2.79 GHz (2 processor) was used in *in-silico* study of the compounds. The operating system used included window-7 and centox-6.5. Software used in the study include latest release of Schrödinger LLC (NY, USA) and ChemBio Draw Ultra-12

3.6 Extraction of plant material

Dried aerial part of plant was grounded to get fine powder. 4.3 kg of powdered plant material was used for extraction purpose. Extraction was done using solvents in increasing order of polarity using petroleum ether, ethyl acetate, methanol, aqueous methanol and finally water. Percolation process was used for extraction where plant material was percolated three times with each solvent. Extracted fractions were dried and weighted. Weight of various extracts obtained included petroleum ether extract (MVE-1) 60 g, ethyl acetate extract (MVE-2) 64 g, methanol extract (MVE-3) 67 g, aqueous methanol extract (MVE-4) 500 g and water extract (MVE-5) 190 g.

3.7 *In-vitro* anticancer assay

MTT assay was performed to evaluate the anti-proliferative activity of plant extracts. All the five extracts of *S. rebaudiana* were dissolved in DMSO (1 mg/ml) to form a stock solution and subjected to *in-vitro* cytotoxic study and showed results in varied range of concentration. Three human cancer cell lines included in the studies were having different origin, tumourogenicity and morphology. A-549 human non-small cell lung cancer cell line (non-metastatic

cells) have adherent property and forms a monolayer (Forest *et al.*, 2005; Kobayashi *et al.*, 1995). H-460 is the large cell lung carcinoma cell lines with adherent and metastatic properties (Forest*, et al.*, 2005). MCF-7 breast cancer cell line used in this study is employed as a standard to evaluate the potential of endocrine based anticancer therapies (Levenson and Jordan, 1997). To study the outcome values of cytotoxicity assay the results were plotted in bar graph in the form of percentage survival cells versus concentration of extract employed. The microplate absorbance readings were converted into percentage survival by considering vehicle control (DMSO) absorbance as 100% viable cells.

3.7.1 Cell culture and treatments

Cancer cell lines A-549, H-460 and MCF-7 were used to establish the anti-proliferative potential of the extracts using *in-vitro* MTT assay. The cell lines were grown in DMEM media supplemented with 10% fetal bovine serum (FBS), 50 U/ml peniciliin G, and 50 µg/ml streptomycin sulphate. The cells were cultured at 37^{o}C with 5% CO_2 and 95% humidity conditions. Approximately, 8000 cells per well of 96 well plate were seeded and the plate was then cultured properly for 24h. After the incubation period of 24 hour the cells were treated with extracts in triplicate with concentration of 10 µg/ml, 50 µg/ml and 100 µg/ml and cells were incubated for 48 h. After treatment media was discarded, cells were washed with 1x PBS and mixed with 100 µl per well of MTT (5 mg in 10 ml of 1x PBS) and incubated at room temperature in dark for 4 h to let production of formazon crystals. Finally added 100 µl of DMSO in each well to dissolve the formazon crystals followed by analysis using microplate reader at 570 nm. Results were then plotted in graphs to calculate cytotoxic potential and IC_{50} values.

3.8 Isolation and characterization of compounds

Isolation of molecules was performed with the help of column chromatography. The characterizations were done using NMR and MASS spectroscopy. Furthermore structure of MVR-5 has been confirmed using chemical degradation methods. MVR-5 is degraded using periodate oxidation and degraded product is characterized using MASS spectroscopy

3.8.1 Isolation

Column chromatography technique was used for the isolation of compounds. TLC of all five extracts was checked using appropriate mobile phase. SGs were not visible in UV chamber so to visualize the presence of SGs five percent sulfuric acid in water was sprayed on TLC and put it on hot air oven at temperature of 100 ^{o}C for half an hour. Results indicated that aqueous methanolic extract (MVE-4) and water extract (MVE-5) have abundance of SGs. MVE-4 was chosen for further isolation. Two hundred grams of extract was subjected to pre-column treatment for the enrichment of SGs using gradient method by increasing solvent polarity with the adjusting percentage of chloroform and methanol in mobile phase. Polarity used ranges from pure chloroform to pure methanol and finally by aqueous methanol (20% water in methanol). On the basis of TLC observations all the fractions are mixed accordingly to get two fractions. A fraction among the two having maximum SGs contents was selected for column chromatography. Fraction was dried and weighted (108 g) and subjected to column chromatography (Column 1). Elution was done using gradient elution technique with increasing order of polarity by adjusting chloroform and methanol concentration in mobile phase. Elution was started from pure chloroform and ultimately by 100% methanol. The column was further eluted using 30% water in methanol. Various fractions were subjected to TLC observation and mixed accordingly. Some fractions of elute at 25% methanol in chloroform and 35% methanol in chloroform was selected, dried and weighted to get dry fraction of 10 gram. It was subjected to another column (column 2) for further separation. The column was eluted using gradient elution method just similar as implemented in column 1. Elute from column 2 was observed using TLC and mixed accordingly and a mixture of fraction was dried (1.2 gram) and subjected to column 3 which was further eluted using similar gradient elution technique as used in column 1 and 2. TLC observation is done and fractions were mixed accordingly. A fraction of mixture of two compounds was dried to get 400 mg of fraction which was subjected to column 4. Using the same gradient elution technique two compounds **MVR-1 (60 mg)** and **MVR-2 (7 mg)** have been isolated from column 4. The compound MVR-1 was pale yellow in colour which was visible in 256 nm wavelength in UV

chamber. MVR-2 was greenish coloured semisolid compound which was visible in 365 nm wavelength in UV chamber. Fractions of column 2 eluted at polarity range of 20% and 30% methanol in chloroform have been dried. Dissolved in methanol and finally recrystalized to get a pure compound **MVR-3 (20 mg)** which is little off white colour and TLC is invisible in UV chamber and can be seen by spraying 5% aqueous sulphuric acid in TLC and putting them in hot air oven at 100 ^{o}C for half an hour. A fraction of column 1 eluted at polarity of 25% methanol in chloroform have been dried and dissolved in methanol and then recrystallized to get another compound **MVR 4** (15 mg). It was also white coloured compound not visible in UV chamber and spot on TLC can be seen by spraying dilute sulphuric acid ad putting TLC in hot air oven at temperature of 100 ^{o}C for half an hour. Three fractions of 250 ml eluted from column 1 at 35% methanol in chloroform was dried and then dissolved in methanol and set for recrystallization. Compound **MVR 5** was crystallized in this fraction which was further recrystallized to get pure compound (3.5 g). The compound appears as clear white powder. TLC spot is invisible in UV chamber but spot can be seen by spraying dilute sulphuric acid and putting TLC in hot air oven for about half an hour. Fractions eluted from column 1 at the polarity of 50% chloroform in methanol were dried and weighted (6 g) and subjected to column 5. The column was eluted using gradient elution technique using mixture of ethyl acetate and methanol as solvent starting from pure ethyl acetate and subsequently increasing the concentration of methanol and ultimately by pure methanol. Fractions eluted at polarity of 100% ethyl acetate were checked using TLC one compound **MVR-6** (15 mg) has been obtained from these fractions. This compound is light blue in colour and visible in 254 nm wavelength in UV chamber. Fractions eluted at 15-20% methanol in ethyl acetate was concentrated and set for crystallization. A compound **MVR-7** (15 mg) which is dark blue in colour got precipitated. Thus total seven compounds have been isolated and among them three are most likely to be SGs as per TLC absorption and four seems to be non-SGs compounds. List of all the compounds isolated from aqueous methanol extract of *S. rebaudiana* along with total isolated amount have been represented in Table 4.

Table 4. Isolated compounds from of aqueous methanol extract of *S. rebaudiana.*

Sr. No	Compounds	Amount	% W/W*	Rf value	TLC solvent system	Characterization
1	MVR-1	60mg	$14*10^{-6}$	0.79	EtOAc:EtOH: H_2O (8:2:1.2)	YES
2	MVR-2	7mg	$1.6*10^{-6}$	0.65	EtOAc:EtOH: H_2O (16:2:1.2)	NO (Less amount)
3	MVR-3	20mg	$4.6*10^{-6}$	0.68	EtOAc:EtOH: H_2O (8:2:1.2)	NO (Less amount)
4	MVR-4	15mg	$3.5*10^{-6}$	0.75	EtOAc:EtOH: H_2O (8:2:1.2)	NO (Less amount)
5	MVR-5	3.5g	$0.81*10^{-6}$	0.58	EtOAc:EtOH: H_2O (8:2:1.2)	YES
6	MVR-6	15mg	$3.5*10^{-6}$	0.73	MeOH:EtOAc (1:5)	NO (Less amount)
7	MVR-7	15mg	$3.5*10^{-6}$	-	-	NO (Less amount)

EtOAcA = Ethyl acetate, EtOH = Ethyl alcohol, MeOH = Methanol, H_2O = Water; * Weight of compound isolated/Weight of plant used in extraction * 100

3.8.2 Characterization of MVR-1

Melting point of MVR-1 was found to be in the range of 314-316^{o}C

^{1}H NMR of MVR-1 (PPM, 400 MHz, DMSO) 0.85 (d), 1.25 (s), 2.57 (d), 2.83 (s), 2.96 (s), 3.31 (m), 3.97 (m) 4.28 (s), 5.41 (s), 6.20 (s), 6.36 (d), 6.93 (d), 7.27 (d), 7.38 (s), 7.57 (d), 7.66 (d) 7.84 (s), 7.98 (s).

^{13}C NMR of MVR-1 (PPM, 100 MHz, DMSO) 93.41, 98.59, 101.34, 104.21, 115.12, 115.20, 120.91, 134, 144.75, 148.75, 156.42, 156.99, 161.98, 164.04, 177.678.

Some peaks of impurities at aliphatic region of ^{1}H and ^{13}C NMR where neglected.

ESI-MS of MVR-5: m/z [M] $^{+}$ 302.1 corresponding to molecular formula $C_{15}H_{10}O_{7.}$

3.8.3 Characterization of MVR-5

Melting point obtained 196-197^{o} C,

^{1}H NMR of MVR-5 (400 MHz, DMSO) showed three anomeric peaks at δ_{Hn} 5.2 (1H, d, *J*=5.5Hz), 4.34 (1H, d, *J*=5.64 Hz), 4.40 (1H, d, *J*=7.64 Hz) and δ^{13}C 94.1, 96.3 and 104.5, respectively.

^{13}C NMR peaks of MVR-5 in (PPM, 100 MHz, DMSO) 37.46, 38.88, 38.91, 39.09, 39.30, 39.51, 39.71, 43.16, 43.55, 46.84, 48.64, 53.13, 56.55, 60.58, 60.81, 61.12, 69.51, 69.70, 70.39, 72.44, 75.11, 75.98, 76.21, 76.71, 76.95, 77.46, 78.43, 78.76, 78.96, 79.09, 82.69, 84.73, 94.09, 96.29, 103.58, 104.66, 153.33, 175.64.

ESI-MS of MVR-5: m/z $[M+Na]^+$ calculated for $[C_{38}H_{60}O_{18}Na]^+$, 827.8620 corresponding to molecular formula $C_{38}H_{60}O_{18.}$.

3.8.3.1 Periodate oxidation of MVR-5

Additional Periodate oxidation of MVR-5 was done for the confirmation of structure. The solution of MVR-5 (25 mg) in dioxan-H_2O (1:1, 6 ml) was treated with 10% aq. $NaIO_4$ (5 ml) at room temperature for 18 hr. The solvent was evaporated at 50^o C under reduced pressure. The residue was dissolved in 5 % KOH in MeOH (5 ml) and refluxed for 2 h. After neutralization with acetic acid, solvent was removed under reduced pressure and the residue diluted with H_2O (5 ml) and extracted with Et_2O. The Et_2O extract was dried under reduced pressure at 50 ^oC. The degraded product of MVR-5 (MVR-5D) (10 mg) was separated by column chromatography by eluting with 5% MeOH in chloroform having mp 110-112 ^oC. The mass of degraded product was further obtained and m/z $[M+H]^+$ was found to be 319.2273.

3.9 *In-silico* study

3.9.1 Ligand preparation

All the compounds (Figure 8) used in *in-silico* study were drawn using ChemBio Draw Ultra-12.0 and saved in sdf format. Preparation of ligands for docking was done using LigPrep application of schrdinger suite 2013. LigPrep application of Schrodinger suite performs the basic functions such as addition of hydrogen atoms, removal of unwanted molecules such as water and small ions. Charged group neutralization, generation of ionization as well as tautomeric state using Epik, generation of stereoisomer if required, generation of low energy ring conformations, removal of any badly prepared structures and finally optimization of geometries. Thus LigPrep wizard produced the representative structural

model of actual ligand which can be used in docking study to get accurate results.

3.9.2 Homology modeling

Homology modeling of ATD of TIR2 and T1R3 subunits of STR had been constructed in the study. The basic steps performed here included getting amino acid sequence of T1R2 and T1R3 subunits, selection of templates, constructing model and finally model refinement.

3.9.2.1 Template selection

Sequences of T1R2 and T1R3 was retrieved from NCBI (Accession: XP_94400 NP_689418.27, GI: 112789566 and NP_689414.1, GI:91206396 respectively). The template required for the homology modeling was provided by the Protein Data Bank (PDB). A BLAST search of PDB database was done for the suitable templates of T1R2 and T1R3. The BLAST search results were further used to create a phylogenetic tree using ClustalW2 to determine the most suitable template for T1R2 and T1R3. A chain of the ligand binding domain of the Metabotropoc glutamate receptor mGluR5 complexed with glutamate (PDB ID 3LMK) which is closest to the sequences of T1R2 and T1R3 in the phylogenetic tree have suitable E-value and sequence identity of 25% with respect to T1R2 and 28% with respect to T1R3 was selected for homology modeling of both the subunits (Drobovetsky *et al.*, 2010).

3.9.2.2 Model building

Homology models of T1R2 and T1R3 were constructed using prime-v34012 module of Schrodinger LLC (NY, USA) which is an automated protein modeling tool. The primary sequence of T1R2 and T1R3 in FASTA format was imported into structure prediction wizzard present in Prime tool. BLAST search was performed and A chain of 3LMK being closest in phylogenetic tree of TIR2 and T1R3, having suitable E-value and having sequence identity of 25% and 28% respectively is selected for homology modeling. Thus using A chain of 3LMK, initial target-template alignment was generated and subsequently secondary structure prediction was performed by secondary structure prediction module (SSP) integrated with prime in order to introduce minimal gaps and optimizing

initial alignment. Prime structure prediction module is provided with two model building methods and we have selected energy based method to build the models. The generated model was further subjected to loop and side chain optimization. The restrain minimization of final models of T1R2 and TIR3 was done using protein preparation wizard using OPLS_2005 force field with default cutoff root mean square distance (RMSD) of 0.30 Å.

3.9.2.3 Model validation

Final homology models T1R2 and T1R3 were evaluated using RAMPAGE (Ramachandran plot) (Lovell *et al.*, 2003), PROCHECK (overall average G factor) (Laskowski *et al.*, 1993) and ERRAT web servers (overall quality factor) (Colovos and Yeates, 1993; Paital *et al.*, 2013).

3.9.3 Protein preparation

The X-rays crystal structure of EGFR kinase domain in complex with iressa (PDB ID 2ITY) has been obtained from protein data bank (http://www.rcsb.org/pdb). It was lung-cancer derived mutated EGFR protein which is generally responsible for the cause of non-small-cell lung cancer (Yun *et al.*, 2007). Crystal structure of P13K-gamma in complex with quinoline inhibitor (PDB ID 3S2A) and crystal structure of mTOR in complexes with rapamycin (PDB ID 4DRI) have also been downloaded from protein data bank (März *et al.*, 2013). For the preparation of proteins 'protein preparation wizard' application of Schrödinger suite 2013 have been used. Protein preparation process consists of two steps which include preparation and refinement. Before refinement water molecule were deleted, addition of hydrogen atoms, bond order assigning, creation of disulfide bonds and zero order bonds to metal and charge fixation were incorporated into the raw PDB protein structures. Afterward refinement component was performed in which restrained impact minimization of target protein takes place where the side chains were reoriented and steric clashes were revealed and made the protein ready to dock. .

3.9.4 Receptor Grid generation

Fully prepared proteins (2ITY, 3S2A and 4DRI) and homology models of T1R2 and T1R3 were used for grid generation. Ligand docking cannot be performed

without the generation of receptor grid. In case of 2ITY, 3S2A and 4DRI the grid were generated at the place of co-crystallized ligand and in case of homology models of T1R2 and T1R3 subunits the site map program of Schrödinger 2013 was run to find out all the possible active sites. It had provided multiple possible active sites. Grids were generated on all the sites and docking was performed on all of them.

3.9.5 Glide Docking

Docking were performed using glide docking module (Grid Based Ligand Docking from Energetics, from Schrödinger LLC) present is Schrödinger suite 2013. There are three docking precision available with Schrödinger glide docking which include High throughput virtual screening (HTVS), standard precision (SP) and extra precision (XP). The HTVS is suitable for screening of millions of compounds, have limited number of conformational search and is fast, SP is suitable for thousands of compounds and has better coverage of conformational space and XP is suitable for tens or hundreds of compounds and most accurate among all of them. We have used XP precision in our study. In case of 2ITY, 3S2A and 4DRI various compounds isolated from *S. rebaudiana* was searched in literature and docking was done in grids generated at the place of co-crystallized ligand. In case homology models of T1R2 and T1R3 only SGs of known sweetness profile were docked on all the generated grids and results were analyzed for best agreement with known experimental sweetness profile of various SGs also taking in account the better rank order of active site as predicted by site map program.

3.9.6 QikProp

QikProp is an application present in Schrödinger suite 2013 which is quick, accurate and easy to use method for estimating absorption, distribution, metabolism and excretion (ADME) property of chemical compounds. It predicts physically significant descriptions and pharmacologically relevant properties of organic molecules. Using this module we have calculated ADME properties of chemical compounds of *S. rebaudiana* used in the study

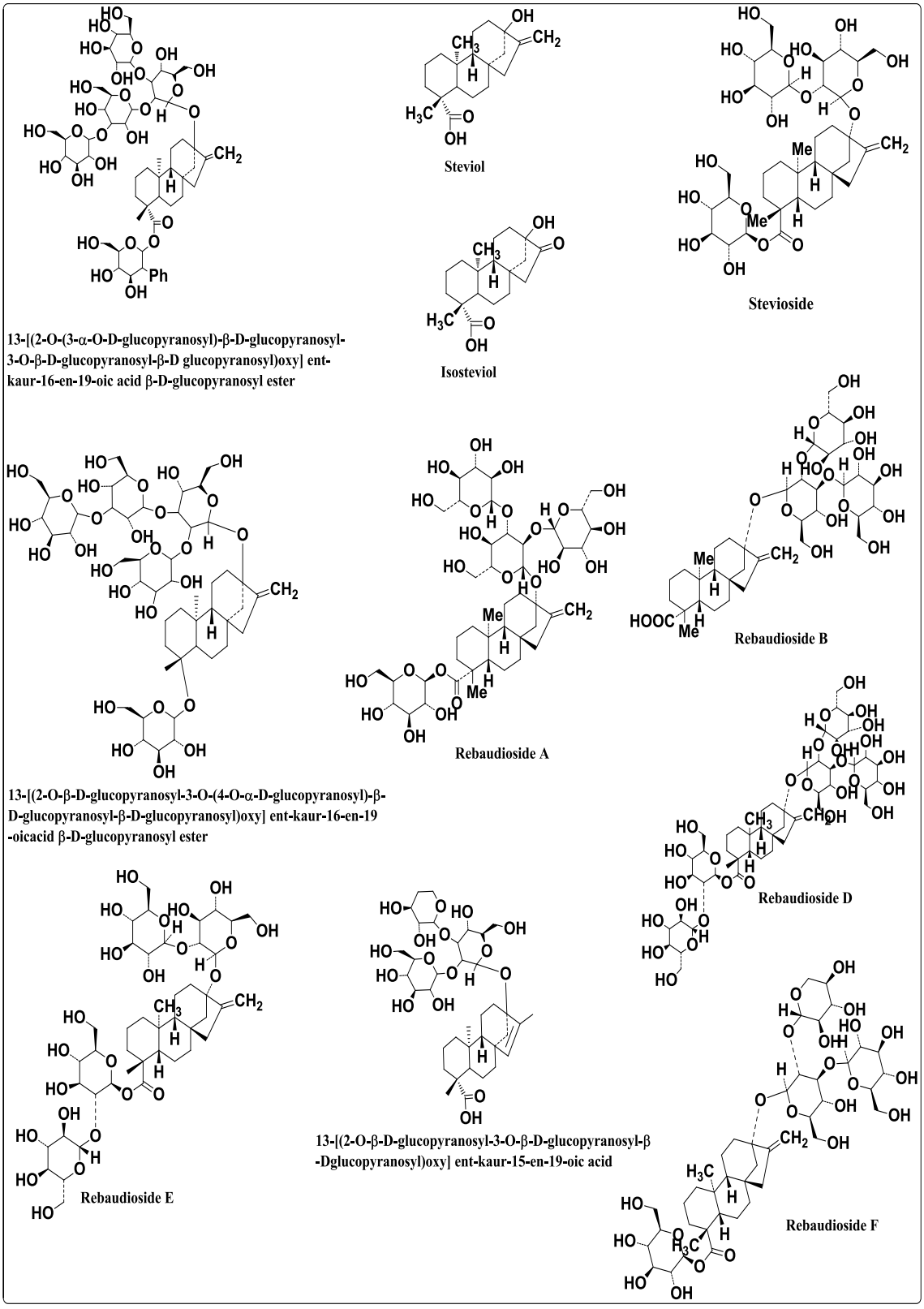


Figure 8. Ligands used in *in-silico* study

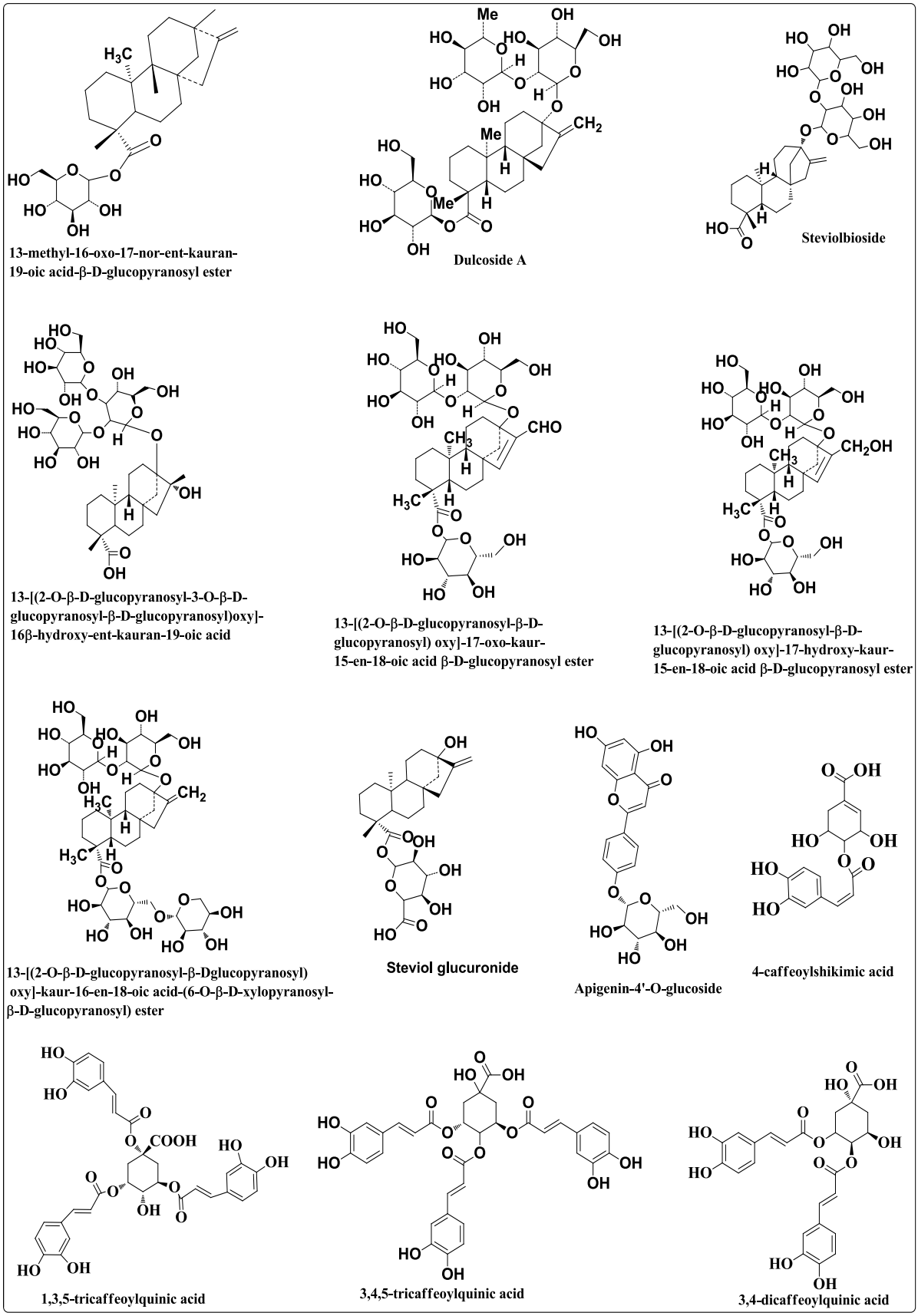


Figure 8. Continued

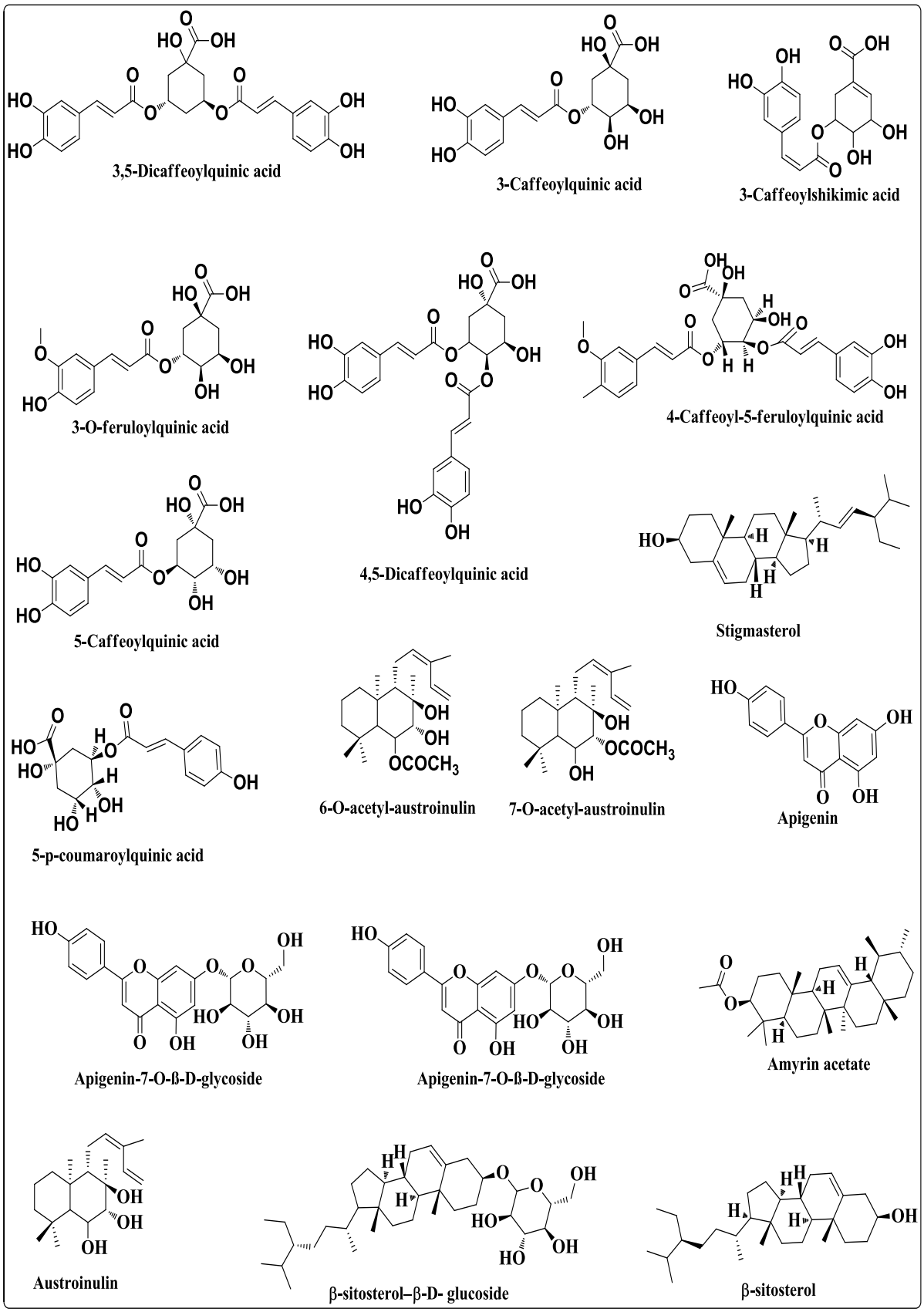


Figure 8. Continued

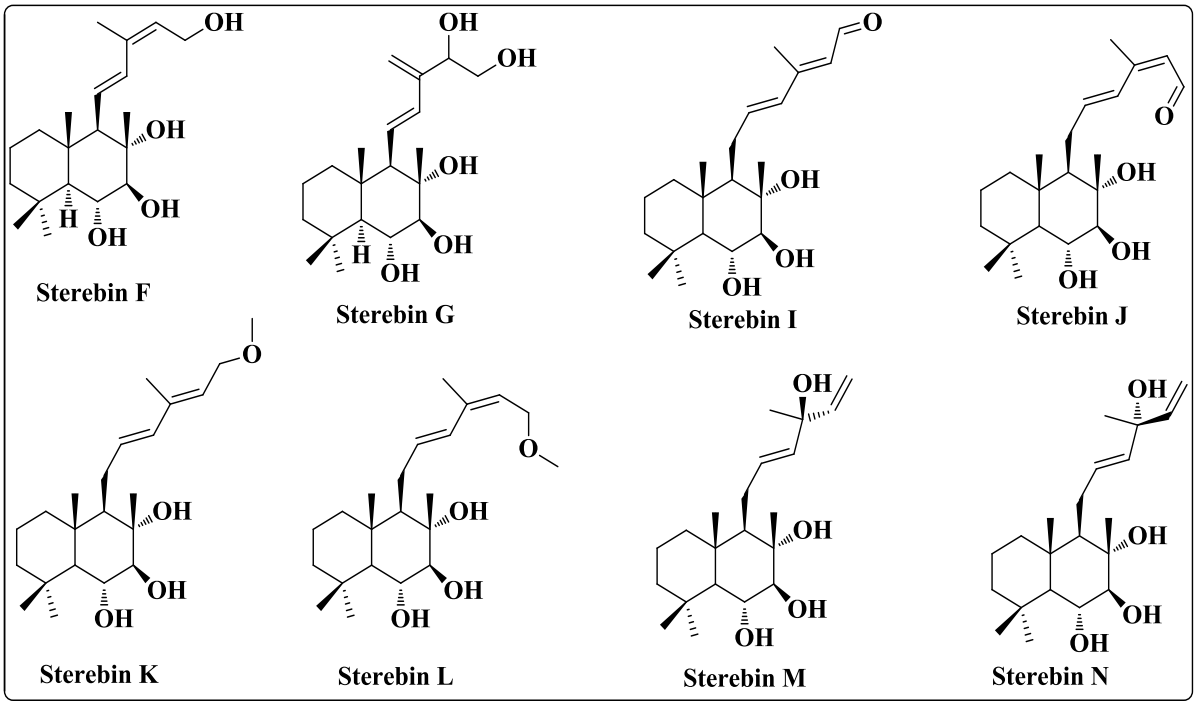


Figure 8. Continued

CHAPTER 4

RESULT AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

Part 1

4.1 *In-vitro* anticancer activity

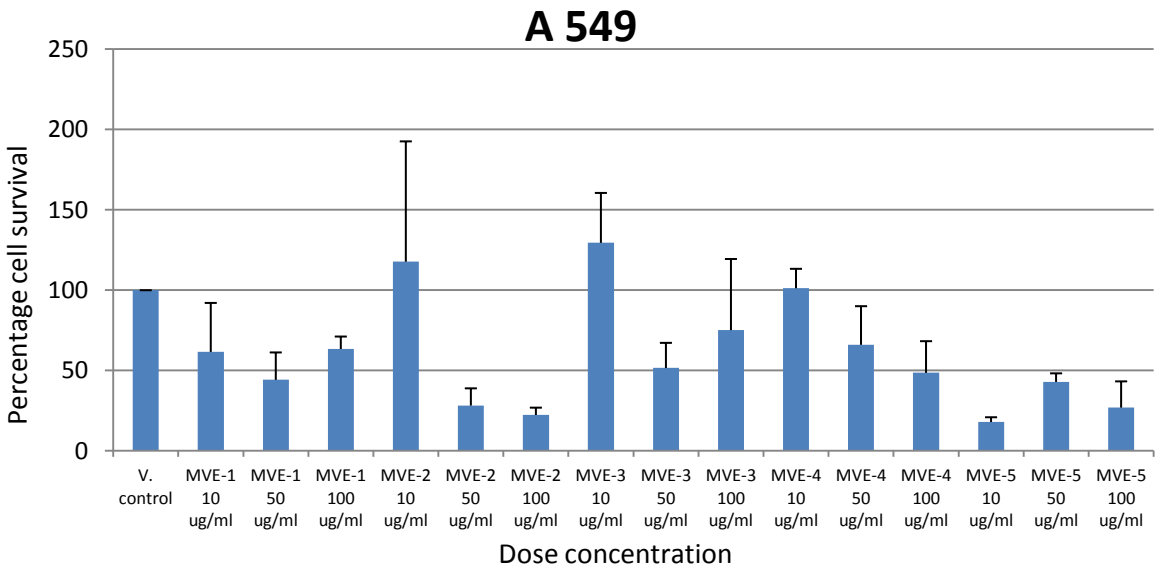
In order to evaluate the anticancer potential of plant extracts of *S. rebaudiana,* MTT assay was performed using multiple cell lines. The cell lines used in our study include non-small cell lung cancer cell lines (A-549 and H-460) and breast cancer cell lines (MCF-7). Approximately 8,000 cells were taken in each well of 96 well plate and further treatment was given as per protocol mentioned in material and method section. The cells were treated with extracts in triplicate of concentration and the experiment was also repeated three times. The results of MTT experiment indicated that the plant *S. rebaudiana* has significant anticancer potential against A-549, H-460 and MCF-7 cell lines.

In A-549 cell lines MVE-5 and MVE-2 showed excellent anticancer potential. MVR-5 was showing good anti-proliferative activity at the dose of 10 µg/ml, 50 µg/ml as well as 100 µg/ml, which was best among all the extracts. Unusual increased survival of cell lines at dose of 50 µg/ml and 100 µg/ml compared to 10 µg/ml of the dose has been observed which might be due some compound in extract with proliferative activity, which slightly dominates at higher dose of 50 µg/ml and 100 µg/ml compared to 10 µg/ml of dose. MVE-2 also showed dose dependent antiproliferative potential which is significantly better at 50 µg/ml and 100 µg/ml of dose. MVE-4 showed significant and dose dependent activity which is only reliable at higher dose concentration. MVE-1 showed best anticancer potential at 50 µg/ml of concentration. The anticancer potential of various extracts against A-549 cell line is represented in Figure 9 (a).

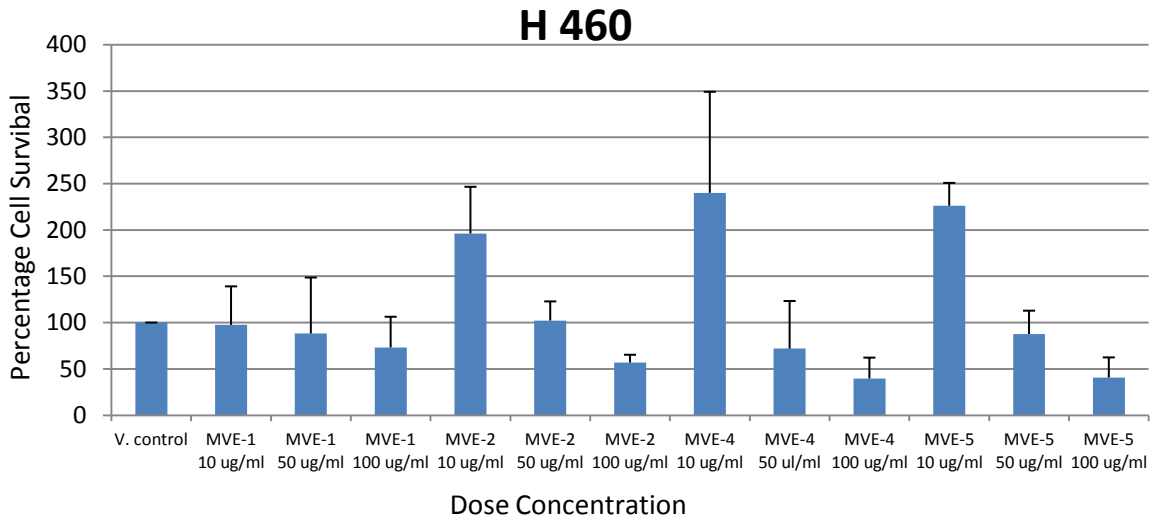
In H-460 cancer cell line MVE-5 and MVE-4 have shown promising anticancer potential at 50 µg/ml and 100 µg/ml of concentration, although at lower dose of 10 µg/ml the proliferative activity has been observed. There might be an interference of multiple components in the extracts having proliferative as well as antiproliferative potential and at lower dose proliferative factors is

dominating. Moreover significant dose dependency has been observed in case of MVE-2, MVE-4 and MVE-5. MVE-1 have shown moderate inhibitory potential at higher concentration which is represented in Figure 9 (b).

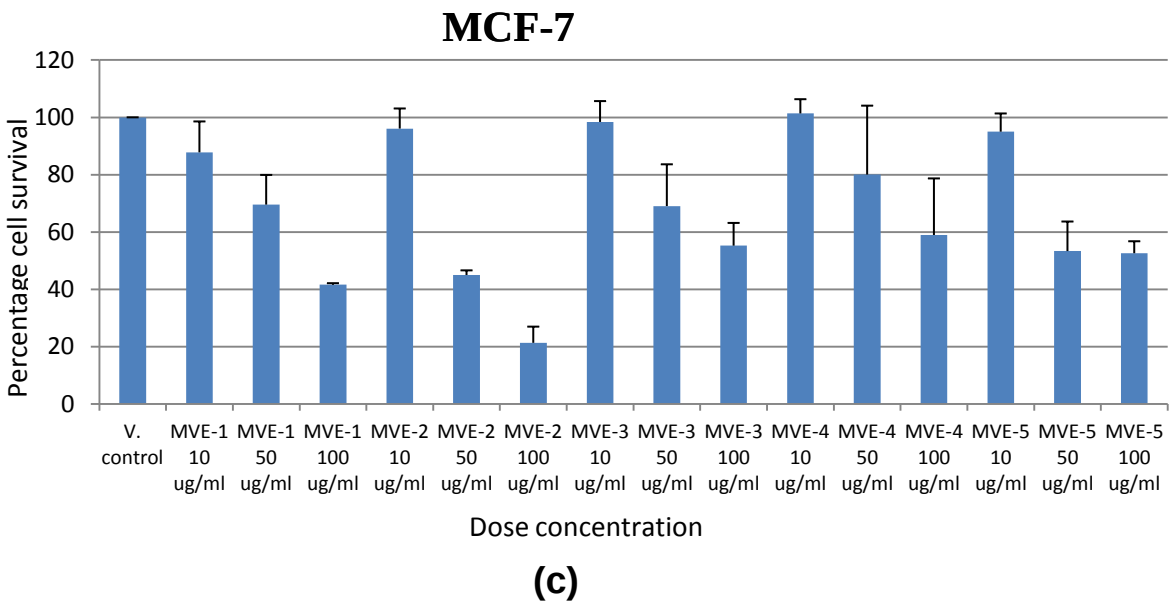
In MCF-7 cell line, MVE-2 showed dose dependent antiproliferative activity. Comparatively better antiproliferative activity was observed at 50 µg/ml and 100 µg/ml of dose. MVE-1 also showed good and dose dependent antiproliferative potential which is significant at higher dose. The activity associated with MVE-3 and MVE-5 was slight and may be significant only at higher concentration. The anticancer potential of various extracts against MCF-7 cell line is represented in Figure 9 (c).



(a)



(b)



(c)

Figure 9. Bar graphs of cytotoxicity potential of *S. rebaudiana* against A549 lung adenocarcinoma cell line **(a)**, H460 large cell lung carcinoma cell line **(b)** and MCF-7 breast cancer cell line **(c)**. It is depicting percentage survival (% viability) of cell at different concentrations of the extracts in comparison to vehicle control.

Furthermore, IC_{50} value of extracts against all the three cell lines has been calculated which is shown in Table 5. The IC_{50} value indicated that in case of A-549 cell lines, MVE-5 have shown best antiproliferative activity and was found to be less than 10 µg/ml. Moreover, IC_{50} values of MVE-2 was also comparatively better and found to be less than 50 µg/ml and MVE-4 had shown IC_{50} value of 90 µg/ml. In case of H-460 reasonably better IC_{50} have been observed for MVE-4 and MVE-5 which is 88 µg/ml and 92 µg/ml respectively. Thus for both the lung cancer cell lines high polarity solvent extracts of *S. rebaudiana* have shown comparatively higher antiproliferative effects. In case of MCF-7 breast cancer cell lines, MVE-1 and MVE-2 have shown reasonable IC_{50} value of 90 µg/ml and 53 µg/ml respectively. Thus in breast cancer cell lines, non polar extracts have shown comparatively good antiproliferative potential. Overall comparison indicated that antiproliferative potential of *S. rebaudiana* is associated with MVE-1, MVE-2, MVE-4 and MVE-5 extracts which may contain multiple moieties which produce antiproliferative effects by different mechanism of actions. Thus *S. rebaudiana* seems to be a good source of antiproliferative

agents and can be explored further for the search of some new anticancer lead molecules.

Table 5. IC_{50} values of various extracts as examined by MTT anticancer *in-vitro* assay

Sr. no	Cell line	Extract	IC_{50}
1	A-549	MVE-1	<50 µg/ml
		MVE-2	<50 µg/ml
		MVE-4	90 µg/ml
		MVE-5	<10 µg/ml
2	H460	MVE-4	88 µg/ml
		MVE-5	92 µg/ml
3	MCF-7	MVE-1	90 µg/ml
		MVE-2	53 µg/ml

4.2 *In-silico* anticancer study

Preliminary screening for anticancer property of *S. rebaudiana* extracts by MTT assays using A-549, H-460 non-small cell lung cancer cell lines and MCF-7 breast cancer cell line indicated that there is significant anticancer potential associated with it. Thus screening of associated compounds seems to be a reasonable way to search some newer anticancer lead compound. Literature survey have been done for the search of various compounds isolated from *S. rebaudiana* (Chaturvedula, Clos*, et al.*, 2011; Chaturvedula, Upreti*, et al.*, 2011; Prakash Chaturvedula*, et al.*, 2011; Wölwer-Rieck, 2012). Among various anticancer target proteins, epidermal growth factor receptor (EGFR) is an important target which is expressed in more than 60% of non–small cell lung carcinomas (da Cunha Santos *et al.*, 2011). Moreover, EGFR signalling is highly involved in the development of BC as well as resistance towards chemotherapeutic agents (Navolanic *et al.*, 2003). PI3K and mTOR proteins work in synchronized manner with EGFR and are also involved in lung cancer and breast cancer (Wong *et al.*, 2010). Taking in consideration above mentioned facts as well as anticancer activity shown by *S. rebaudiana* against

lung cancer and breast cancer cell lines, docking study was performed on EGFR, PI3K and mTOR receptors. Anticancer drug candidates were identified by glide docking of compounds of *S. rebaudiana* on all the three receptors and taking in account the best docked compounds on the basis of lowest Glide score. For the comparison purpose standard inhibitors drug of each receptor was also incorporated in the docking study along with test compounds. Gefitinib, which is used as standard control for EGFR receptor in docking study is a well established for its activity against non-small cell lung cancer as well as breast cancer (Nakamura et al., 2005; Xu et al., 2011). PI3k inhibitors have shown good anticancer activity against lung cancer and breast cancer (Baselga, 2011; Lee et al., 2013). Thus the standard control inhibitor used in the *in-silico* study for PI3k is N-[2-chloro-5-(4-morpholin-4-ylquinolin-6-yl)pyridin-3-yl]-4-fluorobenzenesulfonamide (CID_44608915). Furthermore everolimus which is used as standard control for mTOR receptor is also well known for its activity against non-small cell lung cancer as well as breast cancer (Baselga et al., 2012; Gazdar, 2009)

4.2.1 EGFR receptor

13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β- D-glucopyranosyl) ester which is a SG have shown very good interaction pattern with EGFR (2ITY) receptor. It's Glide score (G score), lipophilicity, H- bond and electrostatic energies were found to be -14.04, -2.84,-9.33 and -2 kcal/mol respectively. The interaction pattern is represented in Figure 10 in which majority of amino acid residues interact with hydroxyl group of ligand by forming hydrogen bond. The main residues showing interaction with ligand include Lys 745, Asp 837, Asp 855, Cys 797 and ASP 800. Next in the rank order according to G score was 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-D-glucopyranosyl ester which showed G score lipophilicity, H- bond and electrostatic energie of -13.46, -3.98 , -7.59 and -2 Kcal/mol respectively. The main interacting amino acids were Gly 719, Lys 745, Glu 758, Gly 762 and Cys 797. All the amino acids interact in the form of hydrogen bonding with hydroxyl group of glucose molecules. On the basis of G score the next compound with higher G score was found to be rebaudioside A having G score, lipophilicity, H-

bond and electrostatic energies of -12.88, -3.55, -7.98 and -2 respectively. Next to rebaudioside A the compounds with higher G score were found to be 13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopy ranos yl) oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester and 13-[(2-O-β-D- glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid- β-D-glucopyranosyl ester respectively. The G score, lipophilicity, H-bond and electrostatic energies of top ranked standard EGFR inhibitor gefitinib was found to be -9.05, -5.03, -2.03 and -0.95 kcal/mol respectively which is comparatively much higher than top ranked compounds. Thus on the basis of G score top five ranks was found to be hold by SGs in case of EGFR receptor. The G score, lipophilicity, H- bond and electrostatic energies of all the compounds included in the study are shown in Table **6**.

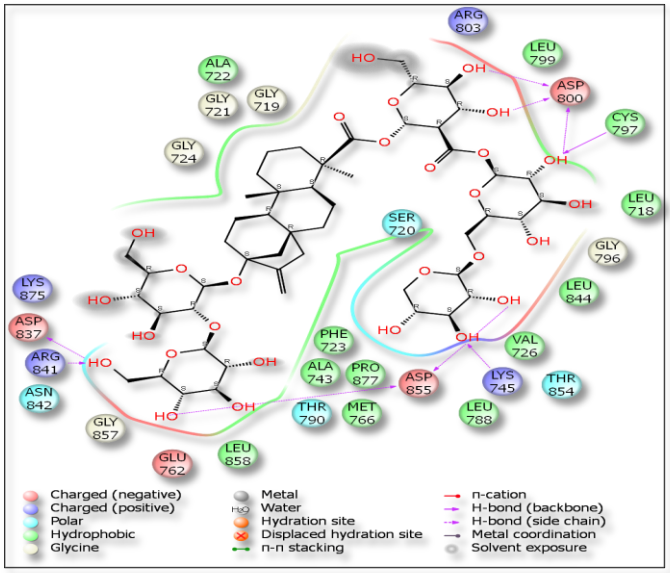


Figure 10. Interaction pattern of 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β- D-glucopyranosyl) ester with EGFR receptor(PDB ID 2ITY) showing best G score

Table 6. G. score, lipophilicity, hydrogen bond and electrostatic energies of various compounds isolated from *S. rebaudiana* docked with EGFR (PDB ID 2ITY)

Ligand	G Score (Kcal/mol)	Lipophilic EvdW	H_{Bond} (Kcal/mol)	E_{Elec} (Kcal/mol)
13-[(2-O-β-Dglucopyranosyl-β-D-	-14.04	-2.84	-9.33	-2

glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β- D-glucopyranosyl) ester				
13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-D-glucopyranosyl ester	-13.46	-3.98	-7.59	-2
Rebaudioside A	-12.88	-3.55	-7.98	-2
13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester	-12.81	-2.22	-8.95	-2
13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid- β-D-glucopyranosyl ester	-12.54	-3.68	-6.75	-2
Quercetin 3-glucoside	-11.56	-4.03	-5.5	-2
Stevioside	-11.45	-3.12	-6.11	-2
Quercetin-3-O-rutinoside	-11.44	-4.46	-5.47	-1.61
Rebaudioside F	-11.36	-2.23	-7.41	-2
Rebaudioside D	-11.23	-2.37	-7.32	-2
Dulcosidea A	-10.97	-2.52	-6.72	-2
Rebaudioside B	-10.92	-1.97	-7.71	-2
Quercetin	-10.9	-3.72	-4	-1.88
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl)oxy]-16ß-hydroxy-ent-kauran-19-oic acid	-10.84	-1.76	-7.19	-2

1,3,5-tricaffeoylquinic acid	-10.59	-3.37	-4.72	-2
Luteolin-7-O-glucoside	-10.52	-3.94	-3.8	-2
Luteolin	-10.48	-3.76	-3.75	-1.64
Apigenin-7-O-ß-D-glycoside	-10.32	-4.5	-4.11	-1.12
Quercetin-3-O-ß-D-rhamnoside	-9.63	-3.93	-4.51	-1.54
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-Dglucopyranosyl)oxy] ent-kaur-15-en-19-oic acid	-9.43	-2.3	-5.41	-1.91
Quercetin-3-O-ß-D-arabinoside	-9.13	-3.78	-3.62	-1.92
3,4,5-tricaffeoylquinic acid	-9.05	-3.46	-4.67	-1.76
3,4-dicaffeoylquinic acid	-8.86	-3.19	-4.22	-1.6
4,5-dicaffeoylquinic acid	-8.86	-3.19	-4.22	-1.6
3,5-dicaffeoylquinic acid	-8.69	-3.41	-4.34	-1.82
Apigenin	-8.65	-3.83	-2.22	-1.3
Gefitinib*	-9.05	-5.03	-2.03	-0.95
4-caffeoylquinic acid	-8.56	-3.22	-3.53	-0.74
4-caffeoyl-5-feruloylquinic acid	-8.55	-3.33	-3.96	-1.86
Rebaudioside E	-8.54	-2.25	-4.62	-2
Gefitinib*	-8.76	-5.67	-1.21	-0.48
Steviolbioside	-8.19	-2.49	-4.32	-1.43
5-caffeoylquinic acid	-8.14	-2.81	-3.34	-1.16
5-p-coumaroylquinic acid	-8.12	-2.92	-3.15	-1.07
Sterebin G	-7.99	-2.21	-4.72	-1.04
Steviol glucuronide	-7.86	-2.61	-3.68	-1.18
3-caffeoylshikimic acid	-7.76	-1.69	-3.99	-2

Apigenin-4'-O-glucoside	-7.2	-3.27	-2.65	-1.43
4-caffeoylshikimic acid	-6.89	-2.97	-2.31	-1.24
Kaempferol-3-O-rhamnoside	-6.87	-3.86	-1.78	-1.68
Luteolin-7-O-glucoside	-6.82	-3.87	-3.32	-2
7-O-acetyl-austroinulin	-6.44	-2.79	-2.88	-0.92
Apigenin-7-O-ß-D-glycoside	-6.13	-4.23	-3.7	-1.59
Sterebin L	-6.06	-3.01	-2.39	-0.78
Sterebin F	-6.06	-3.31	-1.92	-0.66
Sterebin M	-5.97	-2.66	-2.15	-1.09
Sterebin N	-5.97	-2.66	-2.15	-1.09
Sterebin J	-5.96	-2.6	-2.17	-1.03
Sterebin E	-5.82	-3.23	-2.1	-0.51
β-sitosterol-β-D glucoside	-5.72	-4.42	-1.9	-0.54
Luteolin	-5.68	-3.82	-2.07	-1.47
Steviamine	-5.56	-1.46	-2.59	-1.22
Sterebin C	-5.52	-2.63	-2.33	-0.3
Sterebin K	-5.36	-2.69	-1.92	-0.77
Sterebin B	-5.25	-2.68	-1.53	-0.85
Sinapic acid	-5.12	-2.23	-1.31	-0.98
6-O-acetyl-austroinulin	-5.03	-4.17	-0.69	-0.07
Sterebin A	-4.96	-2.44	-1.44	-0.62
Steviamine	-4.95	-1.27	-2.65	-0.86
Sterebin D	-4.68	-2.13	-1.3	-0.85
Sterebin I	-4.68	-1.8	-2.04	-0.86
Stigmasterol	-4.55	-4.26	-0.35	-0.18
13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid-ß-	-4.53	-2	-1.44	-1.07

D-glucopyranosyl ester				
Isosteviol	-4.19	-2.83	-0.57	-0.1
Jhanol	-3.96	-3.23	-0.35	-0.23
Steviol	-3.61	-2.66	-0.02	-0.22
Campesterol	-3.35	-4.16	-0.35	-0.21
β-sitosterol	-3.19	-3.55	0	0.05
Lupeol-3-palmitate	-0.53	-5.05	0	-0.03

* Pubchem ID/name of standard EGFR inhibitors used in the study;

4.2.2 PI3K-Gamma receptor

In PI3K-Gamma receptor (PDB ID 3S2A) quercetin-3-O-rutinoside have shown best binding affinity in the form of G score. G score, lipophilicity, H- bond and electrostatic energies were found to be -13.3, -4.8, -5.83 and -2 Kcal/mol respectively. The main interaction pattern included stacking interaction and hydrogen bonding which is shown in Figure 11. Trp 812 showed π–π stacking interaction with ring A of ligand. Moreover ring C showed π–π stacking interaction with Tyr 867. Ser 806, Val 882, Asn 951, Hip 967 and Asp 964 amino acid residues showed hydrogen bonding of various hydroxyl groups of ligand

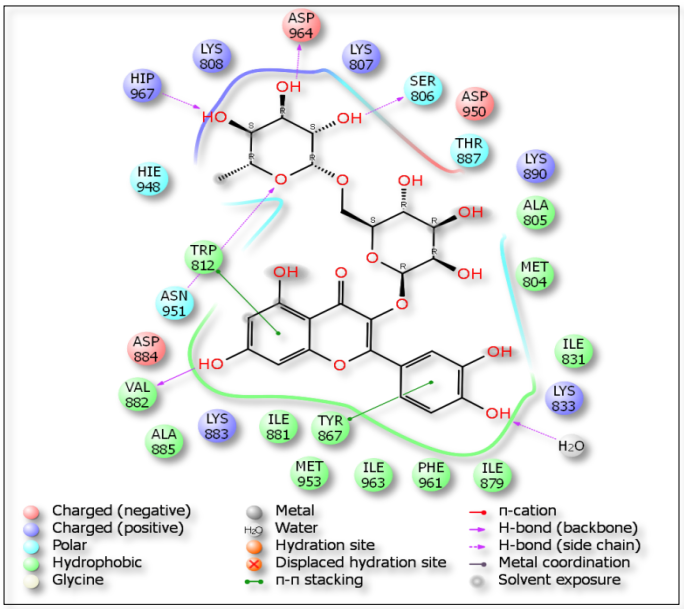


Figure 11. Interaction pattern of quercetin-3-O-rutinoside with PI3K-Gamma receptor (PDB ID 3S2A)

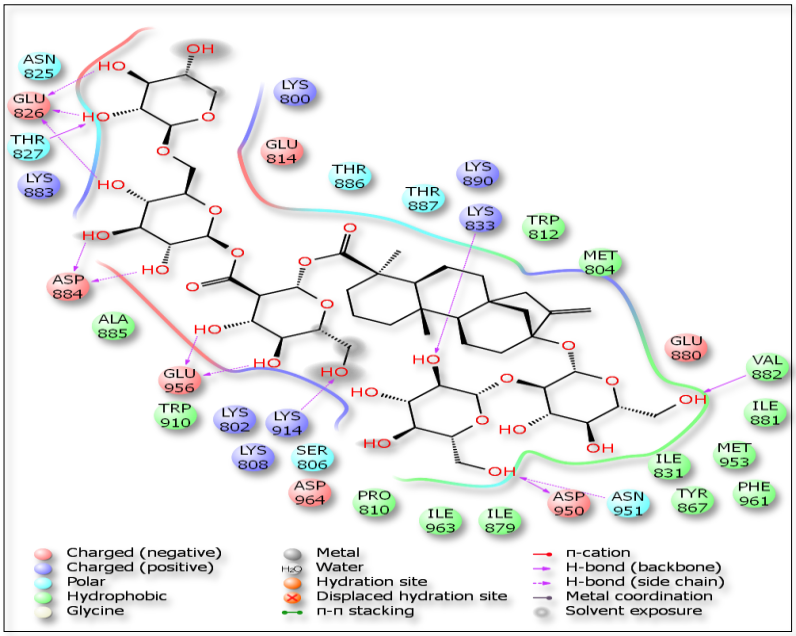


Figure 12. Interaction pattern of SG 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester with PI3K-Gamma receptor (PDB ID 3S2A)

Subsequently SG 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester have shown G score lipophilicity H-bind and electrostatic energy of -13.2, -3.85, -8.3 and -2 Kcal/mol respectively which is almost same to best docked compound quercetin-3-O-rutinoside with G score of -13.3 Kcal/mol. The main interactions (Figure 12) include H-bonding of hydroxyl group of ligand with amino acid residues Glu 826, Thr 827, Asp 884, Glu 956, Lys 914, Asp 950, Asn 951, Val 882 and Lys 833. The next compound showing good docking result was Luteolin-7-O-glucoside showing G score lipophilicity H-bind and electrostatic energy of -12.4, -4.47, -5.63 and -1.53 Kcal/mol respectively. Subsequently SGs rebaudioside D and rebaudioside E have shown good docking results. Rebaudioside D have shown G score lipophilicity H-bind and electrostatic energy of -12.38, -2.47, -7.99 and -2 Kcal/mol respectively and rebaudioside E have shown G score lipophilicity H-bind and electrostatic energy of -12.15, -4.21, -6.31 and -1.8 Kcal/mol respectively. The G score, lipophilicity, H-bind and electrostatic energy of top raked standard inhibitor (Pubchem ID CID_44608915) was found to be -5.52, -5.92, -1.69 and -0.98 Kcal/mol which is much higher than top ranked compounds as per G score. Thus SGs have

shown significant binding affinity towards receptor PI3k-gamma (3S2A). The overall result of various compounds docked with 3S2A receptor is as represented in table 7.

Table 7. G. score, Lipophilicity, Hydrogen bond and electrostatic energies of various compounds isolated from *S. rebaudiana* docked with PI3K (PDB ID 3S2A)

Ligand	**GScore (Kcal/mol)**	**LipophilicEvdW**	**HBond (Kcal/mol)**	**E_{elec} (Kcal/mol)**
Quercetin-3-O-rutinoside	-13.3	-4.8	-5.83	-2
13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester	-13.2	-3.85	-8.3	-2
Luteolin-7-O-glucoside	-12.4	-4.47	-5.63	-1.53
Rebaudioside D	-12.38	-2.47	-7.99	-2
Rebaudioside E	-12.15	-4.21	-6.31	-1.8
13-[(2-O-β-D-glucopyranosyl-β-D glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-Dglucopyranosyl ester	-11.95	-3.25	-6.53	-2
Rebaudioside A	-11.94	-2.85	-7.07	-2
3,5-dicaffeoylquinic acid	-11.87	-3.07	-5.9	-2
3,4-dicaffeoylquinic acid	-11.48	-4.36	-4.32	-1.86
4,5-dicaffeoylquinic acid	-11.48	-4.36	-4.32	-1.86
4-caffeoyl-5-feruloylquinic acid	-11.16	-3.41	-4.85	-1.58
Rebaudioside F	-10.93	-2.57	-6.86	-1.71

3-caffeoylquinic acid	-10.88	-2.84	-4.49	-2
13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester	-10.76	-2.92	-6.22	-2
Quercetin 3-glucoside	-10.68	-4.16	-4.76	-1.27
3,4,5-tricaffeoylquinic acid	-10.63	-3.7	-4.8	-1.42
Luteolin	-10.61	-3.17	-3.11	-1.02
Dulcosidea A	-10.57	-4.26	-5.25	-0.99
Luteolin-7-O-glucoside	-10.40	-3.49	-4.56	-1.9
Apigenin-7-O-ß-D-glycoside	-10.36	-4.45	-3.65	-1.5
Stevioside	-10.23	-3.02	-5.52	-1.78
Quercetin	-10.15	-4.64	-2.88	-0.42
3-O-feruloylquinic acid	-10.09	-3.41	-4.52	-1.68
Apigenin-7-O-ß-D-glycoside	-10.07	-4.72	-3.41	-1.16
1,3,5-tricaffeoylquinic acid	-10.06	-3.65	-4.15	-2
Luteolin	-10.05	-3.91	-3.03	-0.95
Quercetin-3-O-ß-D-rhamnoside	-9.85	-3.1	-3.62	-1.77
4-caffeoylshikimic acid	-9.77	-3.64	-3.19	-0.84
Apigenin	-9.74	-3.05	-2.11	-1.06
13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid β-D-glucopyranosyl ester	-9.64	-2.67	-5.04	-2

Kaempferol-3-O-rhamnoside	-9.58	-4.63	-3.46	-0.95
Quercetin-3-O-ß-D-arabinoside	-9.55	-3.57	-3.74	-1.74
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl)oxy]-16ß-hydroxy-ent-kauran-19-oic acid	-9.39	-2.55	-5.22	-1.91
Apigenin-4'-O-glucoside	-9.37	-4.12	-3.78	-1.01
5-p-coumaroylquinic acid	-9.30	-3.45	-2.19	-1.24
5-caffeoylquinic acid	-9.26	-3.42	-3.58	-1.34
4-caffeoylquinic acid	-9.23	-2.92	-3.71	-1.54
Apigenin-4'-O-glucoside	-8.59	-4.43	-2.41	-1.06
3-caffeoylshikimic acid	-8.36	-4.24	-2.27	-1.01
Rebaudioside B	-8.18	-2.6	-3.78	-2
Sterebin I	-8.11	-3.31	-2.1	-0.73
Apigenin	-8.03	-4.56	-1.79	-0.44
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-Dglucopyranosyl)oxy] ent-kaur-15-en-19-oic acid	-7.95	-2.34	-4.25	-1.59
Steviolbioside	-7.64	-1.53	-4.23	-1.98
Sterebin K	-7.31	-4.06	-2.1	-0.64
Sterebin G	-7.16	-2.99	-3.09	-0.69
Sterebin F	-7.09	-3.07	-2.76	-0.56
Sterebin E	-6.75	-3.1	-2.14	-0.53
Sterebin M	-6.42	-3.36	-2.17	-0.61
Sterebin N	-6.42	-3.36	-2.17	-0.61

Sterebin D	-6.23	-2.49	-1.66	-0.37
Sterebin C	-6.18	-2.68	-1.48	-0.5
Sterebin L	-6.04	-4.01	-1.43	-0.37
Sterebin J	-5.96	-3.43	-1.44	-0.44
7-O-acetyl-austroinulin	-5.81	-2.89	-1.66	-0.35
Sterebin A	-5.5	-2.56	-1.71	-0.52
CID_44608915*	-5.52	-5.92	-1.69	-0.98
Steviamine	-5.44	-2.06	-2.35	-0.52
Steviol glucuronide	-5.41	-1.76	-2.8	-1.12
β-sitosterol-β-D-glucoside	-5.29	-1.76	-2.43	-1.24
Steviamine	-5.04	-2.07	-2.85	-0.51
Austroinulin	-4.97	-3.03	-0.96	-0.38
Sterebin B	-4.81	-2.33	-1.51	-0.45
13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid-ß-D-glucopyranosyl ester	-4.79	-1.36	-2.6	-0.82
6-O-acetyl-austroinulin	-4.73	-3.06	-0.69	-0.47
Jhanol	-4.27	-2.53	-1.05	-0.23
Isosteviol	-4.23	-2.32	-0.59	-0.54
Lupeol-3-palmitate	-4.09	-3.57	-0.66	-0.02
Steviol	-4.08	-2.36	-0.45	-0.47
Beta-sitosterol	-3.77	-3.46	0	0.01
Stigmasterol	-3.62	-2.82	-0.35	-0.26
CID_44608915*	-4.52	-6.19	-0.76	-0.53
Campesterol	-3.12	-3	0	0.07
Amyrin acetate	-2.29	-2.42	0	0.04

*Pubchem ID/Name of standard PI3k inhibitors used in the study;

4.2.3 mTOR receptor

In case of mTOR receptor (PDB ID 4DRI), rebaudioside F have shown best binding energy (Figure 13). The G score, lipophilicity, H-bind, and electrostatic energy of the compound were found to be -16.19, -6.07, -7.46 and -1.76 Kcal/mol respectively. The interaction pattern was shown in Figure 13. The amino acid residues showing interaction included Gly 84, Ser 118, Asp 68 of A chain of receptor and Phe 2039 and Tyr 2038 of B chain of receptor. 13-[(2-O-β-D-glucopyranosyl-β-D glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-Dglucopyranosyl ester is yet another SG showing good binding affinity towards 4DRI receptor. G score, lipophilicity, H-bind, and electrostatic energy of this compound were found to be -15.56, -5.37, -7.93 and -1.41 kcal/mol respectively.

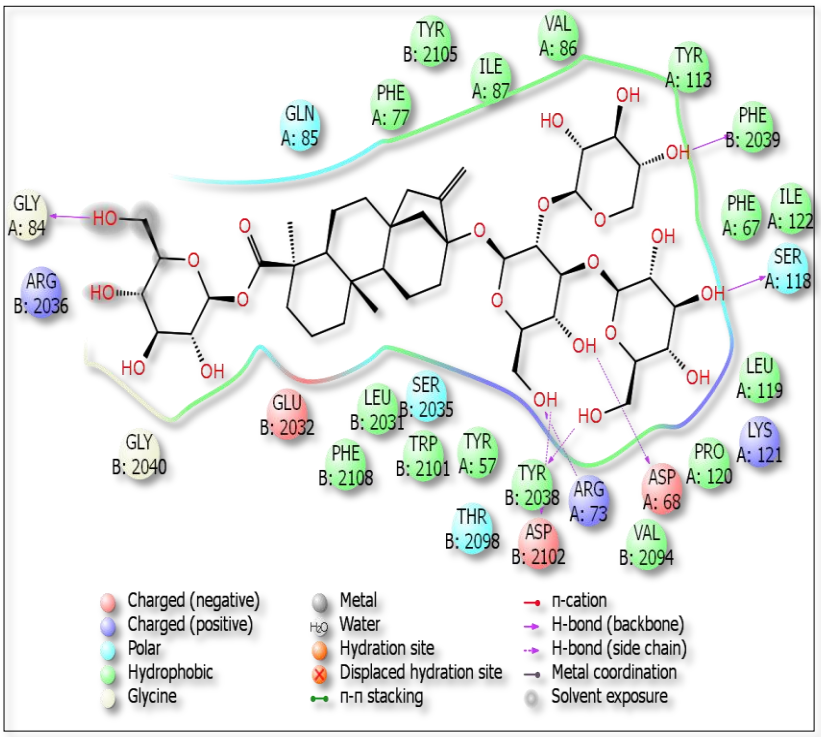


Figure 13. Interaction pattern of Rebaudioside F with mTOR receptor (PDB ID 4DRI) showing best G score

Subsequently another SG 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid β-D-glucopyranosyl ester was also found to have also shown good binding interactions. The G-score of this compound was found to be -15.5 kcal/mol. Moreover lipophilicity, H-bind, and electrostatic of this compound was -5.07, -8.05 and -1.46 Kcal/mol. The G

score, lipophilicity, H-bind, and electrostatic energy standard compound everolimus, (Pubchem ID CID_6442177) were found to be -2.91, -2.35, -2.03 and -.711 kcal/mol respectively which is much higher than top ranked docked componds The overall results of various compounds docked with 4DRI receptor is as represented in Table 8

Table 8. G. score, Lipophilicity, Hydrogen bond and electrostatic energies of various compounds isolated from *S. rebaudiana* docked with mTOR receptor (PDB ID 4DRI)

Ligand	**GScore (Kcal/mol)**	**LipophilicEvdW**	**HBond (Kcal/mol)**	**E_{Elec} (Kcal/mol)**
Rebaudioside F	-16.19	-6.07	-7.46	-1.76
13-[(2-O-β-D-glucopyranosyl-β-D glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-Dglucopyranosyl ester	-15.56	-5.37	-7.93	-1.41
13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid β-D-glucopyranosyl ester	-15.5	-5.07	-8.05	-1.46
13-[(2-O-b-Dglucopyranosyl-b-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-b-D-xylopyranosyl-b-D-glucopyranosyl) ester	-15.43	-4.86	-8.54	-1.99
Rebaudioside E	-15.31	-5.52	-7.09	-1.35
3,4,5-tricaffeoylquinic acid	-15.27	-7.65	-4.76	-1.1
Rebaudioside D	-15.03	-5.21	-7.2	-2

13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester	-14.45	-4.87	-7.64	-2
Rebaudioside A	-14.25	-4.9	-7.68	-1.7
Quercetin-3-O-rutinoside	-13.79	-5.38	-5.46	-1.55
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-Dglucopyranosyl)oxy] ent-kaur-15-en-19-oic acid	-13.74	-4.34	-6.5	-1.99
Stevioside	-13.63	-4.78	-5.99	-1.48
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl)oxy]-16ß-hydroxy-ent-kauran-19-oic acid	-12.55	-5.8	-5.81	-0.61
Dulcosidea A	-12.45	-4.94	-4.8	-1.21
Rebaudioside B	-12.28	-5.66	-5.01	-0.61
Luteolin-7-O-glucoside	-11.88	-5.32	-3.84	-0.81
Steviolbioside	-11.85	-4.93	-4.19	-0.99
Quercetin 3-glucoside	-11.45	-4.04	-4.8	-1.55
Luteolin-7-O-glucoside	-11.44	-5.5	-3.36	-0.65
Quercetin-3-O-ß-D-arabinoside	-11.43	-4.58	-3.76	-1.4
Sterebin J	-11.31	-4.04	-2.94	-0.69

β-sitosterol-β-D-glucoside	-11.09	-5.98	-2.86	-0.6
3,4-dicaffeoylquinic acid	-11.07	-5.46	-3.65	-0.66
4,5-dicaffeoylquinic acid	-11.07	-5.46	-3.65	-0.66
Quercetin-3-O-ß-D-rhamnoside	-10.9	-4.34	-3.85	-1.15
Kaempferol-3-O-rhamnoside	-10.85	-4.47	-3.55	-1.09
Sterebin L	-10.83	-5.04	-1.92	-0.36
1,3,5-tricaffeoylquinic acid	-10.82	-6.1	-4.8	-0.98
Apigenin-4'-O-glucoside	-10.76	-4.78	-3.59	-0.89
3,5-dicaffeoylquinic acid	-10.43	-4.6	-3.95	-1.27
Sterebin G	-10.39	-4.42	-3.23	-0.53
Sterebin K	-10.35	-4.03	-2.98	-0.93
4-Caffeoyl-5-feruloylquinic acid	-10.25	-4.64	-2.95	-1.19
Apigenin-4'-O-glucoside	-10.14	-4.59	-3.36	-1
Sterebin I	-10.12	-4.24	-2.47	-0.65
apigenin-7-O-ß-D-glycoside	-10.12	-4.43	-3.16	-0.74
Sterebin E	-9.84	-3.97	-2.26	-0.66
Quercetin	-9.82	-3.54	-2.76	-0.71
Lupeol-3-palmitate	-9.74	-7.31	0	0.01
3-caffeoylquinic acid	-9.73	-3.83	-3.36	-0.95

Stigmasterol	-9.5	-5.91	-0.7	-0.21
Beta-sitosterol	-9.34	-5.86	-0.65	-0.21
Sterebin M	-9.14	-4.35	-2.4	-0.66
Sterebin N	-9.14	-4.35	-2.4	-0.66
Campesterol	-9.11	-5.54	-0.7	-0.22
Apigenin-7-O-ß-D-glycoside	-9.03	-4.49	-2.17	-0.68
Luteolin	-8.95	-3.91	-1.92	-0.76
Sterebin F	-8.94	-3.21	-2.98	-0.8
Austroinulin	-8.87	-4.06	-1.66	-0.51
5-caffeoylquinic acid	-8.83	-3.18	-2.93	-0.89
3-O-feruloylquinic acid	-8.73	-3.23	-2.95	-0.89
Sterebin B	-8.53	-3.35	-2.1	-0.58
3-caffeoylshikimic acid	-8.52	-3.46	-2.88	-0.84
13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid-ß-D-glucopyranosyl ester	-8.52	-4.29	-2.4	-0.46
Luteolin	-8.46	-4.05	-1.62	-0.54
Steviol glucuronide	-8.43	-4.76	-1.6	-0.66
5-p-coumaroylquinic acid	-8.16	-3.26	-2.27	-0.86
Sterebin A	-8.14	-3.41	-2.07	-0.63
Isosteviol	-8.14	-3.39	-0.83	-0.55
Steviamine	-7.98	-1.81	-2.22	-0.63
7-O-acetyl-austroinulin	-7.91	-3.66	-1.92	-0.6
4-caffeoylquinic acid	-7.69	-2.92	-3.46	-0.53
Sterebin C	-7.47	-3.52	-1.52	-0.4

4-Caffeoylshikimic acid	-7.38	-2.86	-2.33	-0.8
6-O-Acetyl-austroinulin	-7.38	-3.22	-1.18	-0.26
Amyrin acetate	-7.36	-5.46	0	0
Sterebin D	-7.28	-3.35	-1.8	-0.4
Apigenin	-7.11	-3.07	-1.62	-0.84
Steviamine	-6.95	-1.77	-1.72	-0.74
Steviol	-6.5	-3.14	-0.35	-0.55
Jhanol	-6.46	-3.18	-0.7	-0.41
Sinapic acid	-6.41	-3.2	-1.63	-0.25
Apigenin	-5.84	-3.82	-1.33	-0.38
Everolimus*	-2.91	-2.35	-2.03	-0.71
Steviamine	-4.82	-1.98	-1.57	-0.53

* Pubchem ID/name of Standard mTOR inhibitors used in the study

4.3 *In-silico* ADME prediction

ADME properties of all studied compounds have been predicted using Qikprop application and are represented in Table 9. Although SG's along with Quercetin-3-O-rutinoside (in case of PI3k) have shown good binding affinity towards all the three receptors which include EGFR, PI3k and mTOR but pharmacokinetic profile as well as percentage oral bioavailability of all these best docked compounds was found to be unfavourable. The percentage oral absorption of all the compounds was found to be zero. Moreover predicted apparent caco-2 cell permeability was also found to be poorer in all the best docked compounds. Thus from the docking and ADME data it was clear that SGs may have significant anticancer potential towards EGFR, PI3K and mTOR receptor but pharmacokinetics is the major problem in this side. Modification of structure can be tried to improve the pharmacokinetics profile of the agents. 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. Steviol which is the aglycone part of SGs and its oxidised product isosteviol on the other hand have shown good ADME profile. In case of steviol and isosteviol predicted oral absorption is more than 90% and 70% respectively. So there is a chance of modification of these compounds to get good anticancer compounds with good ADME properties. Which can be further justified by the fact that isosteviol have shown multiple anticancer properties.

Table 9. ADME Properties of various compounds from *S. rebaudiana* as predicted by QikProp

Ligand	Mol Wt (130-725)	H-Bond Donner (0-6)	H-Bond Acceptor (2-20)	QPlog Po/w -2 - 6.5	QPP Caco (nm/sec) < 25% poor >500 great	QPlog BB -3 – 1.2	QPlog Kp - 8.0 – -0.1	QPlog Khsa -1.5 - 1.5	% Oral Absorption > 80% high, < 25% poor
13-[(2-O-β-D-glucopyranosyl-3-O-(4-O-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oicacid β-D-glucopyranosyl ester	1129.16	17	45.25	-6.97	0.06	-7.571	-8.495	-3.448	0
13-[(2-O-β-D-glucopyranosyl-β-D glucopyranosyl) oxy]-17-hydroxy-kaur-15-en-18-oic acid β-Dglucopyranosyl ester	983.023	15	38.45	-5.67	0.058	-7.628	-8.96	-2.868	0
13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-17-oxo-kaur-15-en-18-oic acid β-D-glucopyranosyl ester	981.007	14	38.75	-5.61	0.047	-8.051	-9.217	-2.926	0
13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester	1127.14	15	42.85	-6.17	0.006	-9.931	-10.62	-3.232	0
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-Dglucopyranosyl)oxy] ent-kaur-15-en-19-oic acid	804.881	11	28.25	-2.23	0.223	-5.086	-7.413	-1.767	0
13-[(2-O-ß-D-glucopyranosyl-3-O-ß-D-glucopyranosyl-ß-D-glucopyranosyl)oxy]-16ß-hydroxy-ent-kauran-19-oic acid	822.896	12	29	-2.58	0.205	-5.251	-7.449	-1.904	0

13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid-ß-D-glucopyranosyl ester	480.597	4	12.5	1.163	130.25	-1.561	-4.503	-0.201	71.609
Dulcosidea A	788.882	10	26.55	-2.00	1.156	-5.117	-7.362	-1.396	0
Isosteviol	320.428	2	4.75	2.641	63.785	-0.896	-4.427	0.112	74.712
Rebaudioside A	967.023	14	36.75	-4.85	0.133	-7.103	-8.419	-2.618	0
Rebaudioside B	804.881	11	28.25	-2.10	0.369	-4.908	-6.955	-1.8	0
Rebaudioside D	1129.16	17	45.25	-7.00	0.025	-8.634	-9.249	-3.573	0
Rebaudioside E	967.023	14	36.75	-4.75	0.147	-7.033	-8.327	-2.595	0
Rebaudioside F	936.997	13	35.05	-4.21	0.599	-5.491	-7.376	-2.231	0
Steviol	318.455	2	2.75	3.823	193.91	-0.495	-3.389	0.491	90.276
Steviolbioside	628.712	8	19.75	-0.35	1.5	-3.709	-6.396	-1.047	0
Stevioside	804.881	11	28.25	-2.63	0.471	-5.775	-7.947	-1.61	0
Steviol glucuronide	494.581	4	10.55	2.238	11.872	-2.119	-5.267	-0.059	59.284
Apigenin-4'-O-glucoside	432.383	5	12.25	-0.36	10.576	-3.176	-5.533	-0.706	30.174
1,3,5-tricaffeoylquinic acid	678.602	8	14.2	1.924	0.045	-7.465	-7.277	-0.392	0
3,4,5-tricaffeoylquinic acid	678.602	8	13.25	2.252	0.03	-7.653	-7.613	-0.201	0
3,4-dicaffeoylquinic acid	516.457	7	11.45	0.944	0.205	-5.354	-6.987	-0.591	0
3,5-dicaffeoylquinic acid	516.457	7	11.45	0.954	0.211	-5.415	-6.957	-0.599	0
3-caffeoylquinic acid	354.313	6	9.65	-0.25	1.691	-3.254	-6.206	-0.908	16.584
3-caffeoylshikimic acid	336.298	5	8.9	-0.07	2.087	-2.816	-6.178	-0.794	32.217
3-O-Feruloylquinic acid	368.34	5	9.65	0.619	7.025	-2.669	-5.001	-0.784	45.721
4,5-dicaffeoylquinic acid	516.457	7	11.45	0.944	0.205	-5.354	-6.987	-0.591	0
4-caffeoyl-5-feruloylquinic acid	528.512	5	10.7	2.723	1.529	-4.449	-5.481	-0.11	20.273
4-caffeoylquinic acid	354.313	6	9.65	-0.27	1.642	-3.309	-6.226	-0.918	16.258
4-caffeoylshikimic acidf	336.298	5	8.9	0.07	2.33	-3.086	-5.986	-0.82	33.928
5-caffeoylquinic acid	354.313	6	9.65	-0.25	1.691	-3.254	-6.206	-0.908	16.584
5-p-coumaroylquinic acid	338.313	5	8.9	0.328	4.023	-2.84	-5.445	-0.798	39.688

6-O-acetyl-austroinulin	364.524	2	4.45	4.229	1726.3	-0.496	-2.256	0.692	100
Stigmasterol	412.698	1	1.7	7.455	4119.1	-0.182	-1.579	2.019	100
7-O-acetyl-austroinulin	364.524	2	4.45	4.384	1670.0	-0.548	-2.284	0.756	100
Amyrin acetate	468.762	0	2	7.771	4282.6	0.154	-2.063	2.354	100
Apigenin	270.241	2	3.75	1.642	116.37	-1.446	-3.963	-0.028	73.537
Apigenin-7-O-ß-D-glycoside	432.383	5	12.25	-0.36	11.309	-3.173	-5.463	-0.718	30.728
Austroinulin	322.487	3	4.15	3.489	1731.5	-0.488	-2.253	0.379	100
β-sitosterol-β-D glucoside	576.855	4	10.2	5.08	333.91	-1.913	-3.109	0.909	75.94
Beta-sitosterol	414.713	1	1.7	7.569	4118.9	-0.26	-1.498	2.028	100
Campesterol	400.687	1	1.7	7.256	4118.9	-0.194	-1.594	1.933	100
Jhanol	306.487	1	2.45	4.478	4335.9	0.08	-1.838	0.831	100
Kaempferol-3-O-rhamnoside	432.383	5	11.3	0.001	16.988	-2.704	-5.328	-0.537	36.011
Lupeol-3-palmitate	665.137	0	2.45	13.26	6536.5	-0.712	-0.057	4.019	100
Luteolin	286.24	3	4.5	0.96	42.09	-1.946	-4.851	-0.19	61.636
Luteolin-7-O-glucoside	448.382	6	13	-0.99	4.099	-3.748	-6.348	-0.833	6.186
Pyrogallol	126.112	3	2.25	0.102	388.23	-0.738	-3.43	-0.809	73.885
Quercetin 3-glucoside	464.382	7	13.75	-1.43	2.933	-3.609	-6.664	-0.874	0.998
Quercetin	302.24	4	5.25	0.524	21.196	-2.415	-5.346	-0.317	53.748
Quercetin-3-O-rutinoside	610.524	9	20.55	-2.32	3.132	-3.715	-6.321	-1.225	0
Quercetin-3-O-ß-D-arabinoside	434.356	6	12.05	-0.86	8.81	-2.917	-5.906	-0.728	12.898
Quercetin-3-O-ß-D-rhamnoside	448.382	6	12.05	-0.56	8.598	-3.11	-5.891	-0.673	14.439
Sinapic acid	224.213	2	4.25	1.519	64.957	-1.271	-3.743	-0.572	68.284
Sterebin A	310.433	3	6.15	1.927	462.16	-0.948	-3.621	-0.006	85.926
Sterebin B	352.47	2	6.45	2.655	402.75	-1.064	-3.737	0.271	89.117
Sterebin C	352.47	2	6.45	2.76	496.34	-0.984	-3.56	0.286	91.358
Sterebin D	294.433	2	4.45	2.795	802.84	-0.67	-3.25	0.3	95.298
Sterebin E	338.486	4	5.85	2.429	407.63	-1.167	-3.501	0.113	87.885

Sterebin F	338.486	4	5.85	2.466	578.33	-0.99	-3.199	0.091	90.825
Sterebin G	354.486	5	7.55	1.603	235.00	-1.491	-3.697	-0.208	78.767
Sterebin I	350.497	3	6.15	2.635	298.98	-1.387	-3.63	0.183	86.681
Sterebin J	350.497	3	6.15	2.636	319.20	-1.339	-3.594	0.181	87.2
Sterebin K	366.54	3	5.85	3.718	1732.5	-0.675	-2.156	0.393	100
Sterebin L	366.54	3	5.85	3.696	1732.3	-0.665	-2.171	0.389	100
Sterebin M	352.513	4	4.9	3.324	945.09	-0.877	-2.575	0.299	100
Sterebin N	352.513	4	4.9	3.324	945.09	-0.877	-2.575	0.299	100
Steviamine	187.238	3	7.1	-0.78	184.02	-0.151	-5.486	-0.721	62.876

QPP Caco: Predicted apparent Caco-2 cell permeability in mm/sec. Caco-2 cells are the model for gut blood barrier; **Q Plog $P_{o/w}$:** Predicted octanol/water partition coefficient; **Q P log BB:** Predicted brain/blood partition coefficient; **QPP MDCK:** Predicted apparent MDCK cell permeability in nm/sec. MDCK cells are considered good mimic for blood brain barrier; **QP log KP:** Predicted skin permeability; **QP log K_{hsa}** Prediction of binding to human serum albumin**.**

4.4 Characterization of isolated compounds

4.4.1 Characterization of MVR-1

Compound MVR-1 was yellow solid, produced a single yellowish spot on a precoated silica gel60F_{254} TLC plate.

The melting point of compound was found to be 314-316 ^{o}C.

Its m/z ratio indicated an ion peak at m/z [M] $^{+}$ (calculated for $[C_{15}H_{10}O_7]^{+}$, 302.1) corresponding to molecular formula $C_{15}H_{10}O_7$ with eleven degree of unsaturation.

Values of ^{13}C assigned to various carbons include δc 93.41 (C-8), 98.59 (C-6), 101.34 (C-3), 104.21 (C-10), 115.12 (C-2') 115.20 (C-5'), 120.91(C-6'), 134 (C-1'), 144.75 (C-3'), 148.75 (C-4'), 156.42 (C-7), 156.99 (C-5), 161.98 C-2), 164.04 (C-9), 177.678 (C-4) .

Some peaks of impurities were also observed in NMR spectra at aliphatic region showing the presence of grease/impurity

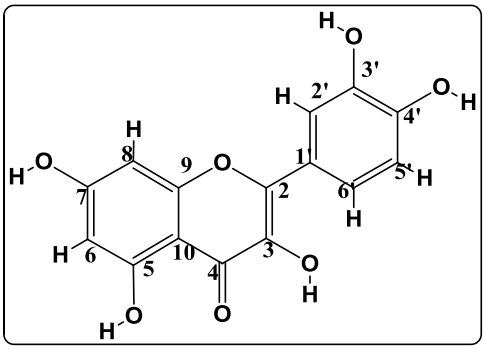


Figure 14. MVR-1

Thus MVR-1 was found to be quercetin which is represented in Figure 14. Further confirmation of structure was done on the basis of literature (Fossen *et al.*, 1997)

4.4.2 Characterization of MVR-5

Compound MVR-5 was a crystalline white solid, produced a single black spot on a precoated silica gel60F_{254} TLC plate. Its m/z ratio indicated an ion peak at m/z $[M+Na]^{+}$ (calculated for $[C_{38}H_{60}O_{18}+Na]^{+}$, 827.8620) corresponding to molecular formula $C_{38}H_{60}O_{18}$ with nine degree of unsaturation. The ^{13}C NMR

spectrum including distortionless enhancement by polarization transfer (DEPT) spectra clearly indicate the presence of 2 – CH_3, 13 – CH_2, 17- CH . Six signals were assigned to quaternary carbons. The ^{1}H and ^{13}C NMR of compound MVR–5 showed three anomeric values at δ_{Hn} 5.2 (1H, d, J=5.5Hz), 4.34 (1H, d, J=5.64 Hz), 4.40 (1H, d, J=7.64 Hz) and δ ^{13}C 94.1, 96.3 and 104.5 respectively assigned to three glucose molecules. In HMBC H-1', H-1'' and H-1''' showed correlation with δc 84.6, 82.56 and 175.6 respectively. The downfield shift of C-4' (i.e δ= 9.2 ppm and HMBC correlation of H-1'' with C-2'clearly indicate the attachment of additional glucose at C-2' position.

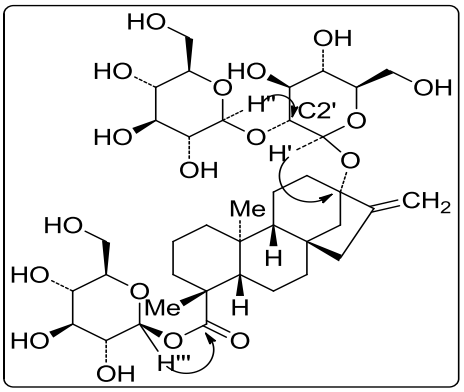


Figure 15. MVR-5

Moreover m/z $[M+H]^{+}$ of Periodate oxidation product of MVR-5 (MVR-5D) was found to be 319.2273. Above mentioned observation confirmed that the compound MVR-5 is stevioside which is represented in Figure 15. Stevioside is a SG containing three glucose molecules attached with steviol moiety which was obtained as Periodate oxidation product MVR-5D having melting point of 110-112 ^{o}C. Further confirmation of structure was done on the basis of literature (Danieli *et al*., 1997)

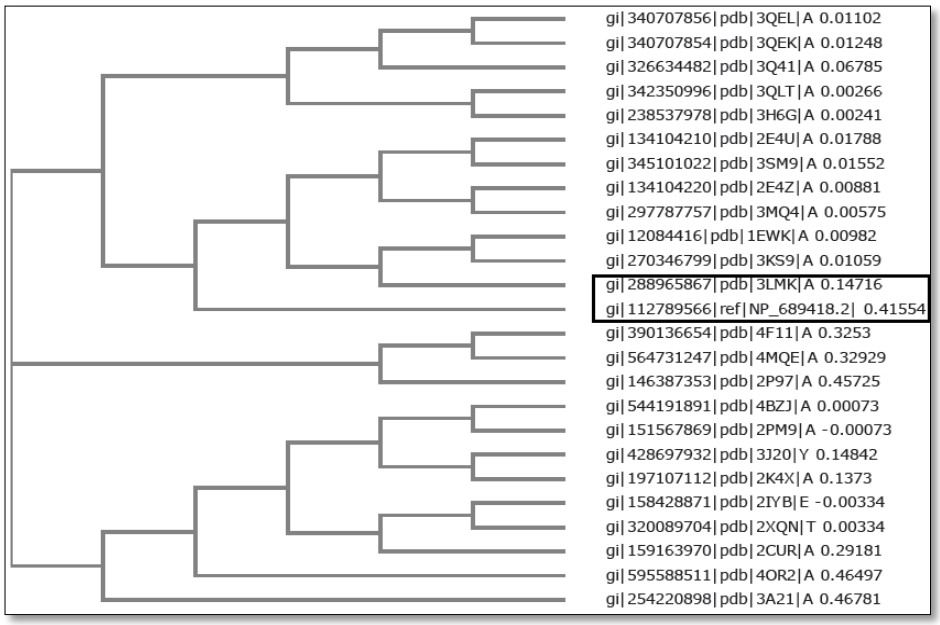
Part 2

4.5 Homology modeling of sweet taste receptor

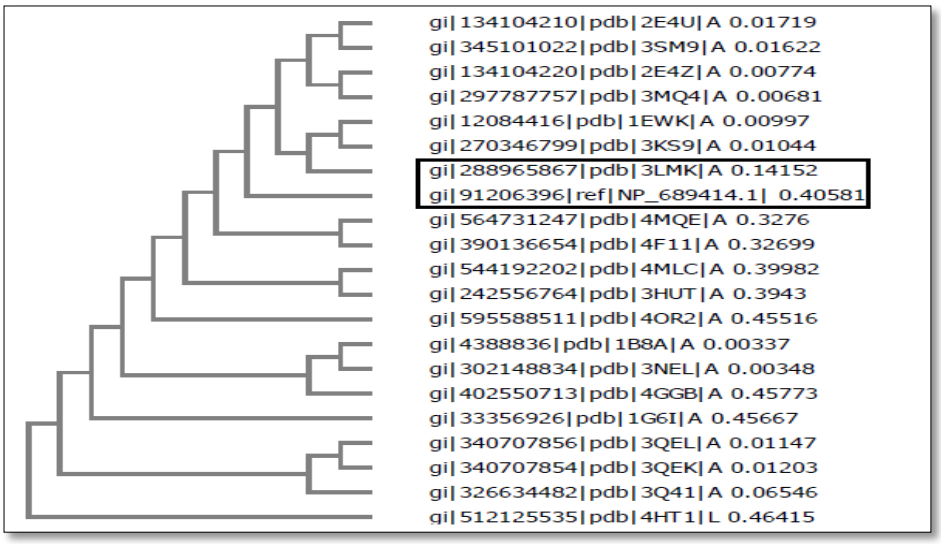
4.5.1 Phylogenetic analysis and template selection

Template for comparative modeling was chosen on the basis of E-value of BLAST search, percentage identity with target sequence and phylogenetic

analysis. The phylogenetic analysis of T1R2 and T1R3 subunits (Figure 16) indicated that both are closely related to A chain of the ligand binding domain of metabotropoc glutamate receptor (mGluR5) complexed with glutamate (PDB ID 3LMK). A chain of 3LMK showed sequence identity of 25% and E-value of 2e-28 with respect to T1R2 and sequence identity of 28% and E-value of 1e-22 with respect to T1R3. Thus, A chain of 3LMK seems to be ideal template and by using this protein chain, homology model of T1R2 and TIR3 was constructed.



(a)

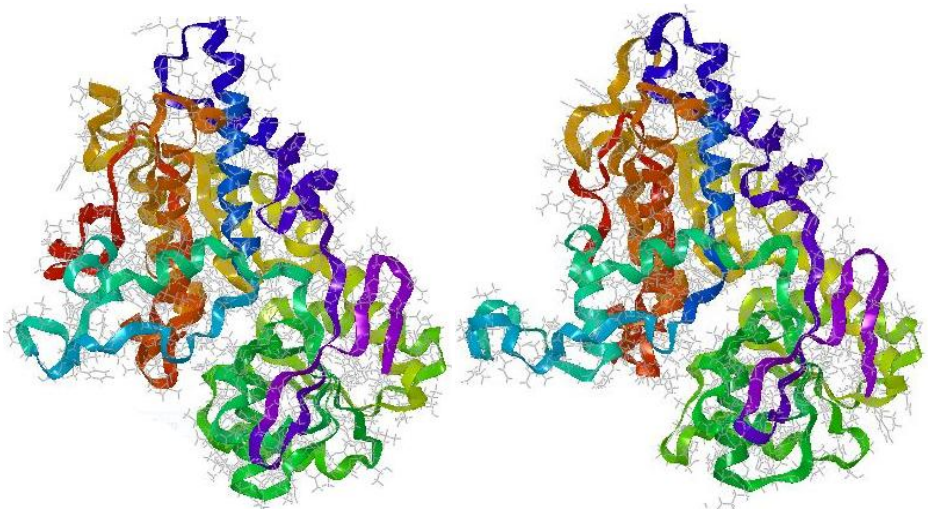


(b)

Figure 16. (a) Phylogenetic tree of T1R2 receptor, **(b)** Phylogenetic tree of T1R3 subunit of human sweet taste receptor

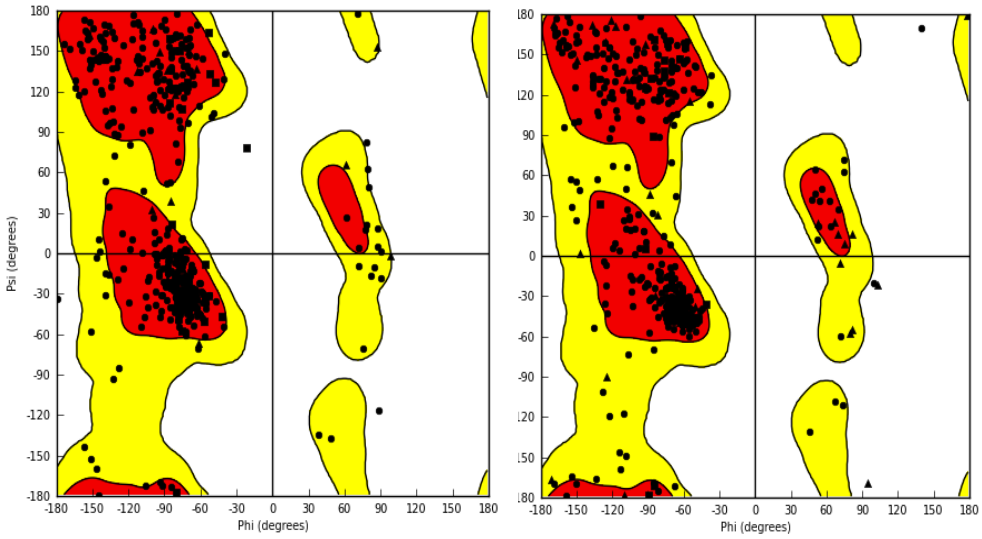
4.5.2 Homology modeling

The homology models of T1R2 and T1R3 (Figure 17) subunits were constructed using prime-v34012 module of Schrodinger LLC (NY, USA) which is an automated protein modeling tool.



(a) **(b)**

Figure 17. Homology model of T1R2 **(a)** and T1R3 **(b)** generated by Prime (Maestro 9.6)



(a) **(b)**

Figure 18. Ramachandran plot of T1R2 **(a)**, Ramachandran plot of T1R3 **(b)** in support of quality of models

Quality of final model was evaluated using Ramachandran Plot (RAMPAGE), PROCHECK (Overall Procheck G-scores) and ERRAT (Overall quality factor). The Ramachandran plot (Figure 18) assessment of T1R2 and T1R3 revealed that 89.8 % residues were present in most favored region, 8.1% in allowed

region and 2% are in outliers region in case of T1R2 and 90% of residues are present in most favored region, 9.1% in allowed and just 0.9% in outlier region. Overall average G factor was found to be -0.41 for T1R2 and -0.15 for T1R3. T1R2 and T1R3 models yielded the ERRAT overall quality factors of 63.830 and 86.486 respectively.

4.5.3 Docking simulation study

Table 10 summarizes the results of docking study in which lowest G score represents the best binding ability of the ligand. Complex formed between ligand and receptor provided a lot of information such as important amino acid needed for key interaction, crucial interactions such as hydrogen bonds, salt bridges, lipophilic interactions π-π interactions and π-cation interactions. In present study, SGs with known sweetness profile was docked against homology model of T1R2 and T1R3 subunits of human sweet taste receptors. Rebaudioside A and E are the sweetest among all SGs of known sweetness profile which is consistent with our dock scores -12.334 and -10.658 respectively. Rebaudioside B, stevioside and rebaudioside D showed fairly comparable sweetness profile and the same trend was indicated by dock scores. Sweetness index of steviolbioside and dulcoside are comparatively less as compared to others compounds which is also found in present study. Dock score of isosteviol and steviol showed poor binding affinity with receptors and in fact these compounds have not shown any sweetness properties. Moreover cumulative dock scores of T1R2 and T1R3 (D1+D2) still preserve the trend of as observed in experiments. Furthermore Pearson correlation coefficient **"R"** have been calculated to determine the correlation between the obtained G score and average of actual sweetness value as mentioned in Table 10. The results indicated that there is strong correlation between obtained G score and average of sweetness value. In case of TIR2 R was found to be 0.9437 which shows that there exists a very strong positive correlation between both the variables. In case of T1R3 R value was 0.854 which also indicated there is strong positive correlation between these variables. Furthermore in case of combined G score of TIR2 and T1R3 (D1+D2) R was found to be 0.939 which is also very strong correlation. Thus statistical data analysis indicated that there

exists a very strong correlation between observed sweetness profile and experimentally obtained G score.

Table 10. Dock scores, protein ligand interactions of T1R2 and T1R3 for SG of known sweetness profile

Ligand	G score of T1R2 (D1) (Kcal/mol)	G score of T1R3 (D2) (Kcal/mol)	D1+D2 (Kcal/mol)	Protein Ligand Interactions (T1R2)	Protein Ligand Interactions (T1R3)	Sweetness as compared to sucrose
Rebaudioside A	-12.334	-7.995	-20.329	Asn 44, Asn 52, Pro 343, Ala 345	Arg 56, Glu105, Glu 148, Asp215, Asp 216, Lys 255,Asp258	350-450
Rebaudioside E	-10.658	-7.841	-18.599	Asn 44, Gly 47, Ile 325, Ala 345, Gly 346	Leu 51,Glu148, Asp 216,Glu 217	250-300
Rebaudioside D	-9.764	-7.767	-17.531	Ile 325, Thr 326, Ser336	Glu 105, Glu 148, Glu 217, Arg 247, Lys 255	200-300
Rebaudioside B	-9.100	-7.814	-16.914	Asn 44, Asn 42	Ser 104, Glu105, Asp 215	300-350
Stevioside	-9.711	-6.664	-16.375	Asn 44, Ile 352, Ala 354	Arg 52, Arg 56, Glu 105, Asp 216, Glu 217, Lys 255	250-300
Steviolbioside	-8.096	-6.657	-14.753	Gly 47, Asn 52, Ser 329, Ala 345	Arg 56, Ser 104, Glu105, Asp 216	100-125
Dulcoside	-6.910	-6.545	-13.455	Asn 44,Trp 341	Arg 52, Arg 56, Glu 105,Asp 215, Lys 255,	50-120
Isosteviol	-4.198	-3.187	-7.385	Gly 346	Glu 217	-
Steviol	-3.426	-2.152	-5.578	Gly 346	Asp 215	-

SGs are among the most investigated compounds because of high sweetness intensity as compared to sucrose along with many other health benefits and high availability in plants. Taste quality of SGs is poor except rebaudioside A, E and D which restricts its use for human consumption and limits its application in food and pharmaceutical products. To solve this problem, knowledge of binding pattern of SGs to STR seems to be quite helpful. The investigation of structure and associated sweetness of SGs revealed that glucose moieties at C-19 and C-13 position have an important impact on the sweetness profile. This fact

indicates that ATD of STR which binds to natural sugars, including glucose seems to play an active role in SG mediated sweetness. Another important point in glucose molecules at C-13 and C-19 subunits of SGs is that they are distant apart from each other. This observation indicates that the ATD of STR have different binding sites distant apart from each other which can be stimulated to get sweetness signaling. Aglycone part of SGs provides glucose moieties at C-13 and C-19 position such an orientation that different binding sites get stimulated in synchronized way to get intense sweet taste. Thus, amino acids residues which get stimulated by SGs are distant apart and may include main active as well as allostearic site.

Docking results showed that rebaudioside A has best G score of -12.334 and -7.995 Kcal/mol for T1R2 and T1R3 respectively. Rebaudioside E, D, stevioside, rebaudioside B showed G score of -10.658, -9.764, -9.711, -9.100 for T1R2 and -7.995, -7.841,-7.767, -6.664, -7.814 Kcal/mol for T1R3 respectively and have fairly comparable sweetness profile. The binding interactions of steviolbioside and dulcoside are less as compared to other. G score of steviolbioside and dulcoside was found to be -8.96, -6.910 and -6.657, -6.545 Kcal/mol for T1R2 and T1R3 respectively Interaction pattern indicates that hydrogen bonding (backbone and side chain) with hydroxyl group of glucose moieties is the key interaction which is responsible for sweetness signaling. It is quite possible that both the subunits triggered signaling independent to each other and the resultant of sweetness intensity is due to cumulative effect of both the subunit. Collective binding affinity can be calculated by adding dock score of both T1R2 and TIR3. It was found to be same trend of experimental results of dockscore after combining of both T1R2 and T1R3.

Protein-ligand interaction profile (Figure 19 & Figure 20) revealed that Asn 44, Asn 52, Ala 345, Pro 343, Ile 352, Gly 346, Gly 47, Ala 354, Ser 336, Thr 326 and Ser329 involves in the hydrogen bond interactions for T1R2 and Arg 56, Glu 105, Asp 215, Asp 216, Glu 148, Asp 258, Lys 255, Ser 104, Glu 217, Leu 51, Arg 52 and Arg 247 involves in the hydrogen bond interactions in case of T1R3

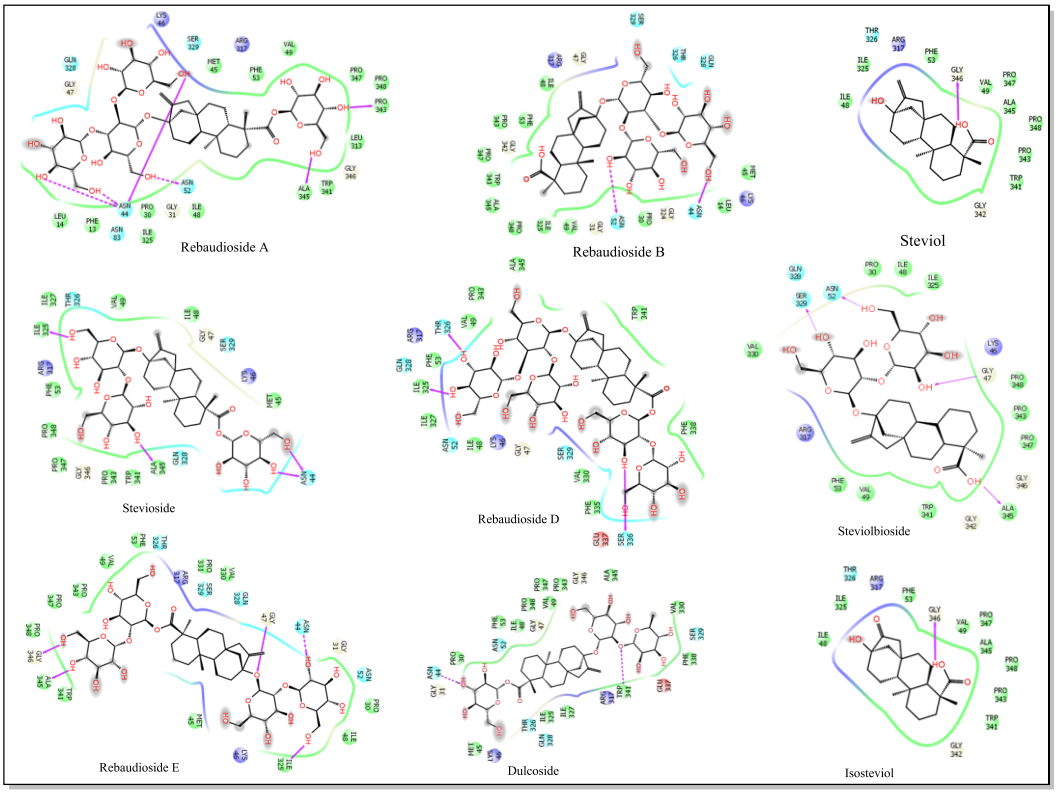


Figure 19. Interaction pattern of SGs with T1R2 receptor

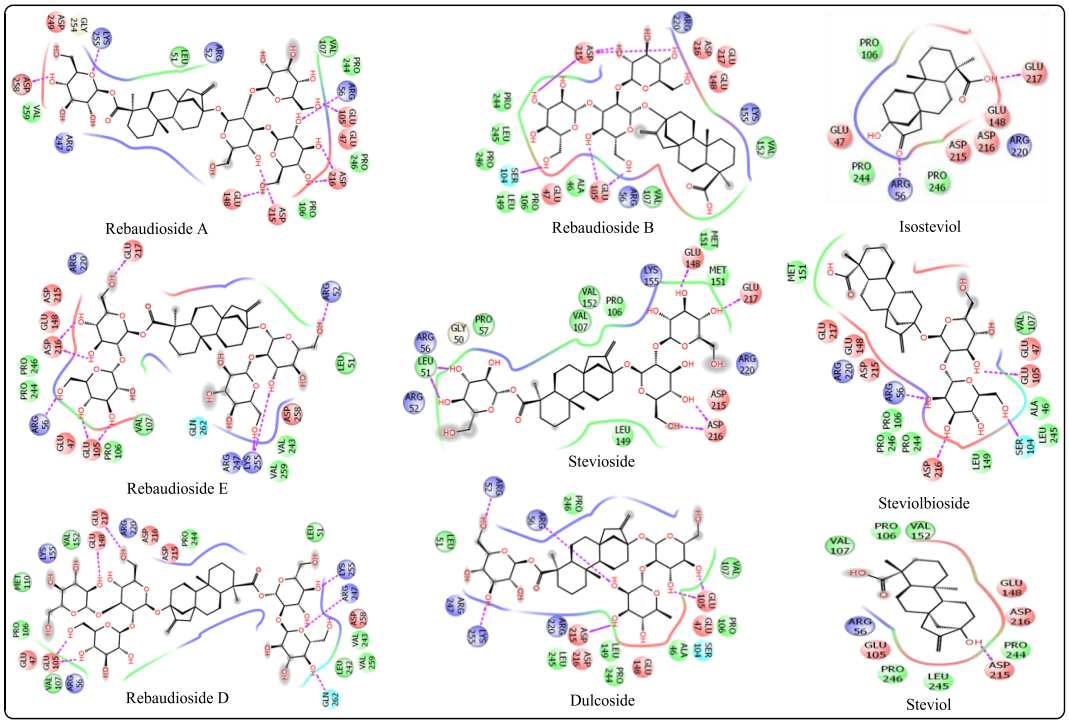
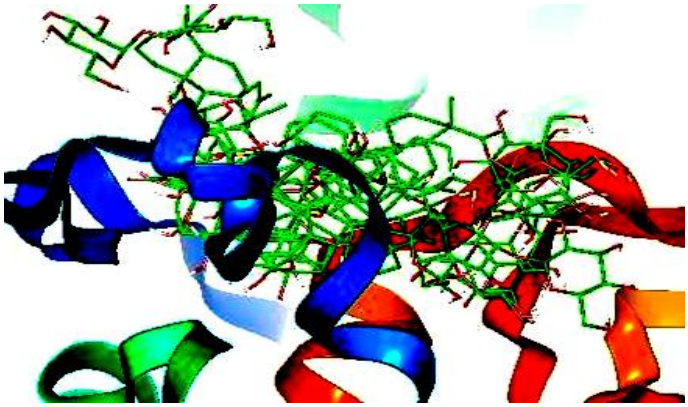


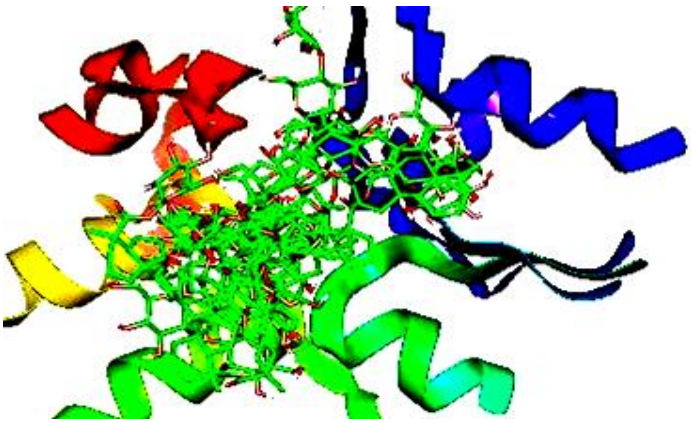
Figure 20. Interaction pattern of SGs with T1R3 receptor

Furthermore interaction pattern and pose of each individual SG is very dissimilar compared to others in the docking study (Figure 21), which is quite reasonable because all of them have unique sweetness properties which is

differing from others. This data also supports the above mentioned assumption of presence of different binding sites at ATD of STR. Thus, several sets of amino acid residues are present in ATD distant apart from each other which produces typical sweet taste and stimulating different set of residues can produce different but characteristic sweet taste. Thus, on the basis of structural aspect as well as docking poses of SGs we can describe the diverse taste of SGs by multiple point stimulation model of SGs for sweetness which is represented in figure 22. Thus diversity in sweet taste of SGs seems to be due to stimulation of main active site residues by monosaccride subunits at C-13 position and some other type of allostearic modulation by C-19 monosaccride subunits or vice versa



(a)



(b)

Figure 21. Non overlapping and diverse poses of SG docked on T1R2 **(a)** and T1R3 **(b)**

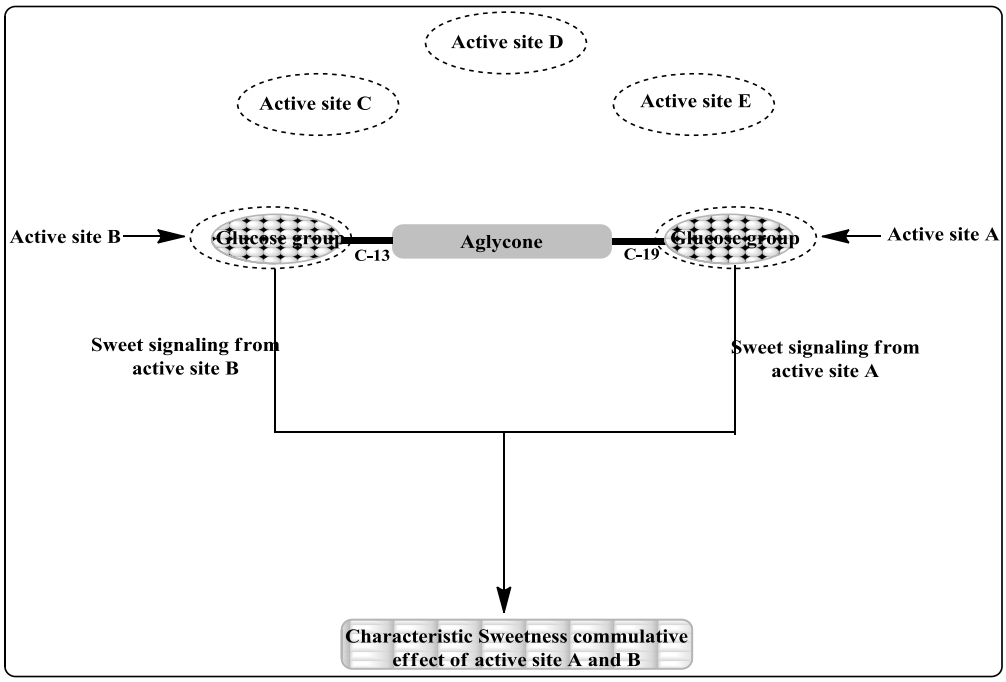


Figure 22. Multiple point stimulation model of the sweetness mechanism of SGs based on its structure, varied sweet taste and diverse docking poses. ATD domain of STR consists of several groups of active amino acids, namely active site A, B, C, D, E and so on. Monosaccride moieties at C-13 and C-19 of SGs stimulate different combination of active sites to produce characteristic sweet taste. The aglycone part may provide glucose molecule such a confirmation that it selectively interact with a set of active sites and produces a characteristic taste.

4.5.4 SAR of SGs based on docking results

1. Steviol glycosides binds to ATD domain of STR to produces sweet taste as docking study at ATD of STR correlated well to the experimental data.
2. Hydroxyl groups of glucose molecules present in steviol glycosides are responsible for key binding interactions.
3. Maximum binding affinity and thus taste intensity may be obtained in SGs having four glucose molecules attached with it. As maximum G score have been obtained in case of rebaudioside A and rebaudioside E.
4. Rebaudioside D with five glucose molecules, stevioside and rebaudioside B with three glucose molecules have shown binding affinity lesser than rebaudioside A and rebaudioside E. Thus increase or decrease in glucose number in SGs from four reduced binding affinity and thus taste intensity.
5. Aglycone part of SGs doesn't participate in binding with STR but provide glucose molecules proper orientation for binding as no key interaction is shown by it in docking study.

6. Glucose molecules at C-13 and C-19 part of SGs interacts with different set of amino acids in ATD which may be termed as active sites and produce different taste and we feel taste which is cumulative effect glucose molecules at both the ends of SGs

CHAPTER 5

SUMMARY

CHAPTER 5

SUMMARY

Dried aerial part of plants powder was extracted thrice by solvents with increasing order of polarity using petroleum ether, ethyl acetate, methanol, aqueous methanol and water respectively. All the extracts were dried using rotavapor. On the basis of TLC observation aqueous methanol extract was selected for isolation process. Total seven molecules have been isolated on the basis of single spot on the precoated silica gel $60F_{254}$ TLC plates. However, only two compounds (**MVR-1** and **MVR-5)** were characterized. **MVR-1** was found to be quercetin and **MVR-5** was found to be stevioside both of which are known compounds. The anticancer potential of various extracts have been determined using MTT based *in-vitro* assay using A-549, H-460 and MCF-7 cell lines and have shown significant potential. In A-549 cell line MVE-5 and MVE-2 have shown excellent activity with IC_{50} value of < 10 µg/ml and 57 µg/ml respectively. Moreover MVE-4 has shown IC_{50} value of 90 µg/ml. In case of H-460 cancer cell line MVE-4 and MVE-5 have shown good activity with IC_{50} value of 88 µg/ml and 92 µg/ml respectively. Furthermore in case of MCF-7 cell line extracts MVE-1 and MVE-2 have shown good activity and IC_{50} value was found to be 90 µg/ml and 53 µg/ml respectively. Thus in case of non-small cell lung cancer cell lines (A-549 and H-460), maximum activity have been found for polar extracts MVE-5 as well as MVE-4 but in case of MCF-7 breast cancer cell lines, non-polar extracts (MVE-1 and MVE 2) have shown significant activity. Apart from -*in-vitro* determination of anticancer potential, *in silico* approach have also been implemented to determine the anticancer potential of compounds isolated from *S. rebaudiana.* Docking study was performed on EGFR, PI3K and mTOR receptors. In case of EGFR receptor (PDB ID 2ITY), SG 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β- D-glucopyranosyl) ester have shown best G score of -14.04 Kcal/mol. For PI3K receptor (PDB ID 3S2A) quercetin-3-O-rutinosid and SG 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl) ester have shown best G score. The G score for quercetin-3-O-rutinosid and SG 13-[(2-O-β-Dglucopyranosyl-β-D-glucopyranosyl) oxy]-kaur-16-en-18-oic acid-(6-O-β-D-xylopyranosyl-β-D-

glucopyranosyl) ester was found to be -13.3 and -13.2 Kcal/mol respectively. Moreover, in case of mTOR receptor (PDB ID 4DRI) best G score of -16.19 Kcal/mol was obtained for Rebaudioside F. Thus SGs have shown good binding affinity for 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. Furthermore homology models and docking study have been done to explore the sweetness mechanism of SGs with known sweetness profile. Homology models of T1R2 and T1R3 subunits constructed using A chain of 3LMK protein have shown good validation results of Ramachandran plot, PROCHECK and ERRAT server. The docking of SGs of known sweetness profile further produced the results which correlate well with experimental data and thus seems acceptable. The important interactions of amino acids have been investigated using docked poses of SG. Asn44, Ans52, Ala345, Pro 343, Ile 352, Gly 346, Gly 47, Als 354, Ser 336, Thr 326 and Ser 329 are the main amino acids showing hydrogen bonding interaction with SGs in case of T1R2 and Arg 56, Glu 105, Asp 215, Asp 216, Glu 148, Asp 258, Lys 255, Ser 104, Glu 217, Leu 51 and Arg 52 amino acid residues have shown hydrogen bonding in case of T1R3. On the basis of dissimilarity in interaction pattern, poses of individual docked SGs, its structural aspect and impact of C-13 and C-19 glucose molecules, we have concluded that there are multiple groups of amino acids so called active sites present on ATD domain of STR which stimulated to get diverse sweet taste. The proposed model of sweetness justified the diversity of taste among SGs. Different SGs interact with different set of active sites by mean of C-13 and C-19 glucose molecules and produce specific taste. It further justified the importance of monosaccride substituent at both C-13 and C-19 end possible role of steviol moiety for intensity and quality of taste. We have further observed that total four glucose molecules attached with aglycone part of SGs produced best binding affinity and thus best taste intensity. Aglycone part only provides proper orientation to glucose molecules but don’t show any important interaction. Multiple point stimulation model although seems specific for SGs is an important aspect and may be implemented in general way to design some

improved sweetener molecules alongwith Its usefulness to enhance the taste quality or sweetness index of SGs by further modifications its structure at target specific positions as guided by docked binding interactions, to attain required taste.

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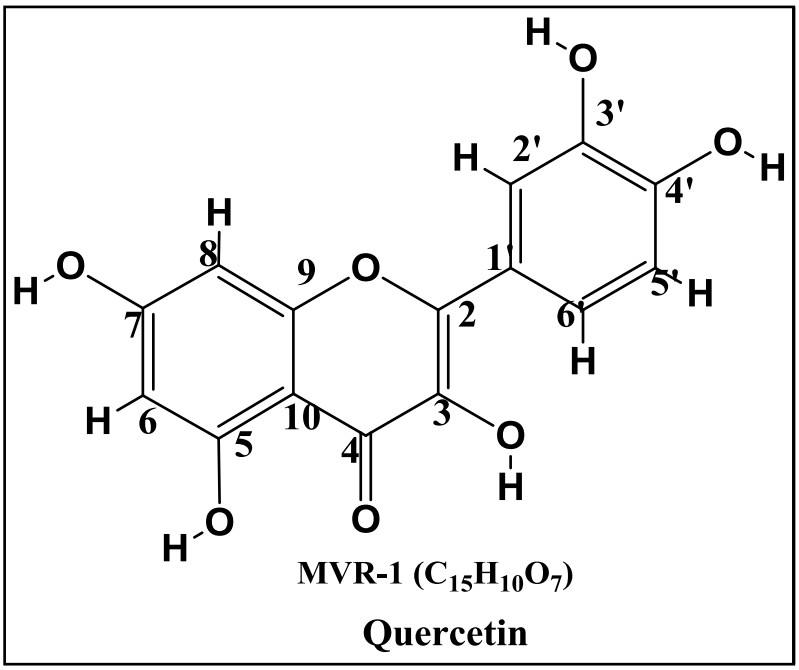
APPENDIX A

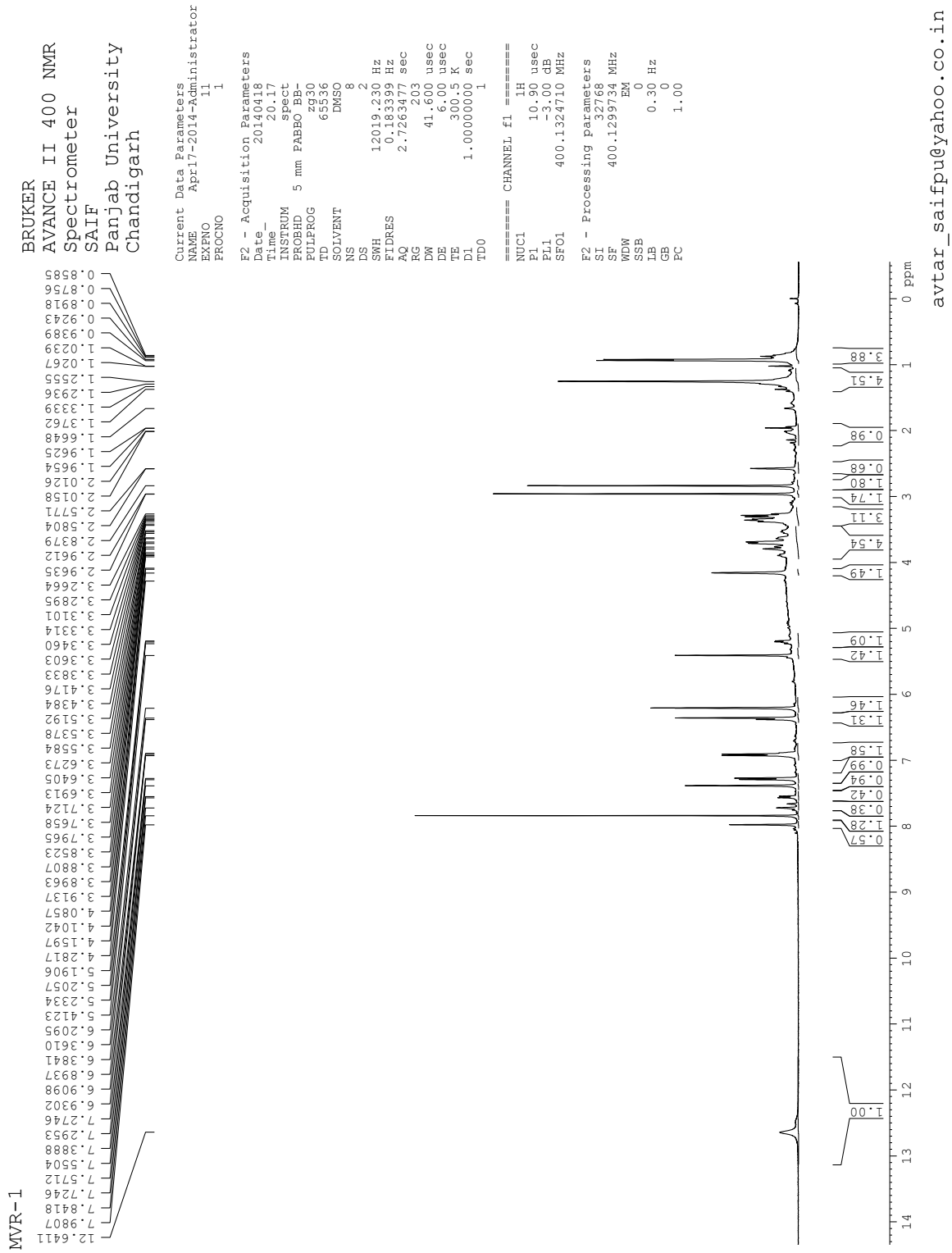
Spectral Data of MVR-1



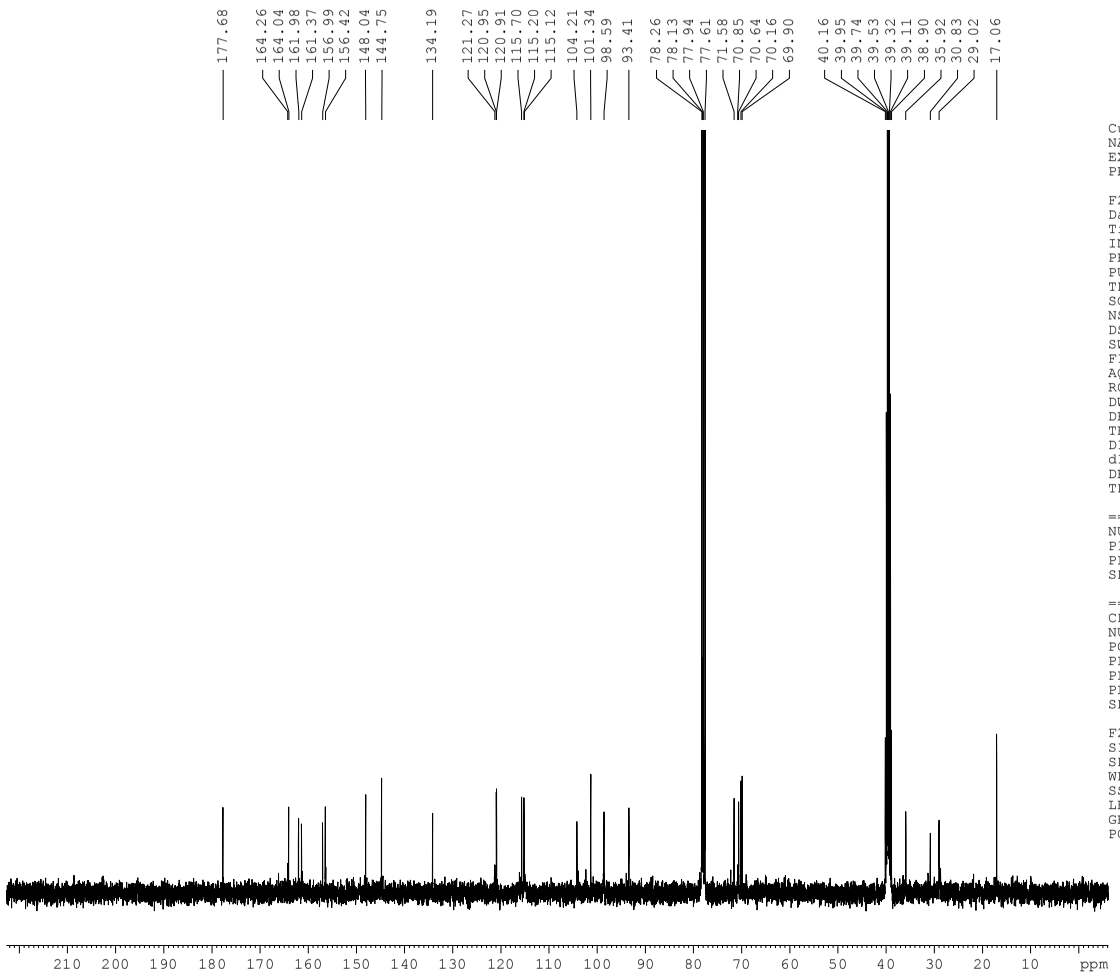
MVR-1 ($C_{15}H_{10}O_7$)

Quercetin

Proton NMR OF MVR-1



MVR-1



BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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EXPNO 12
PROCNO 1

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TD 65536
SOLVENT DMSO
NS 512
DS 4
SWH 29761.904 Hz
FIDRES 0.454131 Hz
AQ 1.1010548 sec
RG 1030
DW 16.800 usec
DE 6.00 usec
TE 300.9 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 9.60 usec
PL1 -2.00 dB
SFO1 100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -3.00 dB
PL12 14.31 dB
PL13 18.00 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
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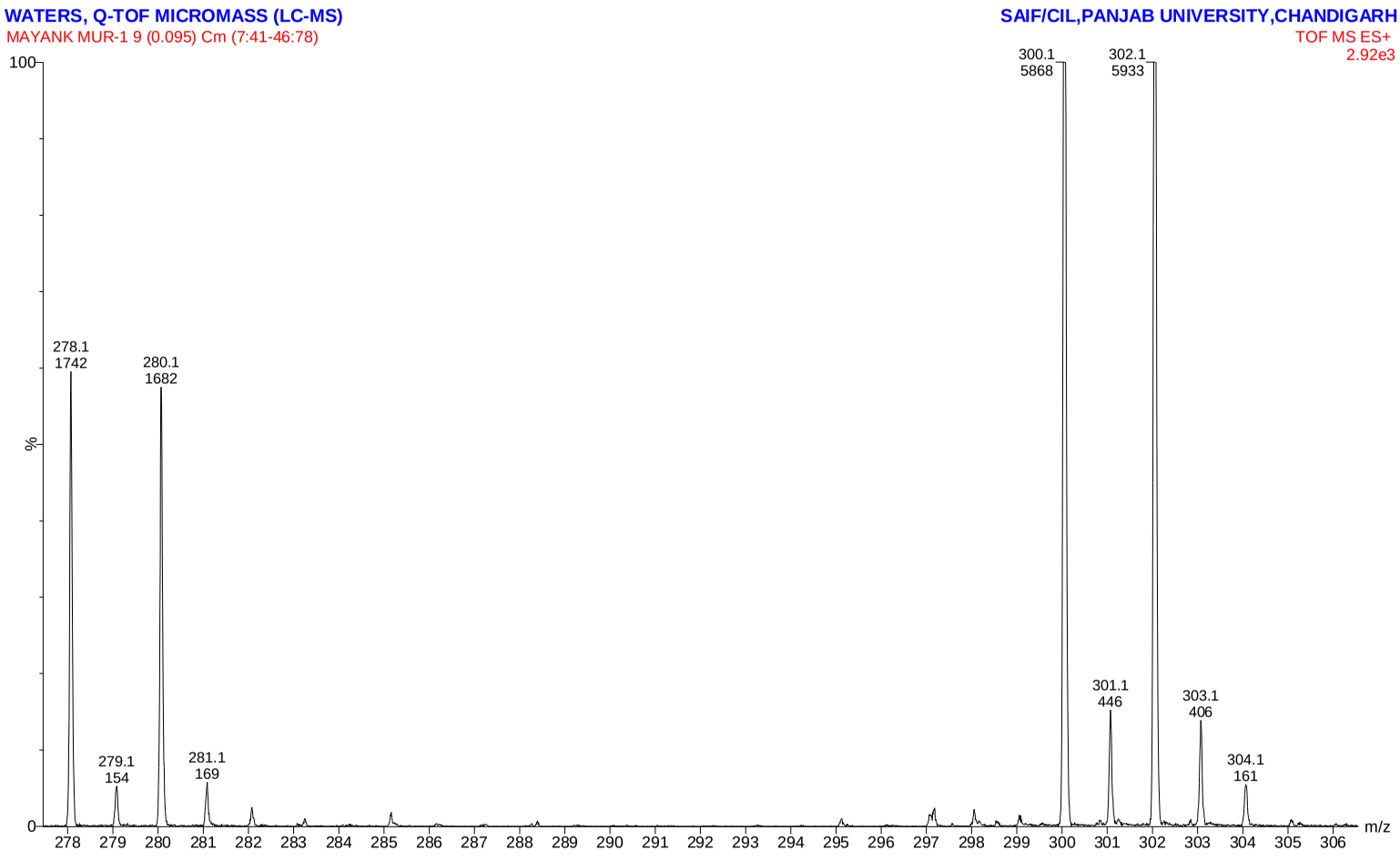
avtar_saifpu@yahoo.co.in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SAIF/CIL,PANJAB UNIVERSITY,CHANDIGARH
TOF MS ES+
2.92e3
100
%
0
278.1
1742
279.1
154
280.1
1682
281.1
169
300.1
5868
301.1
446
302.1
5933
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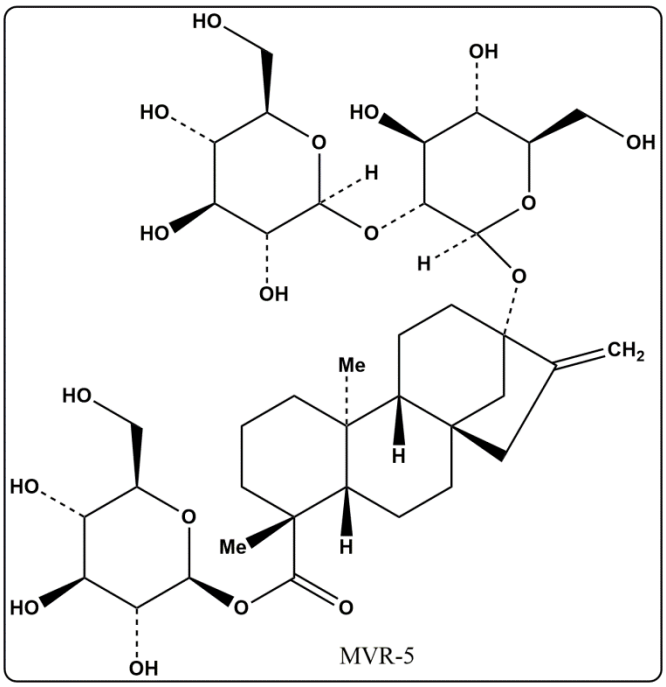
m/z

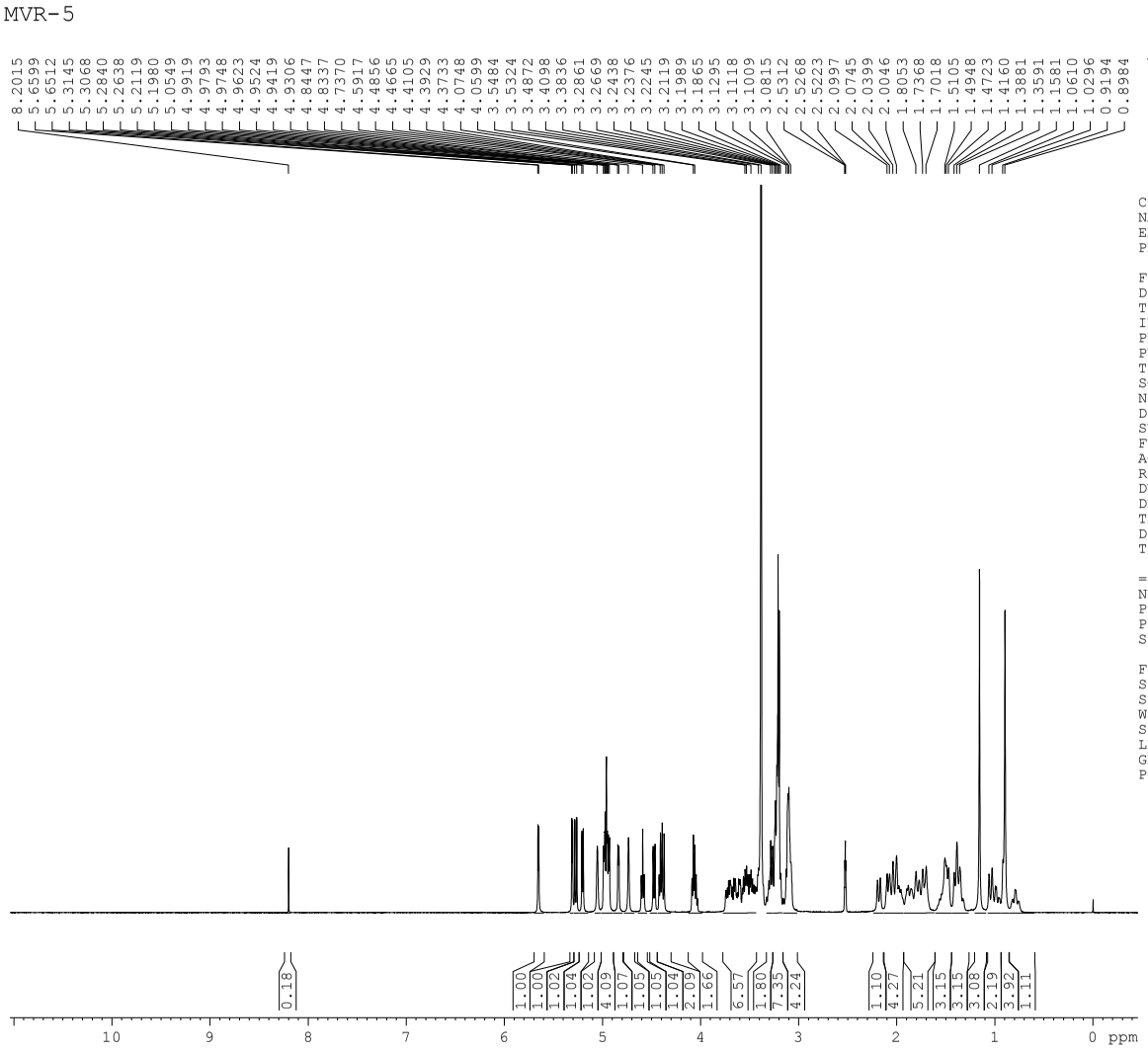
APPENDIX B

Spectral Data of MVR-5



MVR-5

MVR-5



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Spectrometer
SAIF
Panjab University
Chandigarh

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RG                  181
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SFO1        400.1324710 MHz

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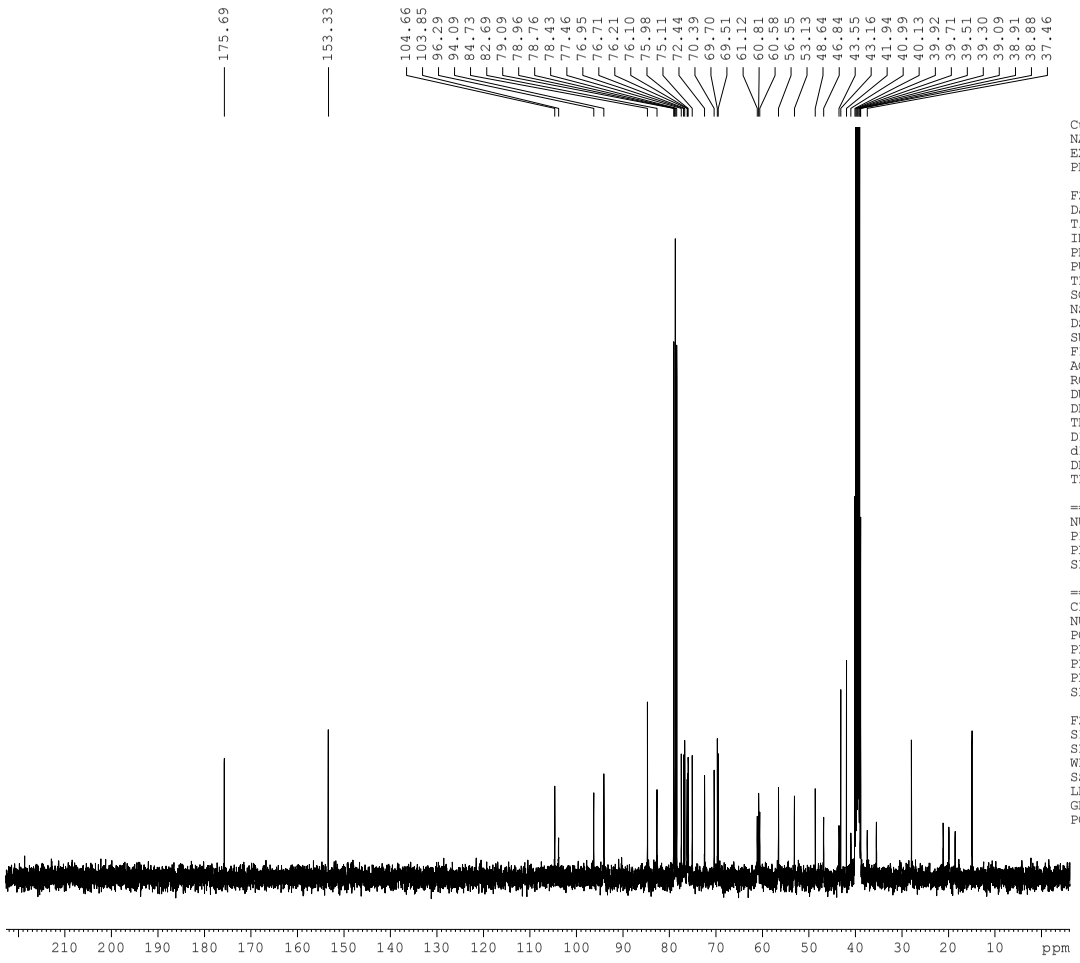

BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

Current Data Parameters

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PROCNO	1

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PULPROG	zgpg30
TD	65536
SOLVENT	DMSO
NS	512
DS	4
SWH	29761.904 Hz
FIDRES	0.454131 Hz
AQ	1.1010548 sec
RG	1150
DW	16.800 usec
DE	6.00 usec
TE	300.6 K
D1	2.00000000 sec
d11	0.03000000 sec
DELTA	1.89999998 sec
TD0	1

======== CHANNEL f1 ========

NUC1	13C
P1	9.60 usec
PL1	-2.00 dB
SFO1	100.6228298 MHz

======== CHANNEL f2 ========

CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
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PL12	14.31 dB
PL13	18.00 dB
SFO2	400.1316005 MHz

F2 - Processing parameters

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SSB	0
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avtar_saifpu@yahoo.co.in

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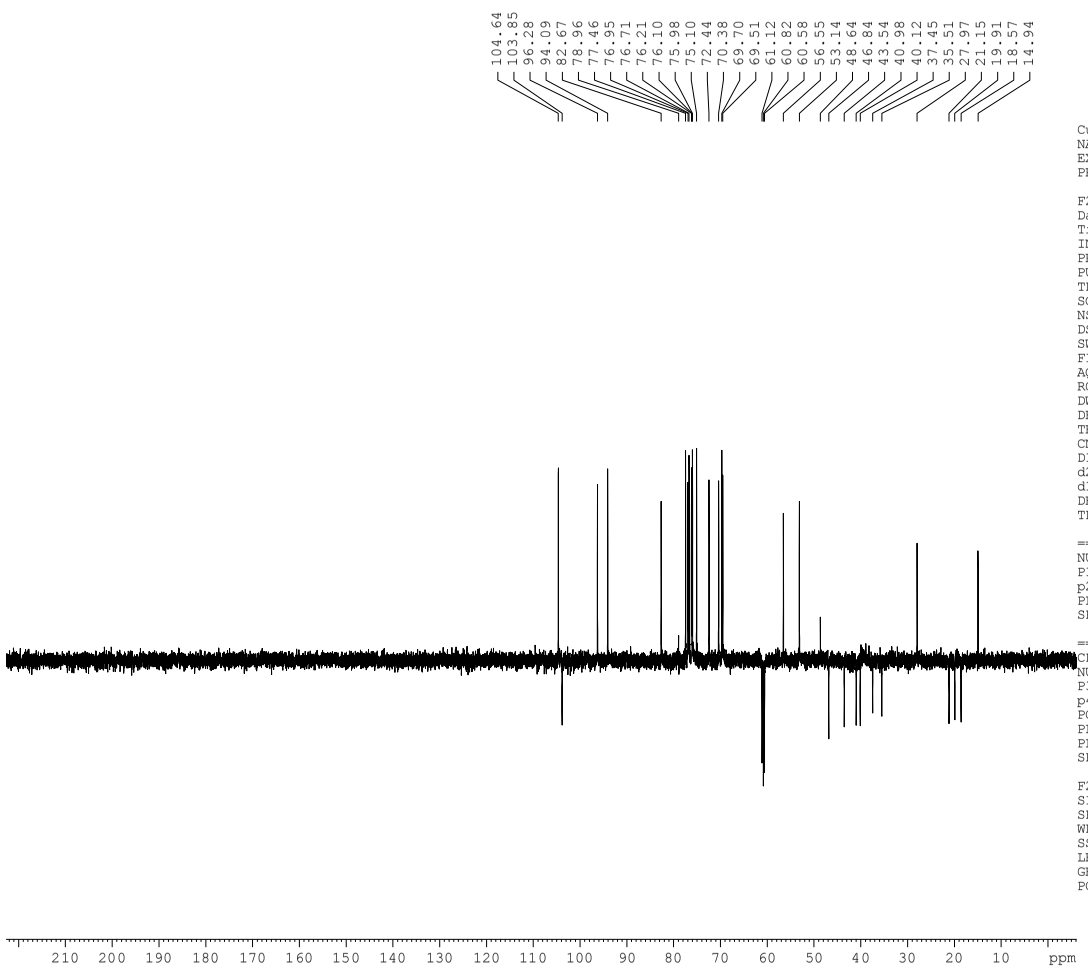

BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

Current Data Parameters
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EXPNO 33
PROCNO 1

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SOLVENT DMSO
NS 256
DS 4
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FIDRES 0.454131 Hz
AQ 1.1010548 sec
RG 1150
DW 16.800 usec
DE 6.00 usec
TE 301.1 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.00001222 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 9.60 usec
p2 19.20 usec
PL1 -2.00 dB
SFO1 100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
P3 10.90 usec
p4 21.80 usec
PCPD2 80.00 usec
PL2 -3.00 dB
PL12 14.31 dB
SFO2 400.1316005 MHz

F2 - Processing parameters
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GB 0
PC 1.40

avtar_saifpu@yahoo.co.in

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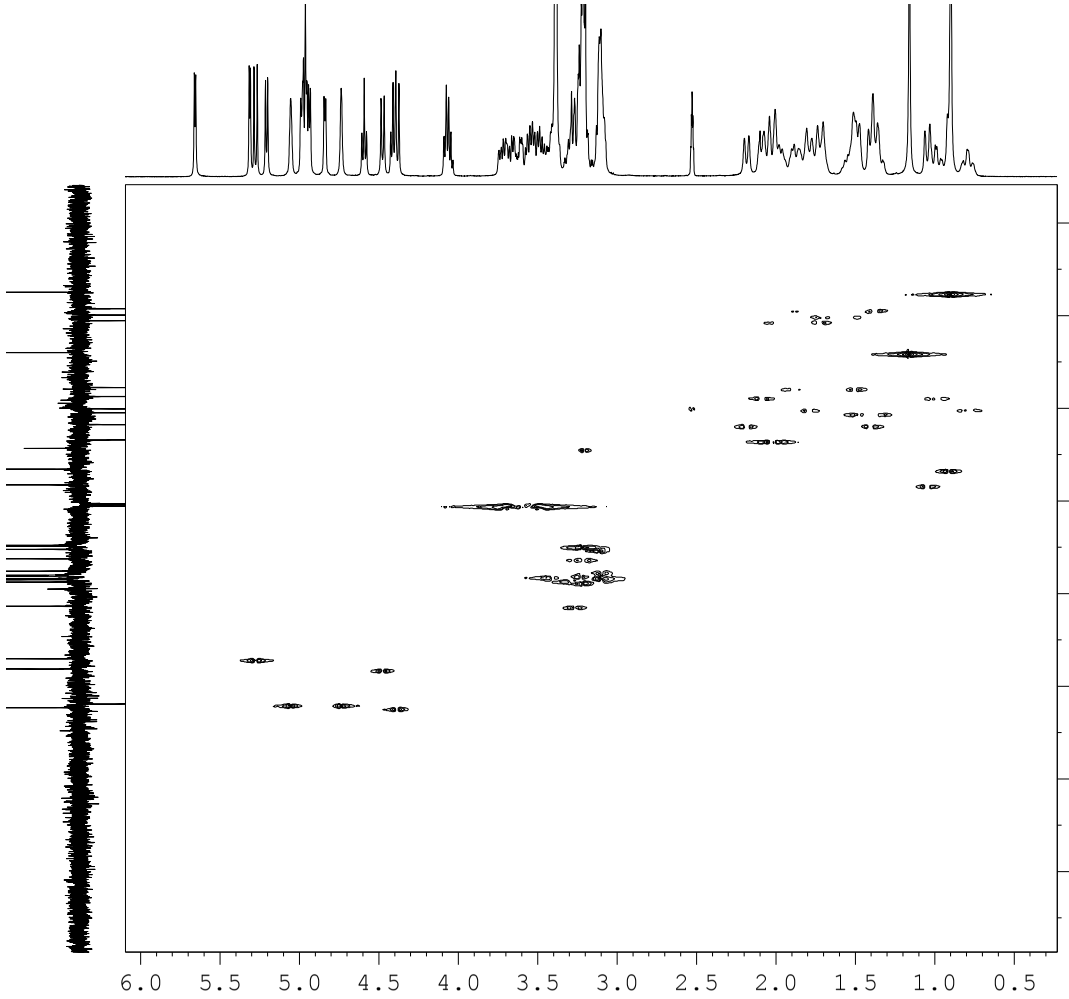


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PROCNO             1

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TD                1024
SOLVENT           DMSO
NS                   2
DS                  16
SWH           2347.418 Hz
FIDRES        2.292400 Hz
AQ            0.2181620 sec
RG                2050
DW             213.000 usec
DE               6.00 usec
TE              301.4 K
CNST2       145.0000000
d0           0.00000300 sec
D1           1.37773395 sec
d4           0.00172414 sec
d11          0.03000000 sec
d13          0.00000400 sec
D16          0.00010000 sec
DELTA        0.00112780 sec
DELTA1       0.00071614 sec
IN0          0.00003000 sec
ST1CNT             128
ZGOPTNS

======== CHANNEL f1 ========
NUC1                1H
P1               10.90 usec
p2               21.80 usec
P28               1.00 usec
PL1              -3.00 dB
SFO1        400.1312583 MHz

======== CHANNEL f2 ========
CPDPRG2           garp
NUC2               13C
P3                9.60 usec
p4               19.20 usec
PCPD2            70.00 usec
PL2              -2.00 dB
PL12             15.26 dB
SFO2        100.6202727 MHz

====== GRADIENT CHANNEL =====
GPNAM1         SINE.100
GPNAM2         SINE.100
GPZ1             80.00 %
GPZ2             20.10 %
P16            1000.00 usec

F1 - Acquisition parameters
ND0                  2
TD                 256
SFO1        100.6203 MHz
FIDRES       65.104164 Hz
SW            165.639 ppm
FnMODE    Echo-Antiecho

F2 - Processing parameters
SI                1024
SF          400.1299930 MHz
WDW               QSINE
SSB                  2
LB                0.00 Hz
GB                   0
PC                1.40

F1 - Processing parameters
SI                1024
MC2       echo-antiecho
SF          100.6127690 MHz
WDW               QSINE
SSB                  2
LB                0.00 Hz
GB                   0
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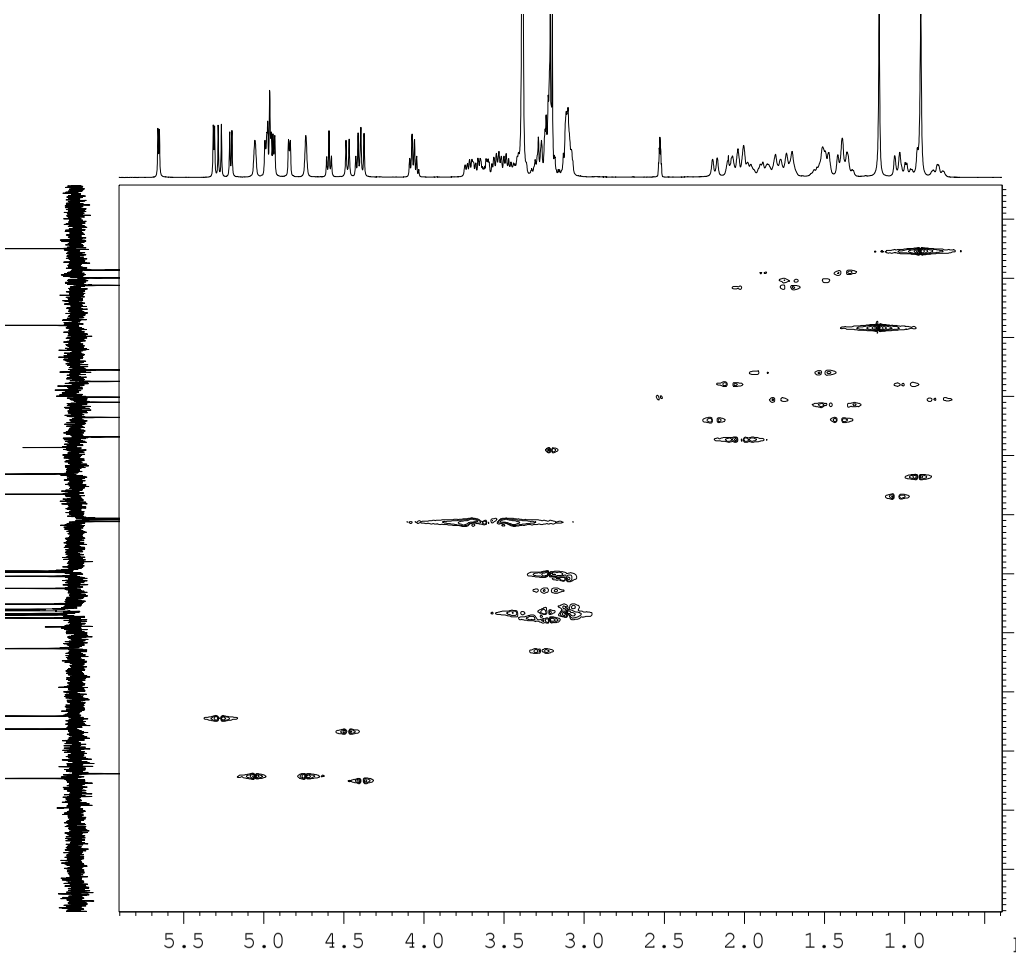
MVR-5

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EXPNO                 34
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PROBHD    5 mm PABBO BB-
PULPROG          hsqcetgp
TD                 1024
SOLVENT             DMSO
NS                     2
DS                    16
SWH             2347.418 Hz
FIDRES          2.292400 Hz
AQ              0.2181620 sec
RG                  2050
DW               213.000 usec
DE                 6.00 usec
TE                301.4 K
CNST2         145.0000000
d0             0.00000300 sec
D1             1.37773395 sec
d4             0.00172414 sec
d11            0.03000000 sec
d13            0.00000400 sec
D16            0.00010000 sec
DELTA          0.00112780 sec
DELTA1         0.00071614 sec
INO            0.00003000 sec
STSCNT                 128
ZGOPTNS

======== CHANNEL f1 ========
NUC1                  1H
P1                 10.90 usec
p2                 21.80 usec
P28                 1.00 usec
PL1                -3.00 dB
SFO1         400.1312583 MHz

======== CHANNEL f2 ========
CPDPRG2             garp
NUC2                 13C
P3                  9.60 usec
p4                 19.20 usec
PCPD2              70.00 usec
PL2                -2.00 dB
PL12               15.26 dB
SFO2         100.6202727 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPZ1               80.00 %
GPZ2               20.10 %
P16              1000.00 usec

F1 - Acquisition parameters
ND0                    2
TD                   256
SFO1          100.6203 MHz
FIDRES        65.104164 Hz
SW              165.639 ppm
FnMODE    Echo-Antiecho

F2 - Processing parameters
SI                  1024
SF           400.1299930 MHz
WDW              QSINE
SSB                    2
LB                  0.00 Hz
GB                     0
PC                  1.40

F1 - Processing parameters
SI                  1024
MC2        echo-antiecho
SF           100.6127690 MHz
WDW              QSINE
SSB                    2
LB                  0.00 Hz
GB                     0
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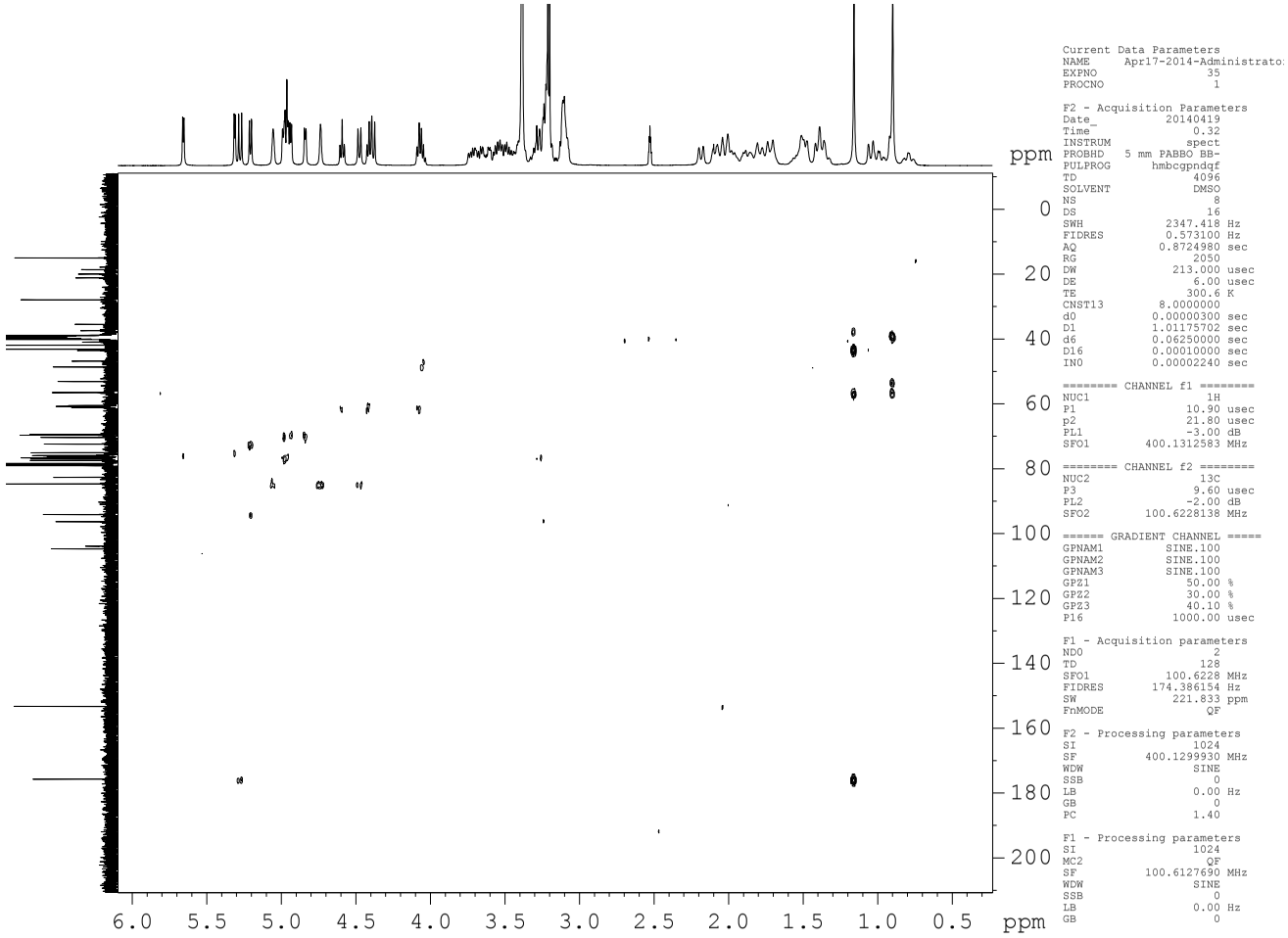


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D16            0.00010000 sec
IN0            0.00002240 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                10.90 usec
p2                21.80 usec
PL1               -3.00 dB
SFO1         400.1312583 MHz

======== CHANNEL f2 ========
NUC2                 13C
P3                 9.60 usec
PL2               -2.00 dB
SFO2         100.6228138 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPNAM3          SINE.100
GPZ1              50.00 %
GPZ2              30.00 %
GPZ3              40.10 %
P16             1000.00 usec

F1 - Acquisition parameters
ND0                    2
TD                   128
SFO1          100.6228 MHz
FIDRES        174.386154 Hz
SW              221.833 ppm
FnMODE                QF

F2 - Processing parameters
SI                  1024
SF           400.1299930 MHz
WDW                 SINE
SSB                    0
LB                 0.00 Hz
GB                     0
PC                 1.40

F1 - Processing parameters
SI                  1024
MC2                   QF
SF           100.6127690 MHz
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SSB                    0
LB                 0.00 Hz
GB                     0
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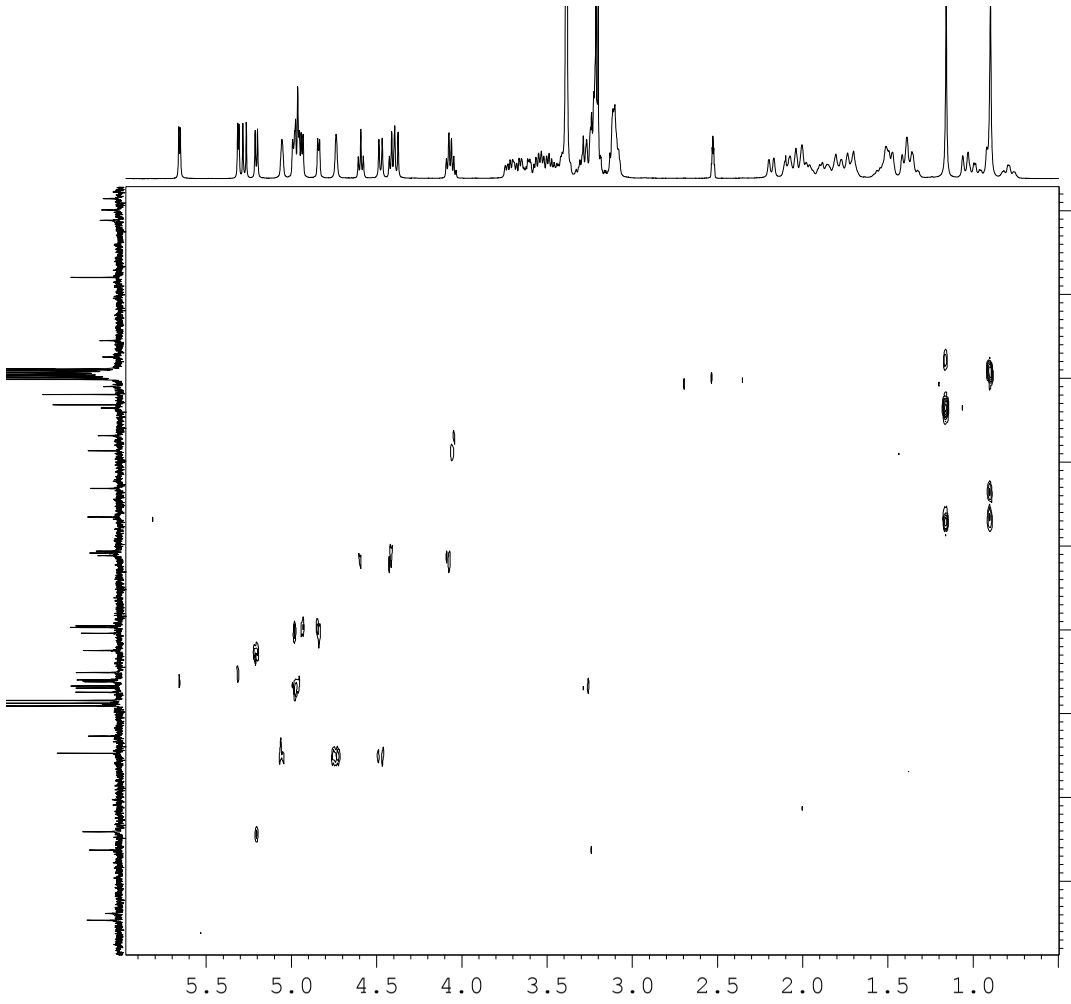


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F2 - Acquisition Parameters
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TD                     4096
SOLVENT                DMSO
NS                        8
DS                       16
SWH                2347.418 Hz
FIDRES             0.573100 Hz
AQ                 0.8724980 sec
RG                      2050
DW                  213.000 usec
DE                    6.00 usec
TE                   300.6 K
CNST13             8.0000000
d0                 0.00000300 sec
D1                 1.01175702 sec
d6                 0.06250000 sec
D16                0.00010000 sec
IN0                0.00002240 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                     10.90 usec
p2                     21.80 usec
PL1                    -3.00 dB
SFO1             400.1312583 MHz

======== CHANNEL f2 ========
NUC2                     13C
P3                      9.60 usec
PL2                    -2.00 dB
SFO2             100.6228138 MHz

====== GRADIENT CHANNEL =====
GPNAM1               SINE.100
GPNAM2               SINE.100
GPNAM3               SINE.100
GPZ1                    50.00 %
GPZ2                    30.00 %
GPZ3                    40.10 %
P16                   1000.00 usec

F1 - Acquisition parameters
ND0                       2
TD                      128
SFO1              100.6228 MHz
FIDRES            174.386154 Hz
SW                  221.833 ppm
FnMODE                    QF

F2 - Processing parameters
SI                      1024
SF             400.1299930 MHz
WDW                     SINE
SSB                        0
LB                      0.00 Hz
GB                         0
PC                      1.40

F1 - Processing parameters
SI                      1024
MC2                       QF
SF             100.6127690 MHz
WDW                     SINE
SSB                        0
LB                      0.00 Hz
GB                         0
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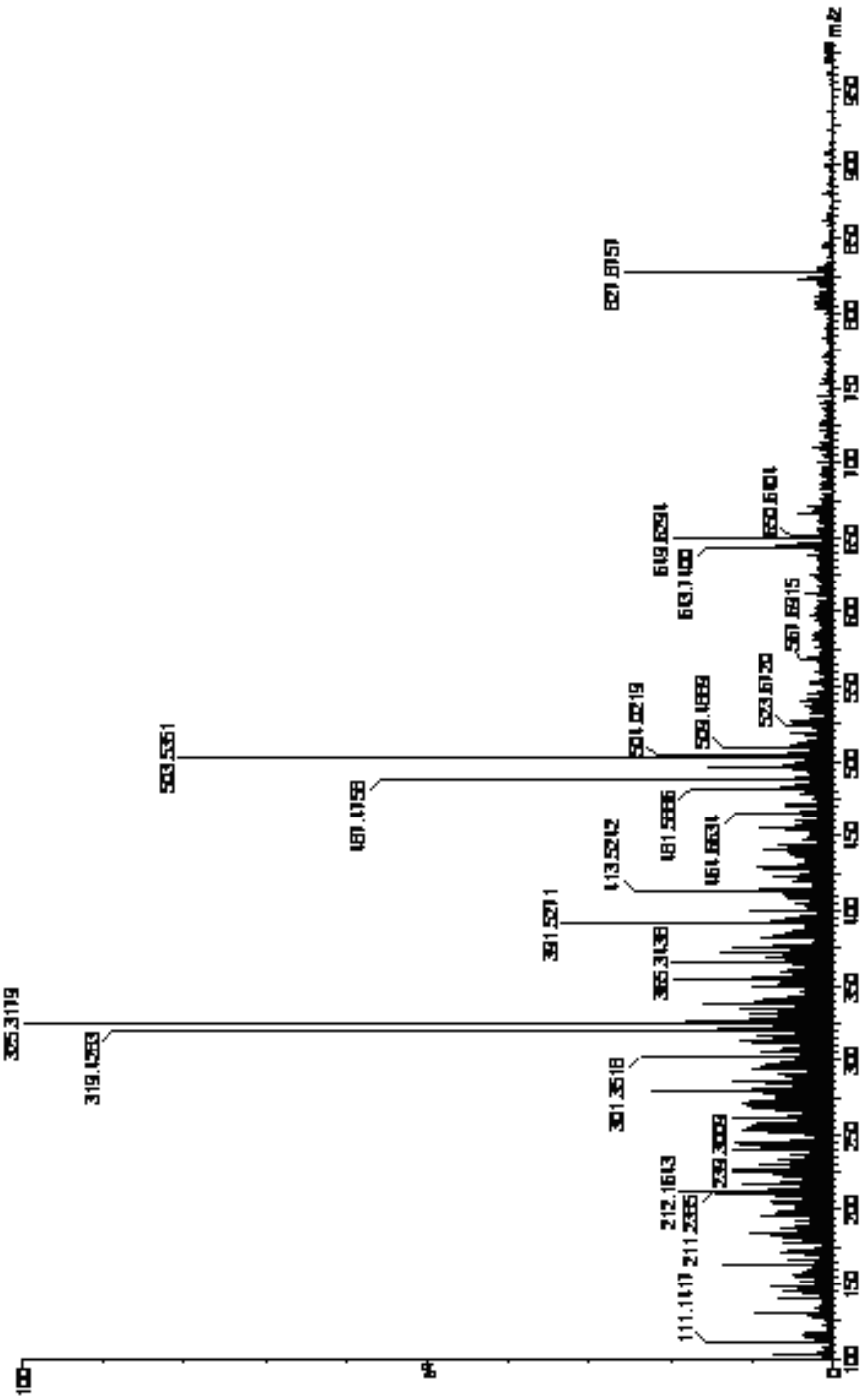

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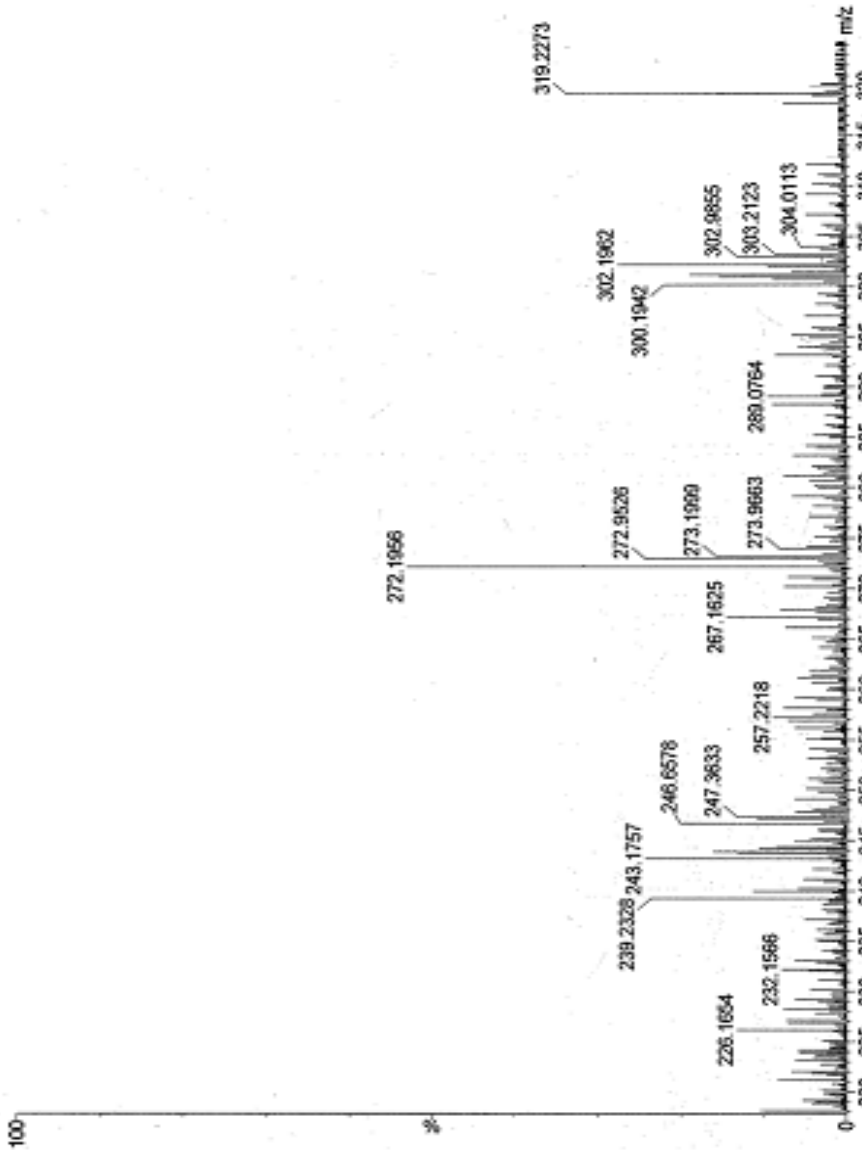
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305
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315
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APPENDIX C

PUBLICATIONS

PUBLICATIONS

1. **Bashyal Insan, M**., and Jaitak, V. (2014). New Approaches to Target Cancer Stem Cells: Current Scenario. *Mini Reviews in Medicinal Chemistry* ***14***(1), 20-34.
2. **Mayank** and Jaitak, V. (2014). Drug Targeting Strategies in Breast Cancer Treatment: Recent Updates. *Anticancer Agents in Medicinal Chemistry* (Accepted, **2014 IF 2.939**)

3. **Mayank** and Jaitak, V (2014). Interaction Model of Steviol glycosides from *Stevia rebaudiana* (Bertoni) with Sweet Taste Receptors: A Computational Approach. ***Phytochemistry*** (Under review, **2014 IF 3.35**)

4. **Mayank** and Jaitak, V. Computational investigation of Natural Alkaloids as Multi-targeted Hedgehog pathway inhibitor in Cancer Stem Cell therapy. ***Medicinal Chemistry research*** (communicated, **2014 IF 1.612**)

5. J. S. Sidhu, R. Singla, **Mayank** and Jaitak, V. Indole derivatives as anticancer agents in breast cancer therapy: A review. ***Bioorganic and medicinal chemistry*** (Communicated, **2014 IF 2.951**)

6. **Mayank**, Jaitak, V Exploration of anticancer potential of *S. rebaudiana* by *in-vitro* and *in-silico* approach (Under process).

7. Jasmin, **Mayank**, Jaitak V, anti-diabetic potential of flavonoids: A Complete study" (Under progress)

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20 *Mini-Reviews in Medicinal Chemistry,* **2014,** *14,* **20-34**

New Approaches to Target Cancer Stem Cells: Current Scenario

Mayank Bashyal Insan and Vikas Jaitak*

Centre for Chemical and Pharmaceutical Sciences, Central University of Punjab, Bathinda (Pb), India-151001

Abstract: Resistance towards chemotherapy and radiotherapy as well as relapse of cancer is the major obstacle in the treatment of cancer. The main factor behind is cancer stem cells (CSCs) which are more resistant to conventional chemotherapy, radiotherapy and are quite able to regenerate whole new tumor again if remain alive during treatment. Targeting CSCs along with actively dividing cancer cells may significantly contribute to the solution of the problem of resistance and relapse. Various approaches are implemented to eradicate CSCs which include CSC markers specific compounds, Drugs which disturb niche and various inhibitors/modulators of signaling pathways. Hedgehog (Hh), Wnt and Notch pathways are modulated/inhibited using various agents and shown beneficial results in multiple forms of cancer. Many inhibitors/modulators of these pathways have been entered in the clinical trials. MicroRNAs have also been developed as anti CSCs agents. In this review, we have covered current status of CSC targeting therapy based on CSC markers, CSC niche, Hedgehog, Wnt, Notch pathway along with MicroRNA based targeting strategies and possibility of implementation multi-targeted anti-CSC therapy for the better outcome of the results.

Keywords: Cancer stem cell, Chemotherapy, CSCs niche, Hedgehog, MicroRNA, Multi-targeted anti-CSC therapy, Notch, Radiotherapy, Wnt.

1. INTRODUCTION

Cancer is a disease which is known from decades but still uncertain about the origin of cancer. It is most likely that cancer originates from a single cell. Various types of mutations cause the cell to divide in such a way to generate a whole tumor capable of invasion and metastasis. Mutations in a normal stem cell can convert it into tumorigenic cell or same may happen to differentiated cell and in either case the resulting tumorigenic cell capable of producing whole malignant tumor may be termed as cancer stem cell (CSCs) [1]. Significant improvement in the survival rate of cancer patients has been noticed due to technical advancement and intensive research in this field. To find a complete and full proof cure is still a challenge as currently used anticancer therapies such as chemotherapy, and radiotherapy are facing major problems such as the rapid emergence of resistance and relapse of disease [2]. Recent findings suggest that cancer stem cells (CSCs) have a significant contribution to this process [3]. The first evidence relating to CSCs obtained in 1994 when a subpopulation of Acute myelogenous leukemia (AML) cells was transplanted to immune-compromised mice and was capable to produce cancer [4]. Subsequently, such cells with stem like characteristics have been reported in many types of tumors including breast [5] and brain cancer [6]. Currently CSCs have been reported in various types of cancers such as skin, head, neck, thyroid, lungs, GIT, reproductive track, cervix, retina and gastrointestinal track [1]. Existence of such cells in wide range of cancers provided a valid proof regarding universal existence of CSCs. CSCs have the ability to regenerate whole tumor after successful therapy that may contribute to relapse of cancer as transplantation of about 100 CSCs produces tumor in immune-deficient mice [5]. The classical chemotherapy targeted towards actively dividing cells ignores CSCs which seems to be the major limitation in permanent cure of cancer. CSCs having properties such as long lifespan, expression of several ABC transporters, more efficient DNA repair capacity, resistance to apoptosis and relatively quiescent nature [3] which directly or indirectly contribute to chemotherapy related resistance. Resistance towards drugs such as mitoxantrone has been reported by Hirschmann-Jax and colleagues in their study which indicated high drug efflux capacity by CSCs [7]. In other study Ho *et al.* mentioned that side population (SP) in lung cancer has the ability to regenerate SP and non-SP cells, more in-vivo tumorigenic ability and has innate chemotherapeutic resistance [8]. Bao *et al.* demonstrated the CSCs mediated resistance of glioma towards radiotherapy because of preferential activation of DNA damage checkpoint response and an increase in DNA repair capacity [9]. To target CSCs can be a noble way to permanently get rid of cancer without relapse and resistance. In this review, we have covered various factors involved in the conversion of normal stem cell to CSCs as well as current status of CSC targeting therapy.

2. CANCER STEM CELL

CSCs represent a subpopulation of tumor cell having self- renewal capacity, ability to regenerate whole tumor, innate resistance to cytotoxic agents and have tumor-propagating ability when serially transplanted into immune-compromised animal model with heterogeneity of cell types similar to primary lesions from where they are derived [10].

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1875-5607/14 $58.00+.00

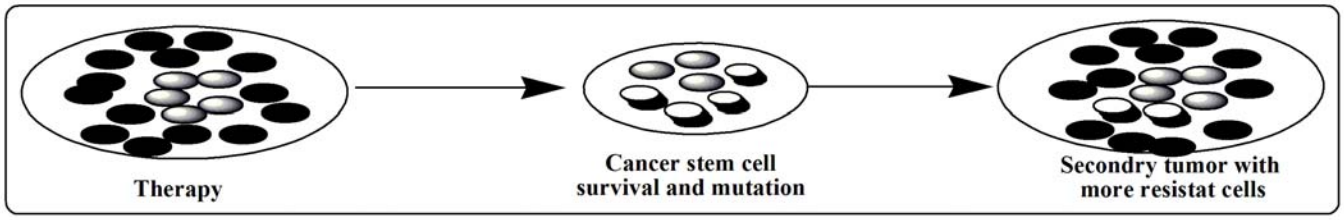

Fig. (1). Classical cancer therapy able to kill actively dividing cancerous cells (black) but cannot kill CSCs (grey). Exposure of drugs or radiations causes some mutations in survived CSCs which produces secondary tumor with more resistant towards conventional cancer therapy (white with shadow).

All these factors indicate that CSCs are the main cause behind resistance and relapse of cancer (Fig. **1**).

2.1. Cancer Stem Cell Markers

Unique pattern of surface proteins as well as cellular activity has been used as a marker for various forms of CSCs. Several reports indicate that these markers may be functionally important. Piotrowicz *et al*. demonstrated the importance of CD44 biomarker as an adhesion molecule and important for cell migration and metastasis [11]. This observation has been complemented by the fact that targetting CD44 is associated with reduction in metastasis in several cancer types [12]. Inhibition of CD177, a receptor tyrosine kinase by imatinam has been found to be effective in case of gastrointestinal stromal tumors and chronic myelogenous leukemia [13]. CSC marker ALDH is responsible of some chemoresistance as in preclinical studies knock down of ALDH1A1 shown to restore chemo-senstivity in ovarian cancer cell lines [14]. Markers are not just the identity of CSCs but are functionally important in maintaining CSCs as well as cancer. CSC markers can be targeted to inhibit the cancer growth. CSC markers are diverse in nature and still lacking of universal marker is the major obstacle in the treatment of cancer (Table **1**).

2.2. Signaling Pathways and Factors Involved in CSC

Signaling pathways Hedgehog (Hh), Notch, and Wnt/β-catenin are well-known embryonic pathways and found to be over activated in CSCs [40]. Over activation of Hh pathway has been found to be associated with tumors such as basal cell carcinoma, glioma and medulloblastoma [41]. Moreover, ligand dependent activation of Hh is associated with various forms of cancers such as lung, pancreatic, prostate and breast cancer [42]. Over-activation of Wnt pathway has been found to be involved in a variety of cancers such as ovarian cancer, sporadic colorectal cancer and medulloblastoma [42-44]. Association of Notch pathway with cancer is clearly understood by the depletion of cancer stem like cell and suppression in embryonal brain tumor by notch inhibitor [45]. About 30% of non-small cell lung cancer is associated with altered notch signaling pathway [46]. Other factors are epidermal growth factor (EGFR)-like/EGF receptor [47], leukemia inhibitory pathway (LIF) [48], liptin [49], telomerase [50], insulin like growth factor-1 [51], prolactin and growth hormone [47] which are involved in governing self-renewal, differentiation in stem cells and their deregulation seems to transfer normal stem cell into CSCs (Fig. **2**).

Table 1. Markers used to identify CSCs in some common tumors.

Cancer Type	Markers	References
Leukemias	$CD34^{+}CD38^{-}$	[15]
Breast cancer	$CD44^{+}CD24^{-/low}$	[5]
	$ALDH\text{-}1^{+}$	[16]
	$CD133^{+}$	[17]
	$CD133^{+}CXCR4^{+}$	[18]
	$CD49F^{+}DLL1^{high}DNER^{high}$	[19]
Liver cancer	$CD133^{+}$	[20]
	$CD90^{+.}$	[21]
	EpCAM	[22]
	$CD13^{+}$	[23]
	$CD44^{+}$	[24]
	$CD24^{+}$	[25]
	OV6	[26]
	GEP	[27]
Glioblastoma	$CD133^{+}$	[28]
	$SSEA\text{-}1^{+}$	[29]
	MET Oncogene	[30]
Pancreatic cancer	$EpCAM^{+}CD44^{+}CD24^{+}$	[31]
	$CD133^{+}/CD133^{+}CXCR4^{+}$	[32]
	c-Met	[33]
	$ALDH\text{-}1^{+}$	[34]
Prostate cancer	CD44+ alpha2beta1^{high} $CD133^{+}$	[35]
	$CD133^{+}$ $CXCR4^{+}$	[36]
	IGR-CaP1	[37]
Head and neck cancer	$CD44^{+}$ BMI-1	[38]
	$ALDH^{high}$	[39]

3. TARGETING CSCs

To targeting CSCs along with actively dividing cells may reduce the risk of resistance development and the relapse rate of disease. CSCs may be targeted by killing or by inducing differentiation [52]. CSC specific targets include CSC specific marker based attack, attacking signaling pathways,

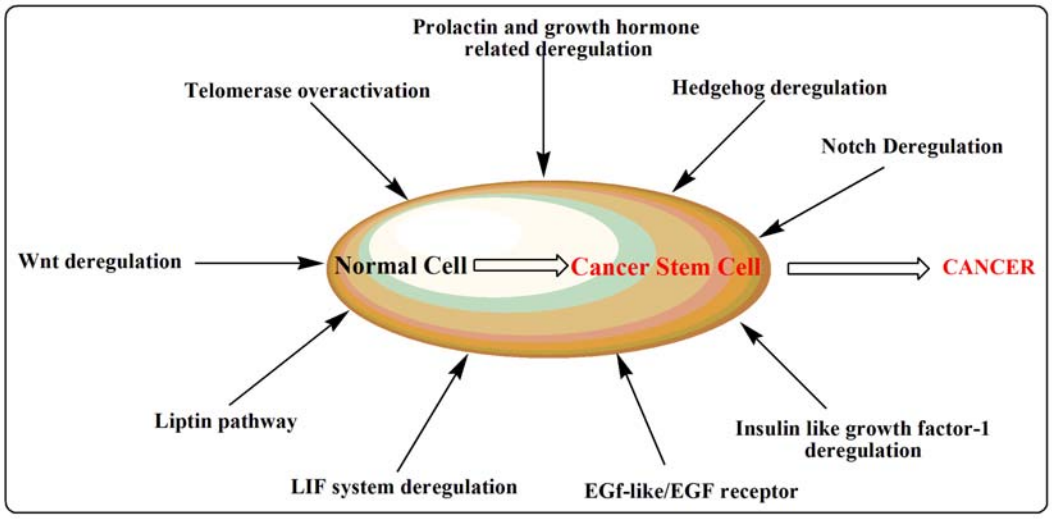

Fig. (2). Factors and signaling pathways involved in conversion of normal cell to CSC.

disturbing niche and microRNA based strategies which either sensitize CSCs towards conventional chemotherapy or directly attack CSCs.

3.1. CSC Markers

CSC markers can be targeted to kill CSC or to induce differentiation. It has been found that adhesion molecule (CD44) targeting provided the positive results in case of AML [53]. Moreover, anti-CD44, antibodies H90 and A3D8 reported to promote differentiation in AML CSCs, inhibit proliferation by stabilizing p27 as well as induce apoptosis [54] which clearly demonstrated the importance of CD44 as CSC target and indicates the function of CD44 in proliferation, survival, apoptosis, and differentiation. Anti-CD44 agents including humanized HuARH460-16-2 anti-CD44 monoclonal antibody have been developed [54]. $CD34^{+}/CD38^{-}$ CSC expresses Siglec-3 (CD33) in abundance response well to CD33-targeting drugs such as gemtuzumab/ ozogamicin (**1**) (Fig. **3**).

The effect of gemtuzumab/ozogamicin was dose dependent, induced apoptosis and found to synergize with nilotinib (**2**) as well as bosutinib (**3**) in inducing growth inhibition in leukemia cells [55]. It has been found that inhibition of c-MET CSC marker by XL184 (**4**) resulted in marked decrease

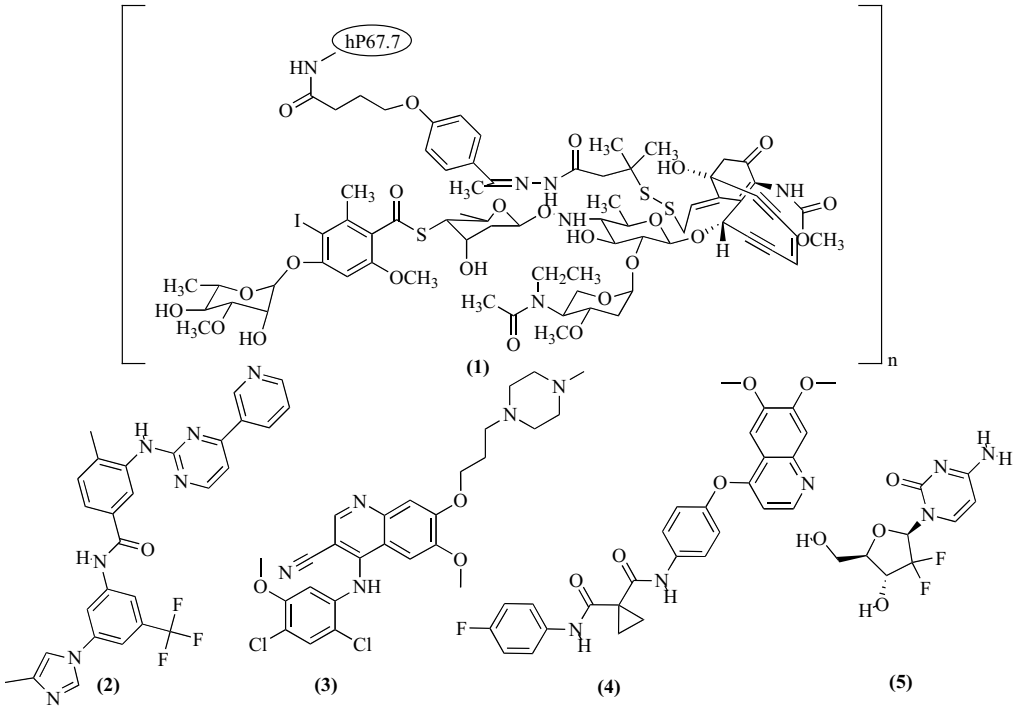

Fig. (3). Compounds used as targeting agent against CSC markers. hP67.7 is humanized monoclonal antibody directed against CD33. hP67.7 is linked with colicheamicin where 'n' represents 1, 2, 3, 4, 5,…

of CSC. Combination of XL184 (**4**) and gemcitabin (**5**) completely inhibits the tumor growth in primary pancreatic cancer xenograft NOD-SCID mices [33]. EpCAM is a recently discovered CSC marker which is targeted by bispecific antibody EpCAMxCD3 results in efficiently reduction in tumor engraftment and retardation in growth of pancreatic carcinoma xenograft [56]. Thus, c-MET inhibitors, EpCAM inhibitors and related physiology may play a crucial role in tough form of pancreatic cancers. Another CSC marker, CD133 was targeted using immunotoxin dCD133KDEL and found to show potent antitumor activity in head neck cell carcinoma xenograft as well as to selectively inhibit MDA-MB-231 ductal breast carcinoma cell that contain minority of $CD133^+$ population [57]. Several other CSC markers have been targeted for *in vitro* or in animal model study in various forms of cancer are mentioned in Table **2**.

CSC markers are not only important for identification but they are also known to be useful targets. Existence of diverse nature of CSC, alongwith associated markers in a typical cancer and occurrence of same surface protein in normal stem cells may produces challenges which may face selectivity related problems in targeting CSC markers. It may be anticipated that these markers are not only the markers but definitely having some function and understanding of which may provide right path towards cancer chemotherapy. Moreover, such markers may assist in proper selection of regimen of therapy and current status of treatment as well as disease. CSC markers appear as a useful tool but lack of universal CSC specific marker is a real challenge still to be worked out.

3.2. Disturbing Cancer Stem Cell Niche

Stem cell niche is a microenvironment where stem cell are formed which interacts with stem cells to regulate cell fate. The understanding of stem cell niche is based on the intensive research on hematopoietic stem cell and its niche i.e. osteoblastic [61] and vascular niche [62]. Stem cells belong to G_0 phase in osteoblast niche and continue cell cycle when in vascular niche probably due to hypoxic condition in osteoblast niche [63] which clearly indicates the importance of niche in stem cell growth and maintenance. Some factors might be responsible to govern the phase of cell cycle by inducing migration of stem cell between this microenvironment. Intensive research has provided various evidences in favor of such niche in case of CSC named CSC niche. The onset of disease in transplanted mice model depends significantly on the activity of CD44 in leukemias. Decrease in its activity is found to be associated with reduced disease progress because CD44 is important for binding and proper establishment of CSC niche [53, 64]. Importance of endothelial cell secreted factors in maintenance of CSCs in case of solid brain tumor provided the evidence that brain tumor microvasculature forms a niche that is critical for the maintenance of CSCs [65]. Proper identification of niche is crucial for the functioning of CSCs which can be targeted by disturbing CSC niche. CSCs in solid tumors are found to be concentrated around blood vessels, and importance of endothelial cell signals makes angiogenesis a suitable target. Vascular endothelial growth factor (VEGF) inhibitors such as antivascular endothelial growth factor antibody bevacizumab [66] seems to be a useful in targeting CSCs by disturbing niche. Hypoxia is one of the major problems in cancer therapy and may be considered as CSC niche which maintains and regulate CSCs by HIF-2, mainly by HIF-2a. The molecular downstream mechanisms of regulation are still unclear but ASPHD2, MAML3 and NFATc2 were involved and regulated by HIF-2 [67]. Topotecan (**6**) and digoxin (**7**) are the HIF inhibitors which were approved for clinical applications in several cancers [52] (Fig. **4**). Molecules like langkamide (**8**), piplartine (**9**),

Table 2. CSC markers target in various cancer types.

Cancer Type	Markers Targeted	Targeting Agents	References
Leukemias	CD44	AntiCD44 antibodies, H90 and A3D8, HuARH460-16-2 anti-CD44 monoclonal antibody	[54]
Pancreatic cancer	EpCAM c-MET	EpCAMxCD3 XL184	[56] [33]
Head and neck cancer	CD133	dCD133KDEL	[57]
Breast cancer	CD133	dCD133KDEL	[57]
Liver cancer	CD133 EpCAM CD44 CD90 CD13 CD24	Anti-CD133 antibody, lupeol Bispecific antibody EpCAMxCD3 Anti-CD44 antibody Anti-CD44 antibody CD13 inhibitor ubenimex, anti-CD13 antibody Anti-CD24 antibody	[58]
Brain cancers	CD133 L1CAM	anti-CD133-SWNTs followed by 8o8 NIR exposure shRNA	[59] [60]

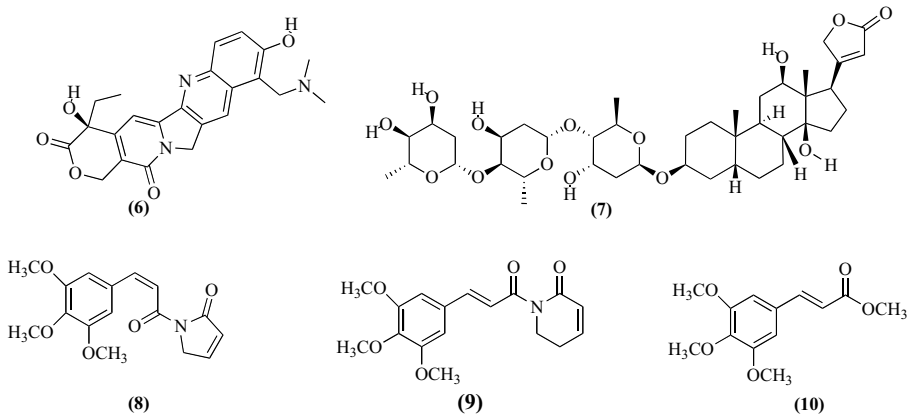

Fig. (4). Natural HIF Inhibitors.

and 3,4,5-trimethoxycinnamic acid (**10**) showed HIF-2 inhibitory activity [68] may be implemented for targeting HIF mediated regulation of CSCs.

3.3. Hedgehog Signaling Pathways

The Hedgehog (Hh) pathway is a developmental signaling pathway involved in numerous developmental processes, including determination of cell fate, patterning, proliferation, survival, and differentiation. In mammals, three Hh proteins i.e sonic Hh, Indian Hh and desert Hh are derived from proteolysis of inactive precursor that contain its own auto-processing domain. In cells that make Hh, newly made protein enters the secretory pathway and undergoes autoprocessing and lipid modifications, resulting in the addition of a palmitoyl group to its NH_2 terminus and cholesterol to its COOH terminus. Hh acts by binding to 12 transmembrane glycoprotein components patched (Ptc) which in the absence of Hh suppress the activity of 7 transmembrane glycoprotein Smo. Hh releases the suppression effect of Ptc on Smo. Surface localization and activation of Smo initiate a signaling cascade and resulting the prevention of degradation of transcriptional regulators Gli2 and Gli3 which induces the expression of

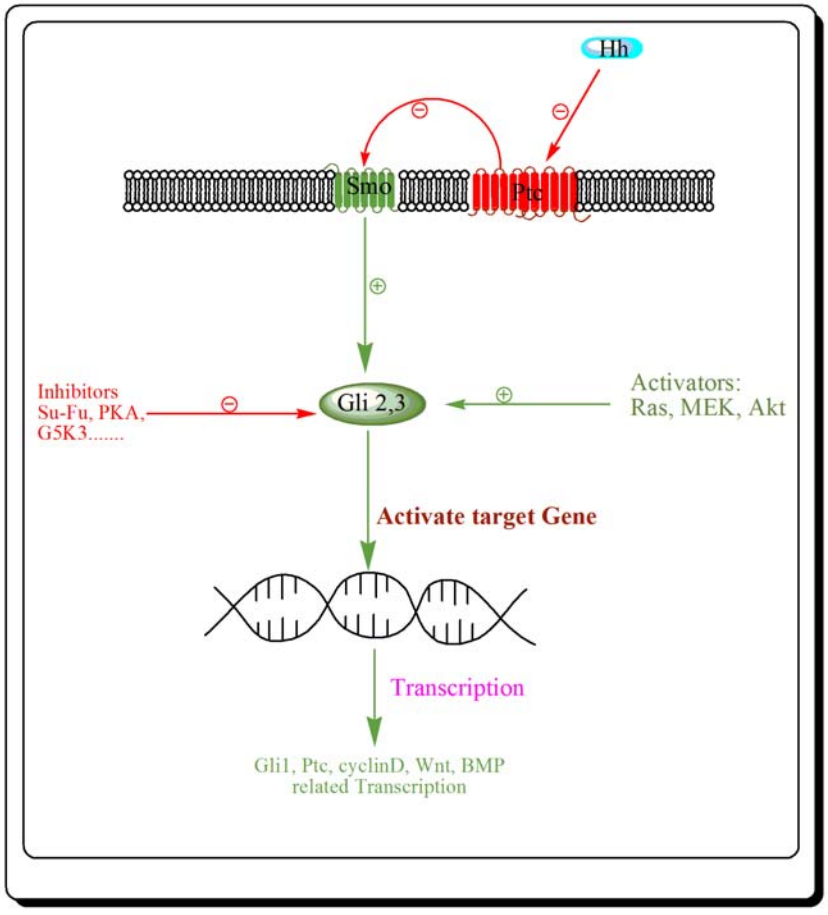

Fig. (5). Hedgehog Signaling Pathway.

genes such as Gli1, Ptc, cyclinD, Wnt and BMP (Fig. **5**). Many other regulators of Gli proteins are known among them Ras, MEK, Akt are the main positive regulators. PKA (cAMP-dependent protein kinase), CK1a (casein kinase 1a), GSK3b (glycogen synthase kinase 3) and SuFu (suppressor of fused) are the negative regulators [69].

Over activation of Hh pathway has been found to be associated with cancer [70]. It was first observed that aberrant Hh signaling due to Ptc mutation was associated with Gorlin syndrome characterizes by numerous basal cell carcinomas (BCCs) and predisposed to other kind of cancers as well [71]. Now the role of Hh pathway in cancer and CSCs is well established [40]. Possible cause of hyperactivation of Hh signaling involves inactivated Ptc, overactivated Smo, improper functioning of inhibitors such as Su-Fu, Pak, G5K3 and activators such as Ras, MEK, Akt but inactivated Hh, over-activated Smo, and Su-Fu related mutations are commonly involved [72]. Hh pathway inhibitors generally categorized into inhibitors of Hh protein, Smo and inhibitor of Gli mediated transcription [73] but majority of Hh inhibitors reported to date binds to Smo protein. Cyclopamine (**11**) (Fig. **6**) a natural alkaloid obtained from *Veratrum californicum* was the first naturally derived molecule which binds and inhibits Smo [74].

Smo inhibitors are under various phases of clinical trials (Table **3**), which include IPI-926 (**12**) [75] a semi-synthetic derivative of cyclopamine, GDC-0449 (**13**) [76], LDE-225 (**14**) [76], XL-139 (BMS-833923) (**15**) [76], LY2940680 (**16**) [76], PF-04449913 (**17**) [77], TAK-441 (**18**) [78] and LEQ-506 (**19**) [79]. Other Hh pathway inhibitors like robotnikin (**20**), its analog (**21**) which inhibits Hh protein and physalin F (**22**), physalin B (**23**) which inhibit Gli mediated activity have been reported [73].

3.4. Wnt Signaling Pathway

Wnt signaling pathway was first discovered by Nusse and Varmus in 1982 [80]. It involved β- catenin dependent canonical pathway and β- catenin independent non canonical pathway. The components of canonical signaling includes 19 Wnt ligands, 10 Fizzled (Fzd) receptors and two co-receptors Lrp5 and Lrp6 belonging to low-density-lipoprotein-related protein (Lrp) family [81, 82]. Canonical Wnt pathway (Fig. **7**) involved the interaction of Wnt ligand with Fzd and Lpr proteins. This interaction results in Dishevelled (Dvi) mediated inhibition of β-catenin destruction complex which consists of glycogen synthase kinase 3β (Gsk3β), axin/ conductin, casein kinase 1α (Ck1α) and adenomatous polyposis coli (Apc) that leads to accumulation as well as translocation of β-catenin to the nucleus. In nucleus, it displaces repressor proteins Groucho, Histone deacetylase and interacts with co-activators such as cyclic AMP response element binding protein (CBP). Formation of activated complex with Tcf/lef class of DNA binding proteins leads to

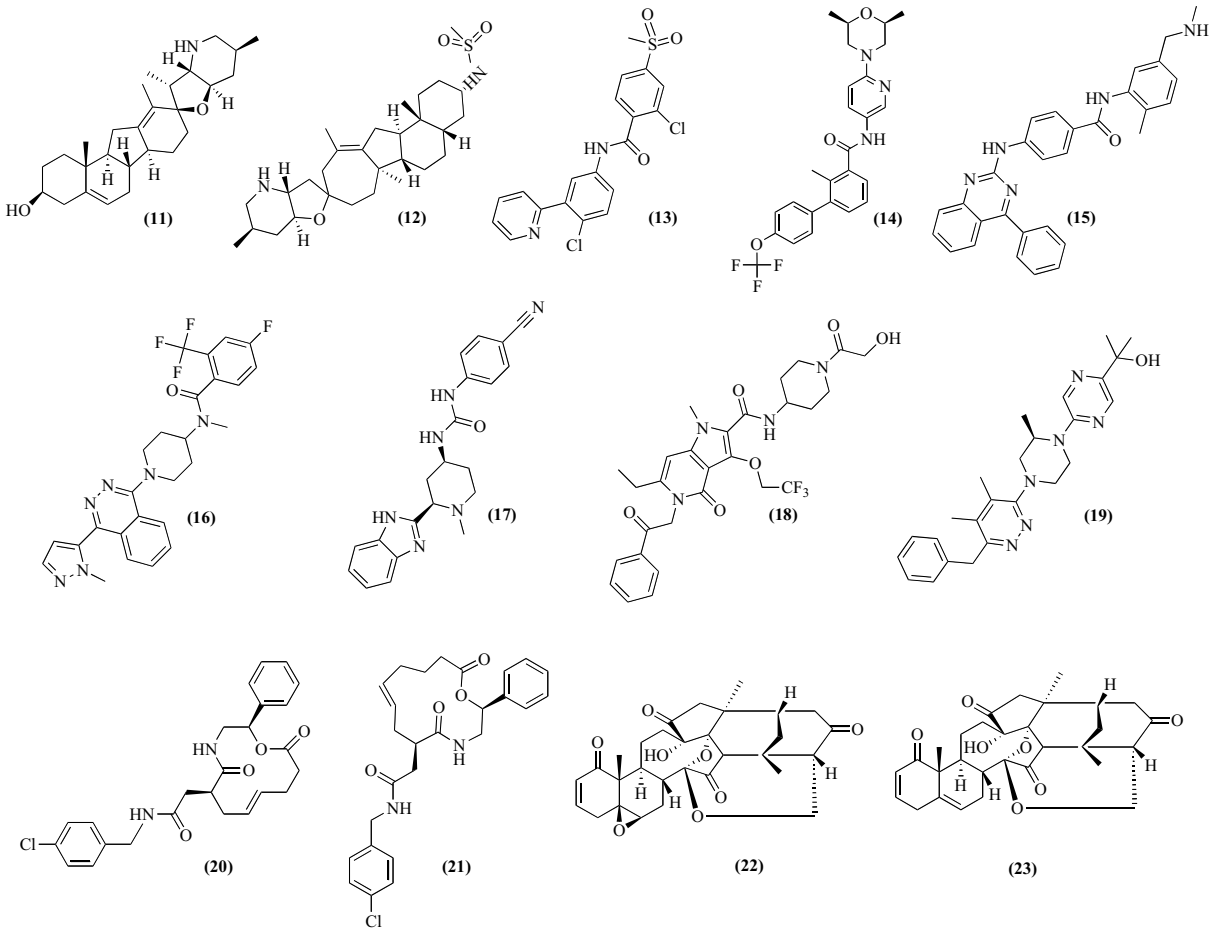

Fig. (6). Hh signaling pathway inhibitors.

Table 3. Current Status of Hh pathway inhibitors in clinical trials (as per clinicaltrials.gov).

Drug	Indications	Phase
GDC-0449	Metastatic pancreatic cancer, extensive stage small cell lung cancer, basal cell nervous syndrome, solid cancer.	Phase II
LDE-225	Extreme stage small cell lung cancer, pancreatic cancers, medulloblastoma, atvancet solid tumors.	Phase I, II
IPI-926	Recurrent Head and neck cancer, myelofibrosis, metastatic pancreatic cancer, chondrosarcoma.	Phase I, II
PF-04449913	Solid tumors, acute myloid leukemia and hematological cancers.	Phase I, II
TAK-441	Advanced nonhematologic malignancies.	Phase I
LEQ-506	Advanced Solid Tumors, Recurrent or Refractory Medulloblastoma, Locally Advanced or Metastatic Basal Cell Carcinoma.	Phase I

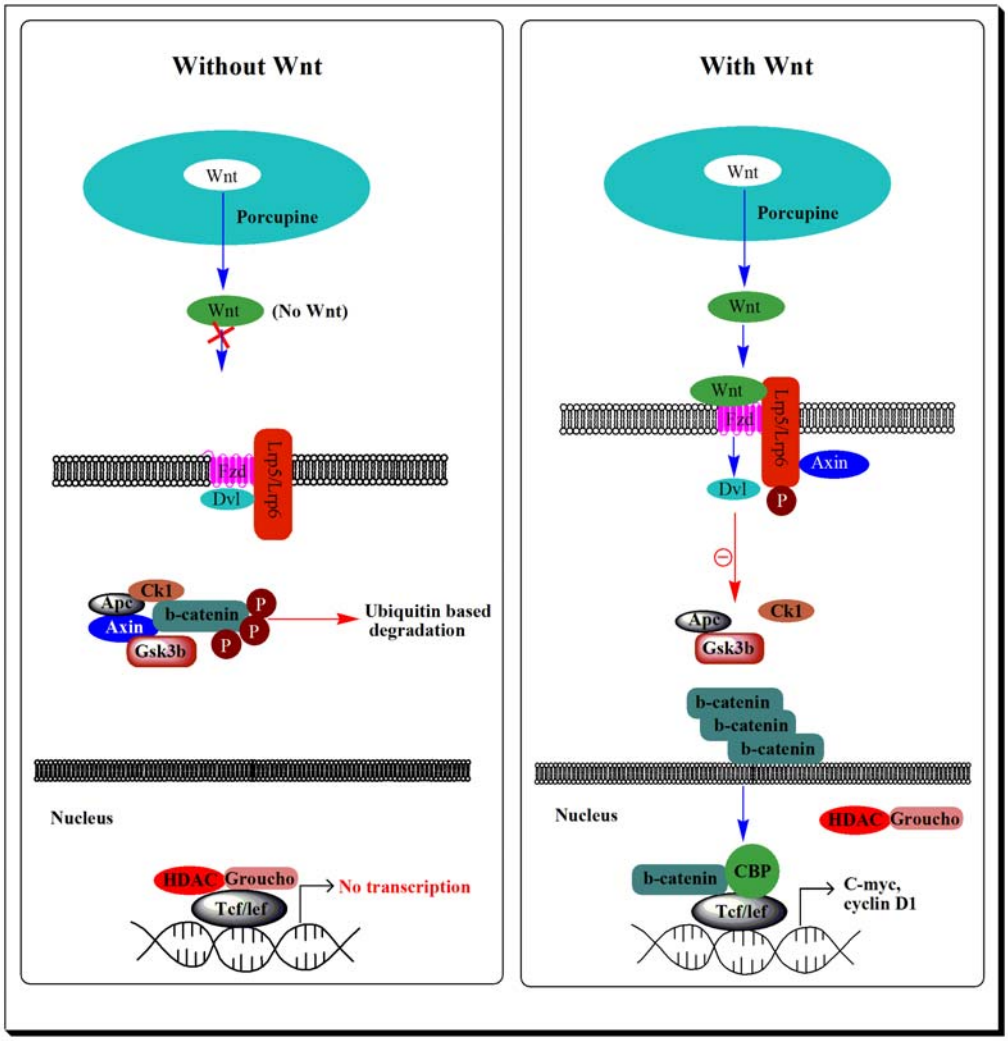

Fig. (7). Canonical Wnt Signaling Pathway.

activate transcription of target genes. In the absence of Wnt, the constitutively active β-catenin destruction complex destruct β-catenin by phosphorylation followed by ubiquitin based degradation [81, 82]. Non-canonical pathway is involved in cell movement as well as polarity and is important in embryogenesis but little is known about its role in cancer [83].

Involvement of canonical Wnt signaling in various types of CSCs is well documented in literature [84]. About 90% of colon cancer has been found to be associated with aberrant activation of Wnt pathway and major fraction of breast cancer is associated with loss of Wnt pathway negative regulator secreted frizzled-related protein1 (sFRP1) [82]. CSC related to skin, intestinal and mammary gland related cancer shows

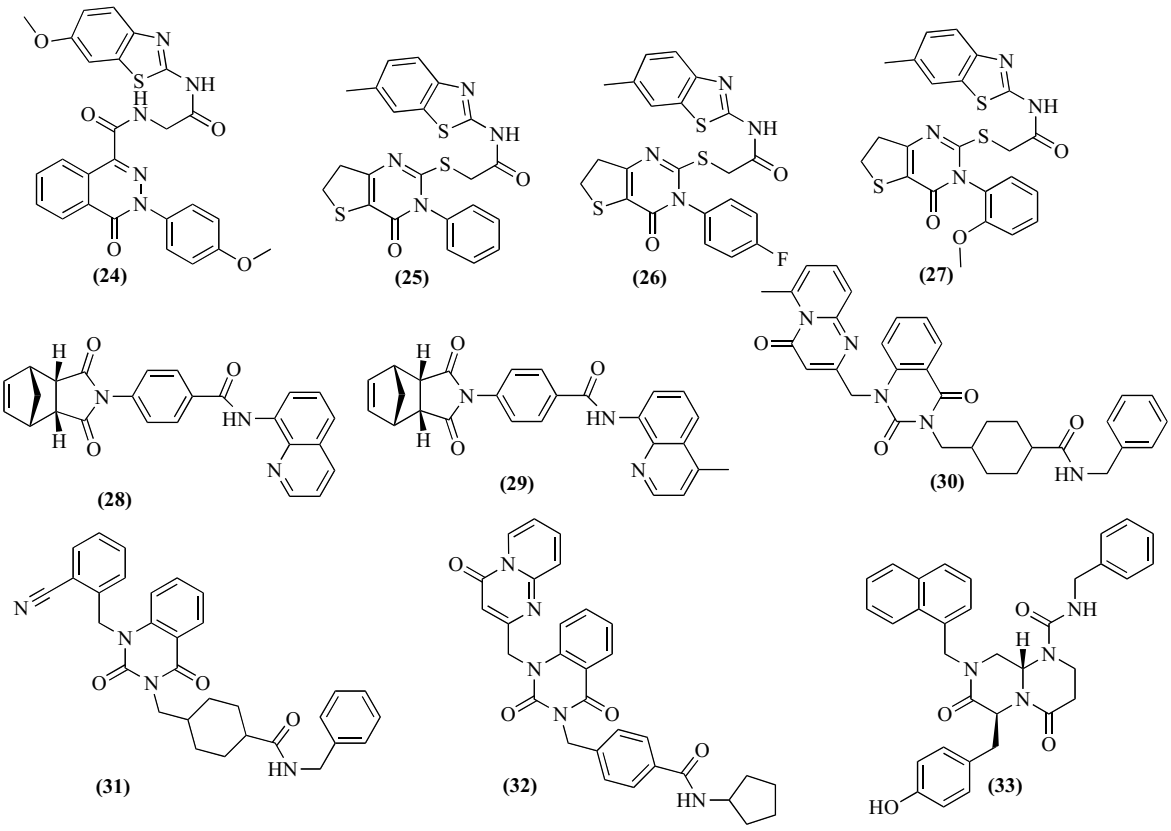

Fig. (8). Wnt signaling pathway inhibitors.

over activated canonical Wnt signaling pathway [85]. Mutations in several components of Wnt pathway such as APC, axin, β-catenin are mentioned in literature and genes related to extracellular Wnt antagonists such as Dickkopf-1 (DKK1), Wnt inhibitory factor1 (WIF1), secreted frizzled-related protein1 (sFRP1) found to be down regulated in various cancers [81]. Targeting Wnt pathway seems to have a lot of therapeutic potential towards cancer therapy specially to eliminate CSCs. Several Wnt pathway inhibitors/modulators have been developed which are responsible for the inhibition or modulation by multiple ways. Wnt production Inhibitor (IWP) inhibits porcupine necessary for the Wnt secretion. IWP1 (**24**), IWP2 (**25**), IWP3 (**26**) and IWP4 (**27**) (Fig. **8**) are small molecules discovered by Chen *et al.* belong to this category [86]. Monoclonal antibodies having Wnt blocking and Fzd blocking ability were also developed and many of them are under clinical trials [82, 87].

Several potent small molecule inhibitors of Wnt response (IWR) i.e. IWR-1 (**28**), IWR-2 (**29**), IWR-3 (**30**), IWR-4 (**31**) and IWR-5 (**32**) which increase the stability of axin were also synthesized by Chen *et al.* [86]. Molecules like FJ9 which disrupt the interaction between the Frizzed-7 Wnt receptor and PDZ domain of Dvl have shown good tumor suppression activity *in vivo* [82, 88]. Another class of Wnt pathway inhibitor belongs to CBP-β-catenin category which inhibits β-catenin/CBP transcription by binding to CBP. Among them ICG-001 (**33**) is in phase I clinical trial [82, 86, 89] (Table. **4**).

β-catenin/TCF interaction antagonists have also been tried among them 2,4-diamino-quinazoline has shown promising results [87]. NSAIDs shown to down regulation of Wnt signaling by COX dependent and COX independent way. It has been demonstrated that PGE_2 generated by COX suppress β-catenin degradation thus suppressing COX is useful strategy to suppress Wnt activity. It has been found that celecoxib a COX-2 inhibitor, inhibits the Wnt signaling independent of COX-2 by degradation of TCF in case of colon cancer cell lines [87]. Resveratrol is under phase 2 clinical trials which inhibit Wnt signaling [81]. Current status of Wnt inhibitors is summarized in Table **4**.

3.5. Notch Signaling Pathway

Notch developmental pathway (Fig. **9**) is an evolutionary conserved pathway which was first identified in *Drosophila melanogaster.* Notch related mutations were associated with notched wings which are passed from parent to progeny [82] and signaling consists of short range communication system between neighboring cells [82]. There is involvement of four Notch receptors (Notch 1-4) and five ligands i.e. jagged (jag) 1, 2 and delta like (Dll) 1, 3 4 [81]. Upon ligand receptor interaction cleavage of receptor first occurs in extracellular domain by metaloproteases of the ADAM (A Disintegrin and Metalloproteinase) and subsequently within transmembrane domain β-secretase presenilin complex which release notch intracellular domain (NICD).

Table 4. Current status of Wnt signaling pathway targeted approach.

Inhibiting Agent	Target	Status	References
OMP-18R5	Fzd	Discovery	[90]
Hydrocarbon-stapled peptide	β-catenin	Discovery	[91]
Salinomycin and Nigericin	LRP6	Discovery	[92]
Sulindac	β-Catenin	Clinical	[93]
Fisetin	Multiple Wnt proteins	Dietary	[94]
Tetrandrine	Wnt/β-catenin Signaling	Discovery	[95]
Streptonigrin	β-catenin/Tcf transcription	Discovery	[96]
Retinoic Acid	Reduces paracrine Wnt-β-catenin Signaling	Dietary	[97]
Quinacrine	Multiple Wnt proteins	Clinical	[98]
Resveratrol	Unknown	Phase II	[87]
Querucetin, Curcumin	Unknown	Preclinical	[87]
1α25,-dihydroxy-Vitamin D3	β-catenin	Clinical	[87]
Differentiation-inducing factors	GSK-3β	Preclinical	[87]
2,4-diamino-quinazoline	β-catenin/TCF	Preclinical	[87]
ICG-001-related analogs	CBP	Phase I	[87]

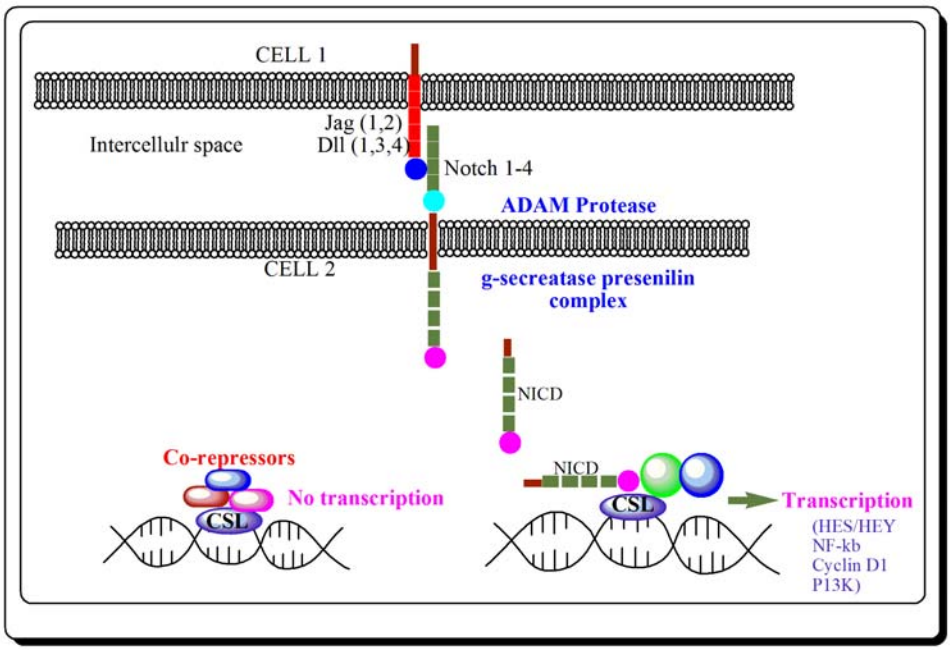

Fig. (9). Notch Signaling Pathway.

NICD migrate into the nucleus and binds to CSL and thus replacing co-repressors such as SMRT, CIR, HDAC by co-activators (MAML1, SKIP) and increases the activity of the target genes [81, 82, 99, 100].

Deregulation of Notch is associated with various forms of cancers. First evidence about the role of Notch in cancer has been observed in abnormal Notch1 lacking its extracellular domain in T-cell lymphoblastic leukemias. Notch receptors lacking extracellular subunit were constitutively activated and have high oncogenic tendency [81]. It was found that aberrant Notch signaling has been associated with various forms of cancers such as breast [101], lung, neuroblastomas, skin, cervical and prostate cancer [102]. Moreover, Notch

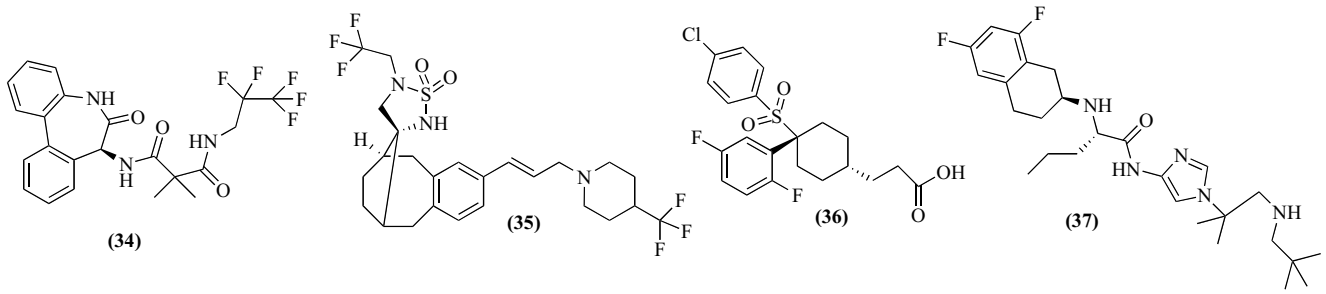

Fig. (10). γ-secreatase inhibitors in clinical trials.

pathway deregulation is also involved in CSC [103]. Thus down-regulating of Notch pathway has been found to target cancer and CSCs. Due to complex nature of signaling pathway multiple molecules may be targeted in Notch signaling like γ-secreatase inhibitors (GSI).

γ-secreatase is composed of four subunits Presenilin, Nicastrin, Aph-1 and Pen-2 [104]. RO4926047 (**34**), MRK-003 (**35**), MK-0752 (**36**), PF-03084014 (**37**) are the some of the GSI inhibitors (Fig. **10**) which are currently in clinical trials [104]. Monoclonal antibodies have also been developed against ADAM10 cleavage domain and thus blocking the receptor in inactive conformation [82]. Other targeting strategies involved blocking Dll by monoclonal antibodies and MIML1 co-activator with cell permeable α-helical peptide was also demonstrated [82]. Anti-Notch monoclonal antibody OMP-59R5, ANTI Dll4 monoclonal antibody OMP-21M18 are currently in phase 1 clinical trial [105, 106].

3.6. MicroRNA based Targeting

MicroRNAs (miRNA) are the non-coding RNAs which are as small as 21-23 nucleotides in size and found in plants, invertebrates, vertebrates which bind imperfectly to the 3'-untranslated region of target mRNA. It inhibit translation and induces degradation of target mRNA [107]. More than thousands of miRNAs have been estimated in human genome which post-transcriptionally regulates many mRNAs some of which are responsible for cell survival, proliferation and thus cancer [107]. Role of miRNAs in cancer was first established in 2002 by the finding that gene related to miRNAs miR-15 and miR-16 is lost in majority of patients with chronic lymphoid leukemia. Subsequently, it has been demonstrated that miR-15 and miR-16 miRNAs act as tumour suppressor by inhibition oncogene BCL2 related translation whose translation product provides survival benefits to the cells [108, 109]. Over-expression of miRNAs may also be associated with cancers. miR-21 is overexpressed in many forms of cancers including breast cancer, lung cancer, glioblastoma lymphoma, leukemias and neuroblastoma [110]. Tumorigenic effect of miR-21 is well established in-vivo and such tumors completely depend on the expression profile of miR-21 [111]. Deregulated miRNAs have been associated significantly with CSCs of various origins and manipulation of miRNAs to counterbalance the deregulated status of CSCs associated miRNAs seems to be the noble way to target CSCs (Table **5**). One such example is the inhibition of let-7 which results in regaining chemosenstivity towards sorafenib and doxorubicin in case of hepatocellular CSCs [114]. It has been demonstrated that silencing miR-181 results in reduced hepatocellular CSCs motility and invasion by controlling abnormal expression of cytokine IL-6 and transcription factor twist [114]. Temozolomide mediated apoptosis was observed in glioblastoma CSCs with downregulated miR 125b-2 expression. The apoptosis results from increased cytochrome c release from mitochondria, Apaf-1 induction, caspase-3, poly ADP-ribose polymerase and proapoptotic protein Bax activation along with down-regulation of BCL-2 expression [119]. Forced expression of miR-124 and miR-137 in glyoblastoma CSCs results in elimination of self-renewal and oncogenic capacity while normal stem cell remains unaffected [120]. Up-regulation or restoring of miR-200c activity by miR-200c mimetic agents seems to be a promising strategy against CSCs. It can impact CSC like properties of ALDH+/CD44+ cells by down-regulating expression of BMI1 and inhibiting metastatic ability of EMT by reducing ZEB1, Snail, N-cadherin expression along with increasing E-cadherin expression [121]. Multiple experiments showed very promising results of manipulation miRNAs against CSCs. miRNAs can be developed as a noble way to target CSCs and till now various strategies have been applied to manipulate miRNA which include synthetic miRNA mimics, miRNA expression vectors and chemically modified anti-miRNAs (antisense oligonucleotides as miRNA inhibitors) in experimental animals [122]. It seems to be a promising candidate in clinical therapeutic agents as miRNAs are the small molecules that can be easily transported by blood fluids [123].

4. MULTI-TARGETED ANTICANCER STEM CELL THERAPY (THE FUTURE PROSPECTIVE)

Multi-targeted drugs seem to be the another aspects with its own significances to be implemented against various diseases including cancer which can be clearly understood by the success stories of multi-targeted drugs such as mematine, NSAIDS, salicylates and metformin [124]. Moreover according to network models partial inhibition of multiple targets is more efficient than complete inhibition of single target [124]. Agents targeting single points (single hits) are more susceptible to get resistant if signaling network system is much complex so that efferent signaling can be easily bypassed. One such example is the tamoxifen related primary resistance in breast cancers [125]. CSCs which are relatively difficult to target using chemotherapy because of having additional survival benefits such as high resistant, extremely high dynamic plasticity according to the need of the environment [126] and have multiple complex signaling. CSCs seem to be more efficient to get resistant toward single-hit

Table 5. Deregulated miRNAs expression in various CSCs.

miRNA	Status	Tumor Type	Mechanism	References
miR-21	Up-regulated	Breast cancer	Tumor suppressor tropomyosin 1 targeting	[112]
miR-495	Up-regulated	Breast cancer	Promotes oncogenesis and hypoxia resistance via down-regulation of E-cadherin and REDD1	[113]
miR-181	Up-regulated	Hepatocellular carcinoma	Directly targets RASSF1A, TIMP3 as well as nemo-like kinase (NLK)	[114]
Let-7	Up-regulated	Hepatocellular carcinoma	Targets SOCS1, caspase-3	[114]
miR-126	Up-regulated	Gastric carcinoma	Inhibit SOX2, and PLAC1	[115]
miR-125b	Down-regulated	Glioma	Down-regulation of cell cycle regulated proteins CDK6 and CDC25A	[116]
Let-7	Down-regulated	Colon adenocarcinomas	Target Lin-28b which promotes cell cancer	[117]
miR-29	Down-regulated	Cholangio Carcinoma	Target anti-apoptotic protein Mcl-1	[118]

targeted therapy as little change in environment leads to change the whole scenario. Multi-targeted anti CSC therapy may have potential to be more reasonable to target CSCs. Various pathways in cancer stem cells which include Hh, notch and Wnt have multiple points which can be targeted simultaneously. The existence of crosstalk among CSCs related signaling pathway creates extra opportunity as well as real challenge to understand relationship among various signaling pathways as well as its implementation in multi-targeted therapy [127]. The clear understanding of various signaling and related cross-talk is the key requirement as number of failures in drug development were associated with undiscovered or underestimated cross-talk effects [128]. Surface markers associated with CSCs are yet another target which creates real challenges such as presence of same surface proteins in normal stem cells and diversity of CSC markers. Targeting a typical CSC marker with high affinity drug may create selectivity issue towards CSCs and normal stem cells. Moreover diverse CSC markers are not easy to be included directly in targeted therapy. The one answer to these problems is multi-targeted therapy where several surface markers can be included simultaneously with low affinity multi-targeted agents. Low affinity may somewhat resolve desired safety issues for the normal stem cells [124]. Till now CSCs targeted therapy is relatively new and multi-targeting approach seems to be quite effective. Multi-targeting drugs for CSCs are not available, but in future multi-targeted anti CSC therapeutic agent if included in CSC targeted therapy may provide real solution to existing challenges.

5. CONCLUSIONS

Various approaches have been tried to target CSC which include targeting CSC markers, niche, developmental pathways, and microRNA based targeting. CSC markers are the promising targets but lack of understanding about its related physiology is still a major obstacle to get rid of cancer. Moreover no universal CSC marker is still available which can be utilized as target. Niche is the important factor for maintaining of stem cells. Disturbing CSC Niche has been tried and some agents have been approved for various cancer. Hedgehog, Wnt and Notch developmental pathways are among the well-studied pathways with respect to its involvement in CSC related pathophysiology. Several inhibitors or modulators of these pathways are under clinical trials. Several microRNAs have the ability to induce differentiation in CSCs and thus manipulation of such microRNA are also tried to target CSC. Multi-targeted approach against CSC is yet another field which is not properly explored yet. In future we are expecting multi-targeted anti-CSC therapeutic agents may provide suitable answers to still unresolved problems associated with CSC targeting approaches. We are also hoping that CSCs specific drugs in combination with classical chemotherapy can significantly reduce the resistance and relapse rate. Thus, it is in fast developing phase and in future we expect the full proof treatment for cancer.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Drug Target Strategies in Breast Cancer Treatment: Recent Developments

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Abstract: Breast cancer (BC) is the leading cause of death among women all over the world. Estrogen receptor (ER) based therapy is one of the major approaches to target BC and is associated with various problems such as primary as well as secondary resistance. ER signaling is a complex pathway as many factors are involved; including several types of ERs and their associated co-regulators. Increasing understanding of ER signals results in new approaches targeting towards BCs. In this context, ER co-regulators have been explored and many modulators of ER co-regulators have been found out. EGFR and mTOR pathways also have significant impact on BC endocrine therapy because of the complex crosstalk mechanism which is responsible for primary and secondary resistance. Triple negative breast cancer (TNBC) is majorly associated with BRCA mutations. Currently there is no approved targeted therapy available in such form of cancer. Although PARP inhibitors seem to be suitable candidates for it. The present review is focused on the current scenario of ER, EGFR, as well as mTOR signaling target therapy. We have also discussed the current status of PARP inhibitors in BC chemotherapy.

Keywords: Breast cancer, chemotherapy, crosstalk, resistance, targeting.

INTRODUCTION

Breast cancer (BC) is the major cancer among women, having world's second highest morbidity rate of 10.9% [1]. Despite being various advancements in cancer chemotherapy, BC is still a major issue as more than three hundred thousand new cases and about forty thousand deaths have been detected in the United States alone during 2013 [2]. BC is a heterogeneous disease and three markers estrogen receptors (ER), progesterone receptor (PR) and erythroblastosis oncogene B2 (ErbB2 or EGFR2) are commonly used to classify BC [3, 4]. The major class is ER/PR positive which comprises of about 75% of all BCs whose growth is influenced by hormones and may be treated with hormonal therapy and thus categorized as a hormonal response positive (HR positive) tumor [5, 6]. Other forms of BC include tumor with overexpressed EGFR2 (20-25%) and triple negative BC (TNBC) (15%) which lacks all three markers [4, 7, 8, 9]. HR positive BC can be further sub-classified as luminal-A and luminal-B type. ER negative BC consists of basal type cells, some of which have overexpressed EGFR2 and other shows normal expression pattern [10]. Thus heterogeneity of BC seems to be much more complex as their response to therapy is diverse and individualization of therapy is required. Proper individualization of the therapy is impossible without a suitable classification system and for that more reliable markers are needed. Primary and secondary resistance towards BC chemotherapy as well as hormonal therapy are major problems in the desired clinical outcome of disease due to cross talk among various signaling cascades [11]. To understand BC and associated signalings, new targets have been explored [12]. In this review, we have discussed the estrogen signaling as a target for BC as well as current status of targeting approaches of EGFR and mTOR signaling as its crosstalk is important in modulating the signaling cascade of ER. The current status of PARP inhibitors and its emerging role in targeting TNBC is further mentioned in the review.

Estrogen Signaling in BC

First indication regarding involvement of steroid hormone signaling in BC comes from the observation that bilateral oophorectomy in patient with metastatic BC results in regression of tumor [13]. Now the involvement of estrogen signaling is well known and hormone therapy is commonly used to treat HR positive tumors. Estrogen signaling is mediated by classical as well as non-classical estrogen receptors (ER) [14]. Classical ER consists of ERα and ERβ which significantly control the cell proliferation and differentiation [15]. ERα is involved in cell proliferation as well as in cancer and ERβ has the opposite effect and thus may be considered as tumor suppressor [14, 16]. ERα which is overexpressed in 60-70% of BC is a ligand dependent transcription factor belonging to the nuclear receptor superfamily of protein with defined functional domains having the ability to activate or repress gene activity [17]. ER without ligand is in inactive state which is sequestered by multichaperone complex that involves heat shock protein 90 (Hsp 90) [18-20]. The complex system is generally present in the cytoplasm of the cell that may be associated partly with nuclear component structure [14]. Once bound to the ligand, which is estrogen in general, it allows the receptor to adopt such a conformation that the associated chaperone complex is dissociated, which releases Hsp90 along with related molecules and dimerization of receptor takes place which finally modifies the target gene expression [21]. Genes related to proteins such as cyclin D1, insulin like growth factor I receptor (IGF-IR), collagenase, PR, PS2, heat shock proteins, TGF-α, IGF-II and vascular endothelial growth factor (VEGF) represent the few among many genes regulated by ER [22]. The interaction of dimer with the promoter region of target genes is a critical step which can bind directly through specific estrogen response element (ERE) or indirectly through contact with other DNA bound transcription factors such as activation protein 1, specificity protein 1 or nuclear factor k-light-chain enhancer of activated B cell [12]. Once bound to the cognate response element in the promoter region of target genes, it allows proper recruitment of a series of co-regulators which control chromatin remodeling [14]. These co-regulators may be classified as a co-activator and co-repressor depending upon whether it activates or suppresses the target gene [23]. Although the classification of co-regulators is not the thumb rule as their behavior is cell specific and some co-repressors such as SMRT are must for the transcription in response to estrogen [14]. The response specificity of ligand depends upon the conformation that the receptor gains after binding to ligand because the recruitment profile of co-regulators depends on conformation gained by ligand bound receptor [14]. Another aspect of estrogen signaling is the existence of estrogen mediated rapid signaling which is known for

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many years [24]. Rapid signaling is mediated through membrane estrogen receptors (mER) and the pathway is commonly termed as a nongenomic pathway [25]. Rapid action of estrogen includes signaling through classical receptors that are present on the cell and through non-classical receptor that involves G protein coupled receptor 30 (GPR30) as well as ER-α36 [26]. Palmitoylation at cystine 447 is the key event in the localization of ERα to the plasma membrane where it activates MAPK and phosphatidylinositol 3 kinase (PI3K)/serine threonine protein kinase (Akt) pathways in BC cells [27]. Another mechanism involved in nongenomic signaling is methylation of ERα at arginine 260 in the DNA binding domain by protein arginine N-methyltransferase 1 (PRMT1) which facilitates Src/focal adhesion kinase mediated downstream transduction cascades [28]. These factors indicate the existence of a functional extranuclear signaling pathway for estrogen in BC cells, which appear to be a new inclusion in targeted therapy. Importance of understanding and exploring extranuclear targeting aspects of estrogen signaling can be realized by the fact that it has significant involvement in cell proliferation, migration, drug resistance and apoptosis inhibition [29, 30]. In a study, blocking of MAPK was demonstrated to show beneficial effects on BC, which further indicates the functional importance of rapid ERα mediated Src/MAPK pathway [12]. Integrin linked kinase is involved in extra-genomic signaling through PI3K pathways which regulates cell migration and thus inhibition of PI3K inhibits BC cell migration [31]. In another study ERα mediated regulation of deacetylation of tubulins in association with HDAC6 facilitate BC cell migration through the extragenomic signaling [32]. Tamoxifen induces tubulin deacetylation, suggesting extranuclear signaling through tubulin deacetylation confers endocrine resistance in BC [26]. Recent findings have revealed that PI3K/Akt activity associated with estrogen extranuclear signaling may be involved in blocking the apoptosis induced by TNF, hydrogen peroxide and serum withdrawal [33].

Another aspect in BC hormonal therapy is *de novo* as well as acquired resistance towards tamoxifen and related agents [11]. The main cause of such resistance includes crosstalk of ER signaling with multiple types of other signaling pathways [34]. Membrane ERα interacts and/or activates several kinases such as IGF-1R, Src, PI3K, MAPK, EGFR and EGFR2 [34-39]. Phosphorylation of co-activator by cytoplasmic kinases leads to modification of ERα activity [40-42]. Temoxifen and related SERM's which are agonists to membrane ERα (mERα) may interact with mER and activate multiple kinases which can phosphorylate ER and mediate ligand independent signaling [34, 39, 43]. Here we conclude that multiple

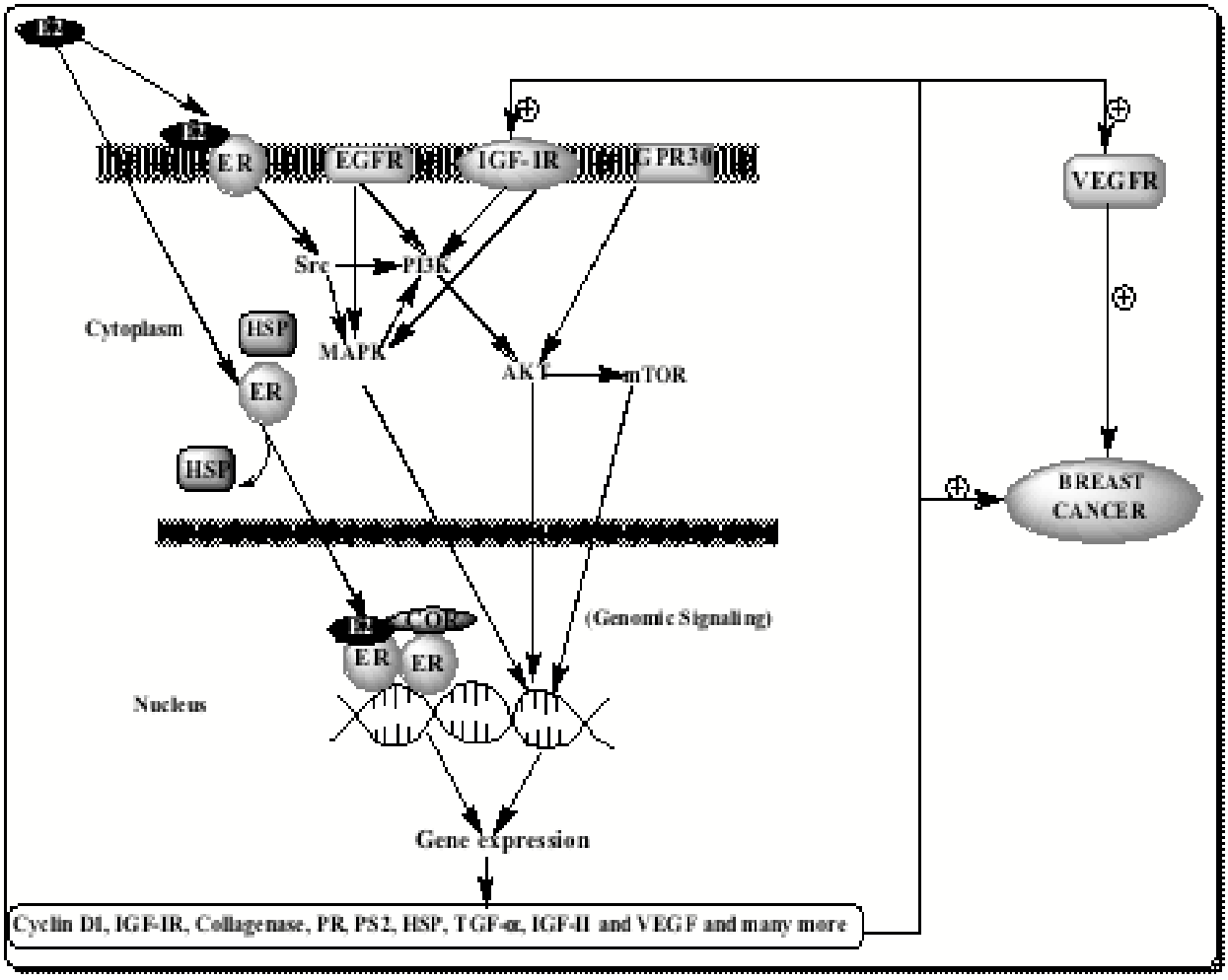

Fig. (1). Simplified model of deregulated estrogen signaling and associated risk of BC. Estrogen receptor signaling pathway is mediated through genomic and extranuclear signaling which is synchronized with various kinases. In genomic signaling, estrogen (E2) binds to ER,which allowes conformational changes in ER , dissociation of heat shock protein 90 (HSP) and related molecules from it, dimerization of ER receptors, interaction with DNA and recruitment of co-regulators (COR) to modify gene expression. The extranuclear signaling induces activation of PI3K/AKT/mTOR and Erk/MAPK pathway which can convey genomic signaling. Multiple induced transcriptions such as cyclin D1, IGF-IR, collagenase, PR, PS2, HSP TGF-α, IGF-II and VEGF may be responsible for cancer and cancer growth.

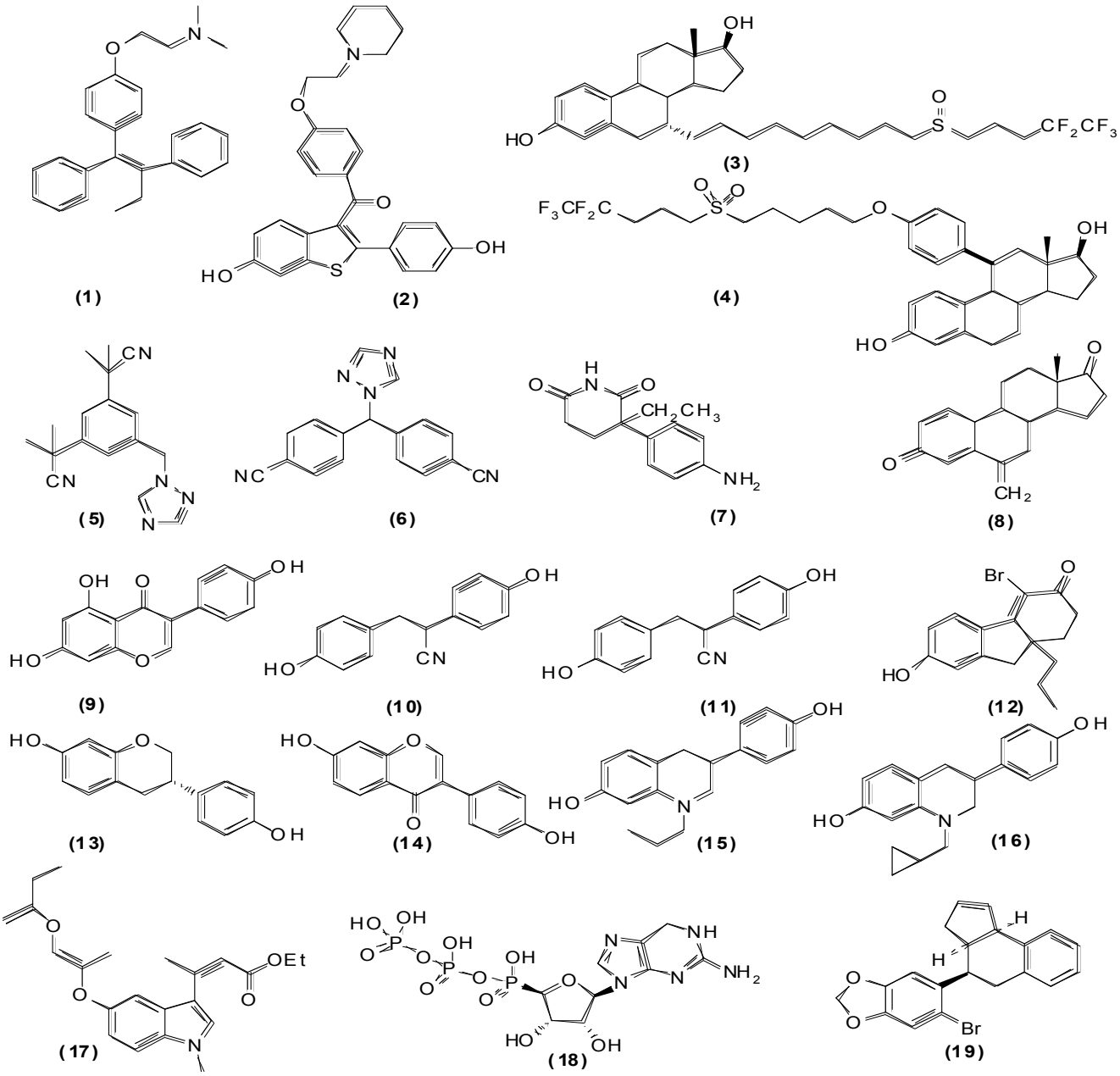

Fig. (2). Inhibitors/modulators of various types of ER mediated signaling.

classes of ER, related co-regulators, growth factors, and other kinases work together. Deregulation of these signalings results in multiple complications for chemotherapy as well as hormonal therapy Fig. (**1**).

Targeting Estrogen Signaling

Number of targeted approaches have been implemented and antiestrogens (AE) are among the oldest examples for BC [44, 45]. Tamoxifen **(1)** Fig. (**2**) is clinically most widely used selective estrogen receptor modulator (SERM) in HR positive BC and mechanism of which involves the recruitment of co-repressor in the final step in breast cell [14, 46]. Raloxifene **(2)** is the SERM which is clinically used for HR positive breast cancer [47]. Another important aspect of tamoxifen and raloxifene is their role in BC preventive therapy which is approved by the US Food and Drug Administration [48]. Another class of antiestrogen belongs to selective estrogen receptor down regulator (SERD) which includes Fulvestrant **(3)** and RU58668 **(4)** [49]. Fulvestrant is an FDA approved drug for treating postmenopausal women with HR positive advanced BC, which prevents ER dimerization, nuclear localization, blocks ER mediated transcriptional activity and induces receptor degradation [50-52]. Aromatase inhibitors (AIs) block the final step in the conversion of androgen to estrogen and thus reduce circulating estrogen level that seem to be the most suitable for postmenopausal women [14]. The agonistic activity of SERM on mER activates the EGFR signaling pathway and hence AIs seem to be superior to SERM. Anastrozole **(5)** and letrazole **(6)** are now commonly used for postmenopausal as adjuvant therapy for advance BC [53, 54]. Aminoglutetimide **(7)** and exemestine **(8)** are the other agents belonging to AIs category that are also in clinical practice [55, 56]. Increased understanding of estrogen signaling and problems associated with SERMs, SERD, and AIs result in evolvement of multiple new targeted approaches which include ERβ, mER, and co-regulators.

ERβ and GPER

ERβ opposes proliferation and tumor propagation associated with ERα and can be considered tumor suppressor. Suppression of angiogenesis and tumor growth in T47-D xenograft model by ERβ is a valid proof regarding its tumor suppressive properties [57]. BC is significantly associated with chromosome 12q loss which encodes ERβ indicating its antitumor action [58, 59]. siRNA mediated knockdown of ERβ results in increased gene transcription related to tumor proliferation [60]. ERβ mediated cell cycle arrest is also demonstrated, which results from the repression in c-myc, cyclin D1, cyclin E, cyclin A cdc25A related transcription as well as increase in expression of CDKp21 $^{waf1/cip1}$ and p27^{kip1} [16, 61-63]. Tamoxifen being antagonistic against ERβ may cause regression in the beneficial effect of SERM related therapy [64]. Thus ERβ seems to be an important target and a ligand which is selective antagonist towards ERα and agonist for ERβ which is theoretically ideal. Many ERβ specific ligands have been mentioned in literature which include genistein **(9)**, DPN **(10)**, ERB-041 **(11)** and tetrahydrofluorenone **(12)** [65].

Equol **(13)** which is a metabolite of daidzein **(14)** is reported to possess modest selectivity for ERβ. Its many derivatives have been synthesized such that compound **(15)**, **(16)** have been reported to showed antagonist activity against ERα and agonist activity against ERβ [65]. GPR-30 which is seven transmembrane G protein coupled estrogen receptor commonly known as a G protein estrogen receptor (GPER) is an important target for BC. Tamoxifen and other classical ER antagonists act as agonists towards GPER and play a critical role in activation of epidermal growth factor receptor (EGFR) as well as ERK. [66, 67]. MIBE **(17)** is a compound with antagonist activity against ERα and GPER [66]. G36 **(18)** and G15 **(19)** are other compounds which showed GPER antagonist activity [68]. Inclusion of ERβ and other non-classical ERs in targeted therapy for BC is comparatively a newer approach and theoretically seems to cause significant impact.

ER Co-Regulators

Co-regulators that include co-activators and co-repressor play an important role in the ER mediated gene expression. Steroid receptor co-activator 1 (SRC 1) was the first co-regulator to be identified [69]. Currently, multiple co-regulators are known and many of them have been found to be deregulated in BC. Over-expression of amplifier in breast cancer-1/SRC3, GRIP1, PELP1, MUC1, breast carcinoma amplified sequence3 (BCAS3) and Ciz1, have been found to be associated with BC [70, 71]. Moreover, overactivation of co-activators of ERα (AIB1 and BCAS3) have been found to be associated with tamoxifen resistance [26]. Deregulation in co-repressors which recruit histone deacetylase, prevent transcription and counterbalance the effect of coactivators have been observed in BC [72]. Thus targeting co-regulators seem to be a noble strategy for BC therapy, which is discussed in subsequent section.

SRC-1

Steroid receptor coactivator-1 (SRC-1) also known as NCOA1 was discovered in 1995 and it has broadened the knowledge about the physiology of ER action [73]. It belongs to p160 SRC family, other members of which are SRC2 and SRC3 [74]. SRC proteins have been found to be associated with BC as it was found that SRC-1 is over-expressed in 19% to 34% of BC cases which is positively correlated with EGFR2 overexpression and with poor clinical outcome of the disease [73]. In MCF-7 BC cells, SRC-1 is over expressed which is associated with increased proliferation in response to the estrogen indicating the importance of SRC-1 intensity in estrogen mediated tumor growth [75]. Moreover MCF-7 cells lacking SRC-1 are unable to show an increase in estrogen mediated SDF-1α expression, and reduce the ability of proliferation and invasion. It indicates the direct link of SRC-1 with SDF-1α/CXCL12 mediated autocrine/paracrine signaling which is responsible for the proliferation and invasion [76]. Furthermore, various studies indicate that SRC-1 coactivates PEA3 mediated Twist expression and promotes epithelial-mesenchymal transition, migration, invasion and metastasis of mammary tumor cells [77]. Role of SRC-2, the second protein of SRC family is still uncertain. Many studies showed its positive correlation with estrogen induced tumor growth and spread where SDF-1α may be involved. Some other reports mentioned that downregulation of SRC-2 modulates estrogen-responsive genes and stimulates proliferation as found in MCF-7 BC cells, suggesting SRC-2 may have proliferative as well as antiproliferative function in BC cells [76, 78]. SRC-3 overexpression has been reported in a variety of cancers, including BC and promotes cancer initiation, expansion and metastasis [79]. Overexpressed SRC-3 status is responsible for the positive crosstalk with IGF-1 pathway and resistance towards classical hormonal therapy [80]. SRC-3 plays a critical role in activating P13K/AKT signaling in mammary tumorigenesis [81]. Role of SRC-2 in BC is still uncertain, but overactivation of SRC-1 and SRC-3 is widely associated with BC and can be a valid target for BC chemotherapy. Gossypol **(20)** Fig. (**3**) is a small molecule that acts as a selective inhibitor of SRC-1 and SRC-3 which has no effect on SRC-2 or other coregulators as observed in MCF-7 BC cells [80]. It reduces the concentration of SRC-3 in prostate, lung and liver cell lines and inhibits the cell viability in same cancer cell line where it promotes SRC-3 down-regulation. Additionally, its effect of sensitizing lung as well as breast cancer cell lines towards chemotherapeutic agents and selective cytotoxicity towards cancer cells indicate the possibility of including SRC inhibitors in clinical consideration. Gossypol **(20)** represent a prototype of new class of chemo-therapeutic agent against ER overexpressed BC and is under clinical trials for several types of cancer [80, 82].

HDACs

Histone deacetylases (HDACs) are the class of enzymes having 18 members, responsible for the removal of acetyl group from lysine amino acid on the histone which restores the positive charge on lysine and ultimately causes more strong wrapping of DNA by the histone [83-85]. Acetylation status of histone is controlled by the opposite activity of histone acetyl transferases (HATs) and HDACs [83]. Irregular HDAC activity is associated with various human cancers, including BC [86, 87]. The repressed state of ER in MCF-7 cells is associated with overexpressed HDAC1 and reactivation of ER takes place by exposure to HDAC inhibitor [88]. Triple negative BC can be sensitized towards tamoxifen by various HDAC inhibitors, which result from the release of HDAC1 from ERα promoter and thus restoring the ERα expression [88]. Impact of HDAC inhibitors on ERα status is somewhat complicated as short time inhibition of HDAC leads to acetylation as well as stabilization of receptor at the protein level, but long time exposure results in delocalization and proteasome mediated degradation of the receptor [14]. Furthermore, HDAC inhibitor mediated increased expression of ERβ has been observed in ER positive BC cells which itself having tumor suppressor activity [89]. Combination of HDAC inhibitor and hormonal therapy has been found to re-sensitize breast cancer resistant to tamoxifen and is involved in down regulating ATK activity with induction of cell death [90]. Inhibition of HDAC results in decrease of EGFR mRNA in ER-negative MDA-MB-221 and *in vivo* along with re-sensitization of tamoxifen related therapy [91]. HDAC inhibitors are found to induce Wnt/β catenin mediated dedifferentiation of human BC cell [92].

HDAC inhibitors are important considerations in the management of multiple forms of BC including HR positive BC. Some HDAC inhibitors are already approved by FDA that includes varinostat **(21)** Fig. (**3**), belinostat **(22)**, panobinostat **(23)**, romidepsin **(24)**

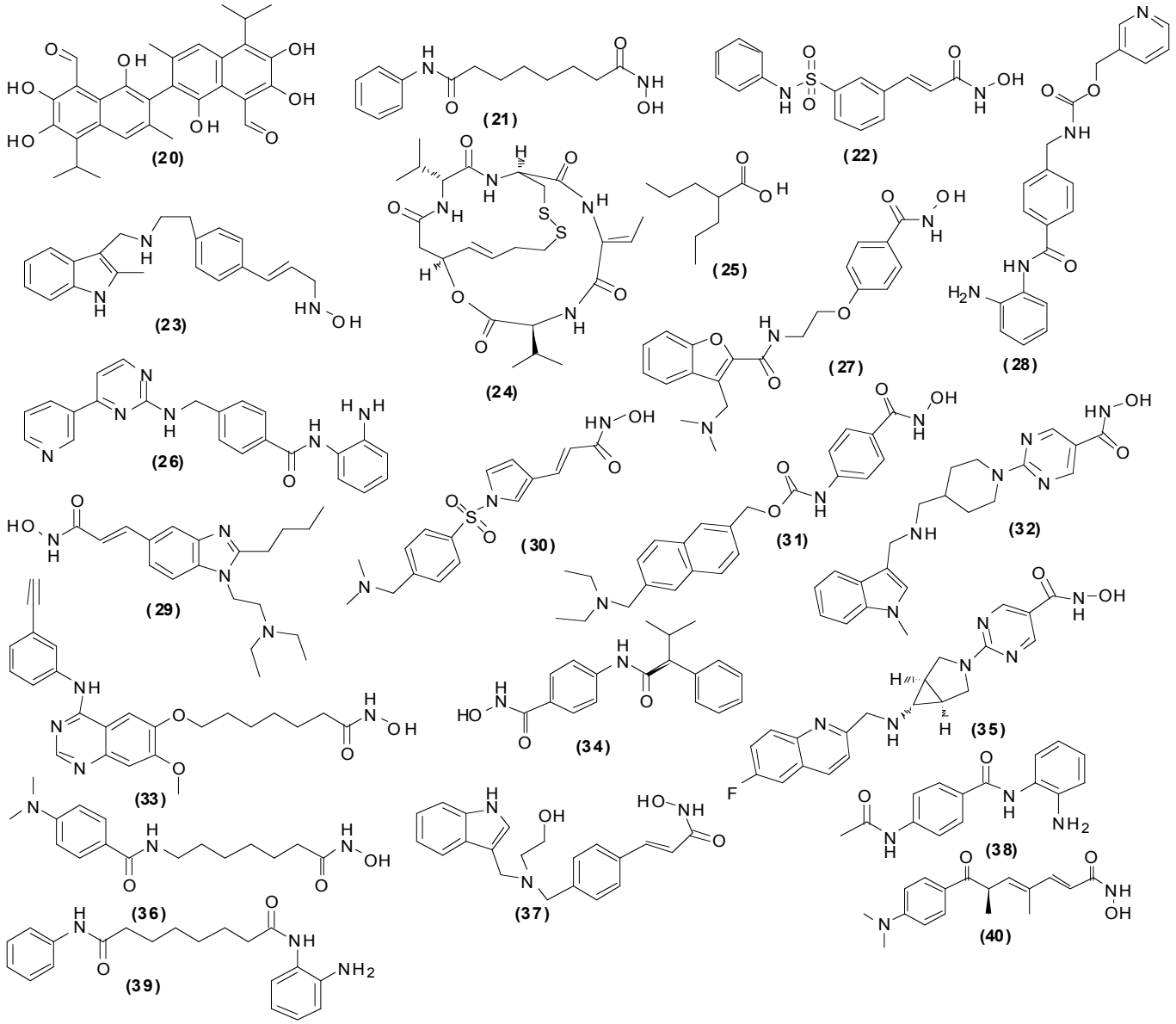

Fig. (3). Gossypol **(20)** a selective inhibitor of SRC-1, SRC-3 and HDAC inhibitors **(21-40)**.

[93, 94]. HDAC inhibitors such as valporic acid **(25),** mocetinostat **(26)**, abexinostat **(27)**, entinostat **(28)**, SB939 **(29)**, resminostat **(30),** givinostat **(31)**, quisinostat **(32),** CUDC-101 **(33)**, AR-42 **(34)**, CHR-3996 **(35)**, M344 **(36),** dacinostat **(37),** tacedinaline **(38),** BLM 210 **(39)** and trichostatin A **(40)** are under clinical trials for various forms of cancer (http://clinicaltrial.gov/).

PRMTs

Nine protein arginine methyltransferases (PRMTs) are responsible for ω-*NG*-monomethylarginine (MMA), ω-*NG,NG*-asymmetric dimethylarginine (ADMA) and ω-*NG,N'G*-symmetric dimethylarginine (SDMA) [95]. PRMT1 is the main member of PRMT family and contribute for more than 90% of the methylarginine residues in mammalian cells and up-regulation of which is responsible for multiple forms of cancer including BC [95, 96]. PRMT2, CARM1 and PRMT7 are the other members, deregulation of which is responsible for BC induction and metastasis [95]. PRMT1 mediated transient methylation of ERα on Arg260 leads to exclusive cytoplasmic localization of receptor and trigger its interaction with p85 subunit of PI3K as well as Src which ultimately results in phosphorylation of AKT and activating the downstream cascade resulting in non-genomic effects of estrogens [14, 28]. Moreover, FAK adhesion protein interacts with R260 methylated ERα which indicates the possible role of the methylated status of ERα in BC migration and metastasis [14]. PRMT especially PRMT1 can be included in BC specific chemotherapy, which may provide better therapeutic outcomes. Many PRMT inhibitors are well documented in literature. Among them AMI1-AMI9 **(41-49)** Fig. (**4**) consists of nine compounds of AMI series identified by random screening having PRMT inhibitory activity [97]. AMI series of compounds have been taken as lead by several groups of researchers and a number of analogs have been synthesized, **(50-53)** which showed significant activity [98-100]. Another class of PRMT inhibitors belong to pyrazlo amide series. Compound **(54)** alongwith its analogs **(55-58)** has shown significant action [101, 102, 103]. Allantodapsone **(59),** stilbamidine **(60),** RM 65 **(61),** NS-1 **(62)**, TBBD **(63)** are other compounds reported in the literature with PRMT inhibitory activity [104]. Multiple PRMTs inhibitors are available which can be used to target PRMT in BC specific chemotherapy.

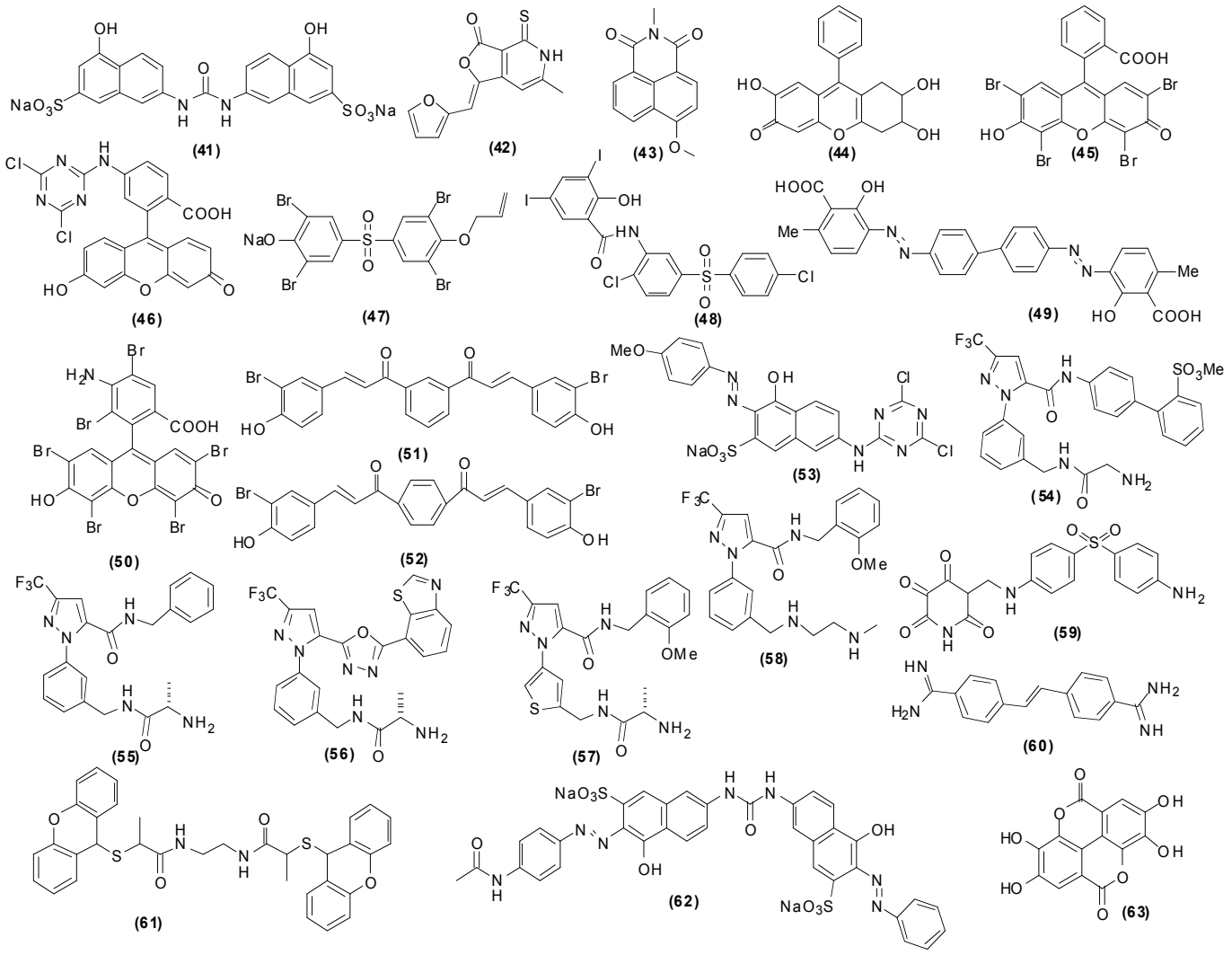

Fig. (4). Various PRMT inhibitors.

PAX-2

Paired Box-2 gene product (PAX-2) is a critical tamoxifen recruited transcription repressor of ErbB2 gene. Elevated AIB-1can lead to competition with PAX-2 binding of tamoxifen-ER complex which results in elevated ErbB2 expression. AIB-1 and ErbB-2 is related to the aggressive nature of BC. Thus PAX-2 may be the connecting link between BC and possible outcome of SERM related therapy [105]. PAX-2 seems to be an important target against BC, but our current understanding is very limited as no drug is known till date. Moreover, reports indicate that it could have tumor suppressor as well as oncogenic function [106]. Thus, proper generalization and validation of PAX-2 is the major requirement for its inclusion in chemotherapy.

TLE1

Transducin-like enhancer protein 1 (TLE1) is the modulator of transcription activity of ER, which exerts survival as well as anti-apoptotic function in various cell types and is involved in pathogenesis of cancer [107]. TLE1 hasg a regulatory effect on anoikis and it has been demonstrated that anoikis in untransformed mammary epithelial MCF10A cell was associated with significant down-regulation of TLE1 expression. Resistance in these cells towards anoikis has been found to be associated with forced expression of exogenous TLE1. In BC, significant up-regulation of TLE1 in a cell after detachment from the extracellular matrix has been seen [107]. TLE1 is responsible for anoikis resistance and anchorage-independent growth of BC cells, which is also correlated with genetic manipulation data of TLE1 *via* overexpression and down-regulation [107]. TLE1 inhibits Bit1 anoikis pathway by reducing the formation of proapoptotic Bit1-AES complex in part through sequestration of AES in the nucleus [107]. Significant overlaps of TLE1 binding sites in MCF-7 cells with ER target is associated with cell proliferation and can be down regulated by siRNAs [94, 108]. Thus, TLE1 is the novel target which can be implemented in the BC specific chemotherapy.

FOXA1

Forkhead box protein A1 (FOXA1) also called hepatocyte nuclear factor 3-alpha (HNF-3α) is encoded by Foxa1 gene [109, 110]. FOXA1 is highly correlated with ER/PR and GATA3 positive BC along with hormonal signaling [111]. FOXA1 is the major factor behind estrogen-ER activity and endocrine response in BC cells as lack of FOXA1 in ERα positive BC is related to resistance to endocrine therapy [111-113]. In ER negative cancer, expression of FOXA1 is associated with the responsive nature of tumor to some endocrine therapy [114, 115]. Thus being a necessary factor for estrogen mediated signaling in BC and endocrine therapy, FOXA1 modulators may be investigated as a noble strategy against BC.

E6-AP

E6-associated protein (E6-AP) is a ubiquitin proteasome pathway enzyme which functions as a co-activator of steroid hormone receptor including ER [116, 117]. Expression of E6-AP decreases in human invasive breast as well as prostate cancer and down-regulation of which is associated with up-regulated ERα in BC and androgen receptor (AR) in prostate cancer. The E6-AP knockout animal model shows overexpressed ERα in mammary gland and AR in prostate [118]. In transgenic mice with overexpressed E6-AP, estrogen is unable to initiate mammary tumor, but tumor development occurs if overexpressed E6-AP is mutated [119]. All these findings suggest that E6-AP may have tumor suppressor activity.

HOXB7

Homeobox gene HOXB7 is overexpressed in multiple forms of cancer, including BC and promotes tumorigenesis by effecting proliferation, survival, invasion and angiogenesis [120]. In BC, overexpressed HOXB7 is associated with SERM resistance [121]. Long term exposure of MCF-7 cells to tamoxifen results in increased HOXB7 expression which ultimately up-regulate the EGFR activity by binding to EGFR promoter region. Furthermore, over-expressed HOXB7 significantly correlates with poor disease-free survival in ERα-positive breast cancer on adjuvant tamoxifen monotherapy [121]. Antagonist of HOXB7 can be an important tool to overcome SERM related resistance which may also result in increased disease free survival but no such lead is still available.

EGFR

Epidermal growth factor receptor (EGFR) family consists of four surface receptors, which include EGFR/ErbB1/HER1, ErbB2/HER2/EGFR2/Neu, ErbB3/HER3, and ErbB4/HER4 [122]. EGFR2 is the most important among them and is amplified in 20% of BC in general and about 10 % in ER positive BC [123]. EGFR2 amplified ER positive BC is harder to manage by endocrine therapy compared to EGFR2 negative ER positive breast cancer [124]. The hetero or homo-dimerization of EGFR allows autophosphorylation of receptor tyrosine to initiate downstream signaling which requires a proper ligand. EGFR2 is unique in itself as no ligand has been reported till date and can dimerize with the other three members to initiate downstream signaling [125]. Dimerization and autophosphorylation of receptors result in activation of multiple intracellular signaling such as phosphatidylinositol- 3 kinase (PI3K)-Akt, Ras-Raf- MEK-MAPK which maintains several cellular functions including cell division. HER signaling also plays an active role in resistance to therapy, especially endocrine therapy, which results from crosstalk between ER and HER signaling Fig. (**5**) [34]. EGFR2 amplified ER positive BC generally shows poor response to endocrine therapy which can be overcome by incorporating anti-EGFR2 therapy [5]. EGFR2 is highly explored in breast cancer and many agents have been approved for treatment. Trastuzumab was the first commercially available EGFR2 targeting monoclonal antibody for the treatment of BC [126]. Trastuzumab binds to the extracellular segment of EGFR2/Neu and inhibit proliferation of EGFR2 overexpressed BC [127]. Major drawback for trastuzumab

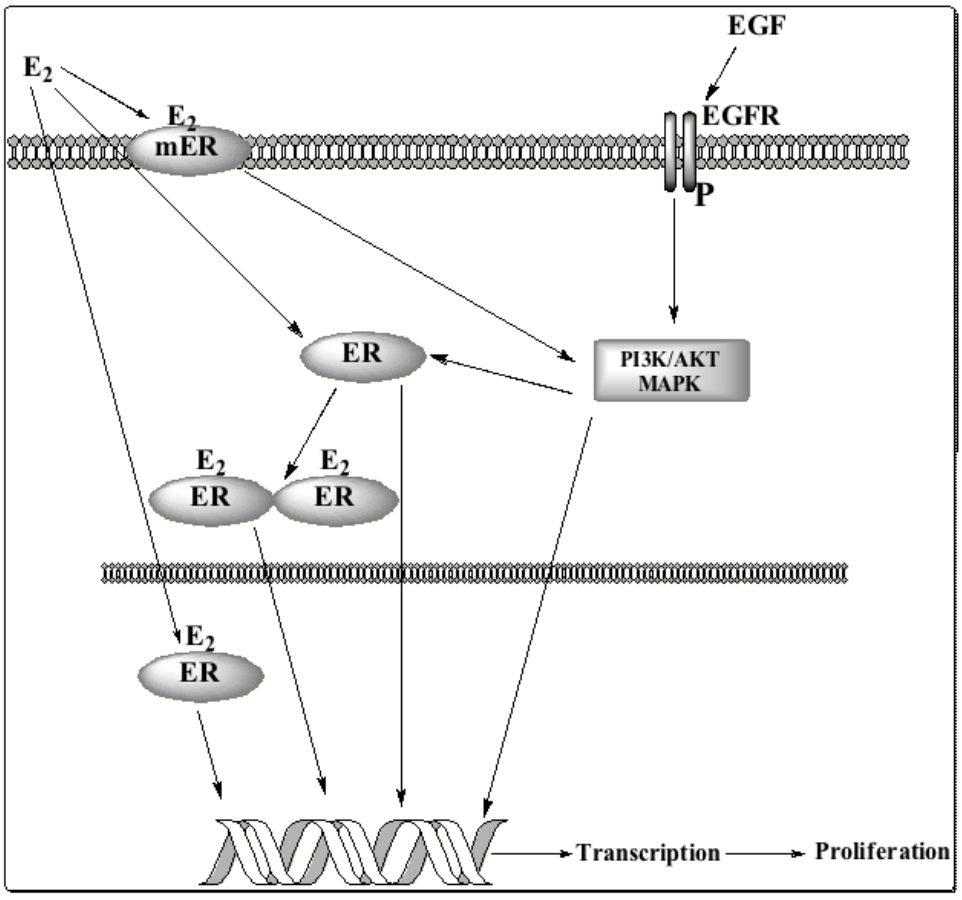

Fig. (5). Simplified model representing cross talk between ER and EGFR signaling. Activation of EGFR by epidermal growth factor (EGF) or by another ligand resulting in dimerization and autophosphorilation of EGFR, which activates multiple signaling pathways such as phosphatidylinositol- 3 kinase (PI3K)-Akt, Ras-Raf- MEK-MAPK and leads to ligand independent activation of estrogen receptor (ER). Membrane ER (mER) mediated signaling can also activate the downstream proteins of EGFR pathway. Thus the crosstalk is bidirectional and is the main cause of resistance towards endocrine cancer therapy.

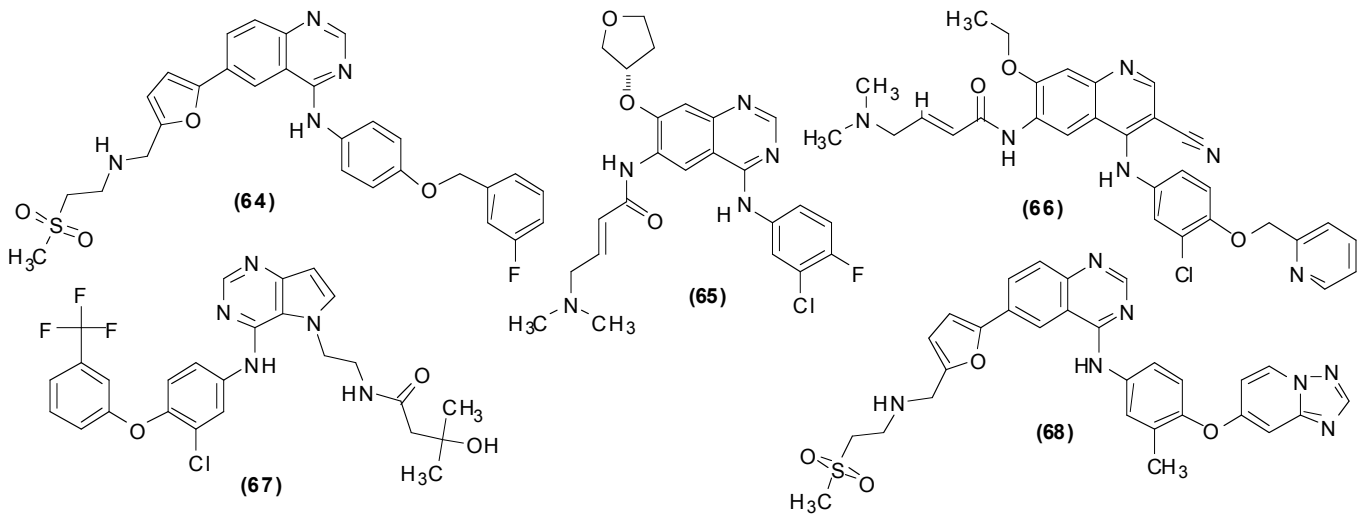

Fig. (6). Small molecule inhibitor/modulator of EGFR signaling in BC.

is the primary resistance as 68-88% of the patients show primary resistance and others may also get secondary resistance towards it [128]. Thus little fraction of patients with EGFR2 overexpressed BC can get a beneficial effect towards trastuzumab therapy that can be a good carrier for other drugs which may be conjugated with it. Trastuzumab as a carrier has been conjugated with emtansine which is a potent microtubule based cytotoxic agent allowing selective delivery of drugs to the EGFR2 overexpressed cells and is under various phases of clinical trials for BC [129, 130].

Pertuzumab is another humanized monoclonal antibody which binds to the extracellular domain of EGFR2, blocking dimerization and is approved by FDA in combination with docetaxel and trastuzumab for the treatment of metastatic BC [131, 132]. MM-302 is a liposome encapsulated doxorubicin attached to antibodies to selectively target EGFR2 overexpressed BC cells which are under clinical trials [133]. Ertumaxomab is a unique monoclonal antibody with dual target of CD3 antigen on T cell and EGFR2 on the tumor cell. It has the ability to form EGFR2-ertumaxomab-CD3 complex resulting in aggregation and activation of T cells, macrophages, dendritic cells and natural killer cells which ultimately lead to phagocytosis and death of tumor cells. It is under phase I and II of clinical trials for EGFR2/Neu positive advanced solid tumors [134]. MM-111 is an antibody fusion protein, which targets HER3/HER3 dimer and inhibit downstream signaling which is also under clinical trials [135]. All these antibodies and antibody conjugates attack the outer domain of the HER receptor. Small molecules have been reported which modulate EGFR signaling intracellular. Lapatinib **(64)** (Fig. **6**) is a small molecule that binds to ATP-binding pocket of EGFR2 protein kinase domain and inhibits self-phosphorylation which is critical for activity [7]. It is approved for the treatment of various forms of cancers, including EGFR2 over-expressed metastatic BC [136]. Afatinib **(65)**, Neratinib **(66),** TAK-285 **(67),** ARRY-380 **(68)** are the other small molecule inhibitors of EGFR, which are under clinical trials for HER overexpressed BC [137, 138, 139]. Other HER targeting agents include MM-121 which is a human monoclonal antibody against HER3 and neratinib that inhibit HER1 as well as HER4 receptor [140, 141].

PI3K/Akt/mTOR Signalling

mTOR signaling is critically involved in normal processes in many cell types including breast cells [139]. It involves PI3K mediated phosphorylation of certain components which ultimately phosphorylate and activate Akt. Once activated, Akt trigger several downstream signaling by activating mTOR results in increased cell survival, proliferation and cell growth [142]. mTOR maintain the resources needed by cells to increase nutrient uptake and angiogenesis when required [142]. It also involved in up-regulation of Akt activity resulting in increasing downstream effects. Proper regulation of mTOR signaling seems to be critical and is done by PTEN, which reverses the action of PI3K and inhibits activation of Akt [143].

This pathway has been found to be highly activated in many forms of cancer, including 50% of BC cases which are associated with mutations that activate this pathway [144, 145]. The major type of mutations involve amplification of PI3K, Akt and loss of PTEN function [146]. Thus PI3K/Akt/mTOR pathway is highly involved in BC and its modulation may provide benefits . Several mTOR inhibitors have been developed and among them Sirolimus **(69)**, everolimus **(70)** and temsirolimus **(71)** have been approved by FDA (Fig. **7**) [147]. Everolimus has been approved by FDA for the treatment of ER/PR positive, HER negative BC in combination with exemestane when 10 mg/day of letrozole or anastrozole failed to produce its effect [147]. Ridaforolimus **(72)**, NVP-BEZ235 **(73)**, SF1126 **(74)**, XL765 **(75)** and BGT226 **(76)** are other mTOR inhibitors, which are under clinical trials [147]. PI3K inhibitors can also be implemented for BC therapy. Some PI3K inhibitors such as SAR245408/SAR245409 **(77)** andGDC-0941 **(78)** are under clinical trials for BC [148]. Akt inhibitor can also be utilized to down-regulate mTOR signaling. Miltefosine **(79)** is an Akt inhibitor, which is under clinical practice [149]. MK-2206 **(80)**, AZD5363 **(81)**, Triciribine **(82)**, AR-67 **(83)**, GSK690693 **(84)** and VQD-002 **(85)** are the Akt inhibitors in clinical trials for various forms of cancers (http://clinicaltrial.gov/).

Poly (Adenosine Diphosphate [ADP])–Ribose) Polymerases (PARPs) Inhibitors

Poly (adenosine diphosphate [ADP])–ribose) polymerases (PARPs) are a family of enzymes that actively participate in DNA repair, gene transcription, chromatin architecture and apoptosis in normal cells [150-152]. PARP1 is the most abundant enzyme among them, which plays a critical role in single strand DNA base-excision repair. PARP1 inhibition results in single stranded break and subsequently double strand break, which ultimately leads to apoptosis [148].

Normal cells with intact homologous recombination double strand DNA repair pathway, the PARP blockage is bypassed. BC cells lacking BRCA1 and BRCA2, the inhibition of PARP leads to cell death [148]. BRCA1 mutation is majorly associated

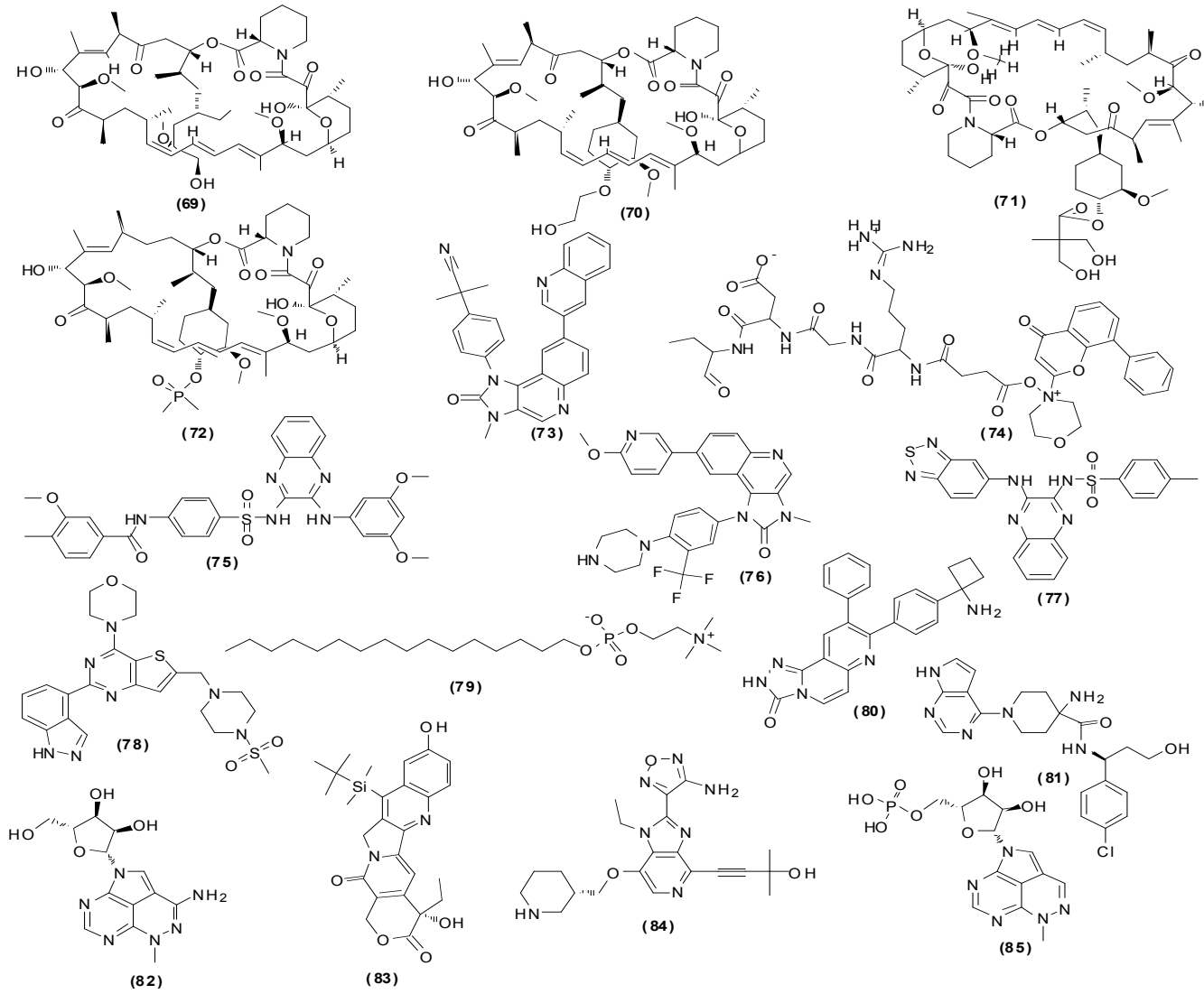

Fig. (7). Various inhibitors/modulators of PI3K/Akt/mTOR signaling.

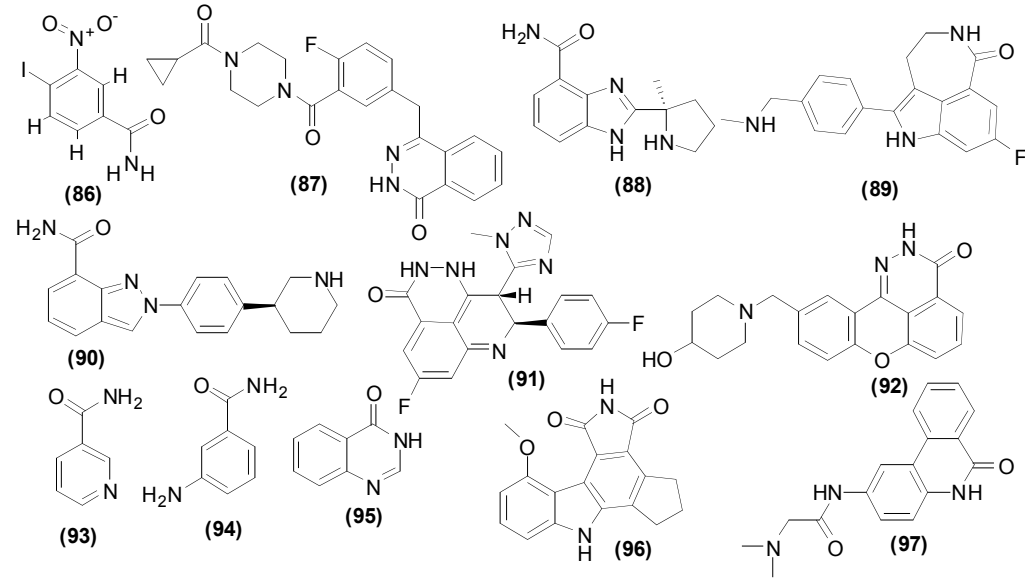

Fig. (8). PARP inhibitors against BC lacking BRCA1/BRCA2 mediated DNA repair.

with TNBC which is an aggressive form of cancer and still no proper targeting approach is available [148]. Moreover PARP is overexpressed in various forms of cancers, which seem to provide extra survival benefits to cancer cells from DNA damage and apoptosis [153]. It has also been found that cancers, especially BC with overexpressed PARP1 is associated with poor overall survival [153]. PARP inhibitors are the noble way to target BC cells by inducing DNA damage and apoptosis. Many PARP inhibitors are under clinical trials which include iniparib **(86)**, olaparib **(87)**, veliparib **(88)**, rucaparib **(89)**, MK-4827 **(90),** BMN-673 **(91)** and E7016 **(92)** (Fig.**8**) [151].

Thus other reported PARP inhibitors include nicotinamide **(93),** 3-AB **(94)**, quinazolinone **(95)**, CEP 8983 **(96)** and PJ-34 **(97)** [154, 155]. Although some PARP inhibitors have reached clinical trials, but phase 3 clinical trials are still lacking and data is still insufficient to compare with standard therapy [156]. Another limitation for implementing PARP inhibitors in clinical practice is the lack of well defined approach for selecting patients that are suitable for PARP inhibition approach [156]. Moreover the emergence of resistance towards PARP is still a key concern in the development of PARP inhibitors. Restoration of BRCA function, up regulation Non-Homologous End Joining, P-glycoprotein mediated efflux and loss of 53BP1 protein are among the causes of resistance towards PARP inhibitors [156, 157].

CONCLUSION

BC therapy is heading from nonspecific approach to targeted approach. Various new targets are identified and implemented for treatment. ER and hormonal therapy is an age old concept, but now various new downstream molecules are included. Many more targets such as co-regulators exist that need further investigation. Nonclassical ERs need to be explored in-depth so that can be utilized properly as anti-BC targeting approach. Interrelationship among different pathways is another issue to be explored as proper understanding of crosstalk among multiple signaling that will help to overcome primary resistance as well as increased efficacy of treatment. PARP inhibitors are an important advancement in cancer chemotherapy that can be combined with other DNA damaging agents to get synergic effect. Selectivity towards BRCA mutated BC cells as BRCA1 lacking cells may almost lose their ability to repair damaged DNA if PARP is inhibited, but normal cells have intact BRCA based repair machinery. Moreover, TNBC can be specifically targeted using PARP inhibitors. BC is a heterogeneous disease for which continuous evolution is going on regarding its targeting approach. In future with the availability of proper targeting approaches and marker based classification for proper selection of regimen can provide full proof treatment for BC.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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