Phytochemical investigation, *In vitro* Anti-mutagenic activity of *Potentilla fulgens* Lodd. and *In-silico* Study of Flavonoids with CDK-2, CDK-6 receptors

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BY

Prakriti Monga

Adminstrative Guide: Prof. (Dr.) P.Ramarao

Supervisor: Dr. Vikas Jaitak

Centre for Chemical and Pharmaceutical Sciences
School of Basic and Applied Sciences
Central University of Punjab, Bathinda

October, 2013

CERTIFICATE

I declare that the dissertation entitled “**Phytochemical investigation, *in vitro* anti-mutagenic activity of *Potentilla fulgens* Lodd. and *in-silico* study of flavonoids with CDK-2, CDK-6 receptors** has been prepared by me under the guidance of 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.

Prakriti Monga
Centre for Chemical and Pharmaceutical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda - 151001.

Date:

CERTIFICATE

I certify that Prakriti Monga has prepared her dissertation entitled **"Phytochemical investigation*, in vitro* anti-mutagenic activity of *Potentilla fulgens* Lodd. and *in-silico* study of flavonoids with CDK-2, CDK-6 receptors**" for the award of M.Pharmacy degree of the Central University of Punjab, under my guidance. She has carried out this work at the Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

(Dr. Vikas Jaitak)
Supervisor
Assistant Professor
Centre for Chemical and Pharmaceutical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda - 151001.

Date:

(Prof. (Dr).P.Ramarao)
Adminstrative guide
Dean, COC
Centre for Chemical and Pharmaceutical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda - 151001.

Date:

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Prakriti Monga

ABSTRACT

"Phytochemical investigation*, in vitro* anti-mutagenic activity of *Potentilla fulgens* Lodd. and *in-silico* study of flavonoids with CDK-2, CDK-6 receptors"

Name of student	:	Prakriti Monga
Registration Number	:	CUP/MPhm-PhD/SBAS/CPS/2011-2012/08
Degree for which submitted	:	Master of Pharmacy
Name of Supervisor	:	Dr. Vikas Jaitak
Centre	:	Chemical and Pharmaceutical Sciences
School of Studies	:	School of Basic and Applied Sciences
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Plants have been used for thousand years in the treatment of various diseases. Plant secondary metabolites have proved to be an excellent source of new medicinal compounds. They offer protection against variety of chronic diseases including diabetes, cardiovascular diseases, obesity and cancer. Mutation is an important factor that is linked to carcinogenesis. It has been found that occurrence of cancer can be reduced by decreasing the incidence or rate of mutation. Plants are promising source of antimutagens agents which are present in them as secondary metabolites such as flavonoids, alkaloids, terpenoids, glycosides etc. *Potentilla fulgens* is an important medicinal plant of higher Himalayas that is known globally for its therapeutic importance. A number of antioxidant constituents have been reported from the plant which mainly consists of polyphenolic compounds. It has been observed that diet rich in polyphenolic compounds such as flavonoids can reduce the risk of cancer. *P.fulgens* reported to have polyphenolic compounds such as flavonoids which are potent bioactive molecules that possess anticarcinogenic effects as they can interfere with initiation, development and progression of cancer by the modulation of cell cycle, apoptosis, and angiogenesis. Anti-mutagenic activity on different fractions of *P.fulgens* was carried out, which indicates that *n*-butanol and water fractions of the plant are strongly anti-mutagenic. Inhibitory activity for *n*-butanol fraction was 60.4% and 35.4% in co-incubation and pre-incubation respectively in TA 98 tester strains while 55.6% and 62.0% inhibition was observed in TA100 tester strains respectively for co and pre-incubation. For water fraction 56.6% and 60.7% inhibitory activity in co-incubation and pre-incubation mode of treatment respectively against TA98 strain and 34.5% and 50.6% inhibition in TA100 strain for co-incubation and pre-incubation treatment respectively. Results specify the importance of *P.fulgens* as a new source of anti-mutagenic agents. Isolation of molecules from ethyl acetate and *n*-butanol

fractions led to the characterisation of one molecule namely catechin out of total six isolated molecules. *In-silico* study of various reported flavonoids were performed on CDK-2, CDK-6 as these receptors are linked to cell cycle and mutation in cell cycle may lead to cancer. *In-silico* study indicates that natural as well as synthetic flavonoid molecules can be considered as a treatment for cancer by inhibiting CDK-2 and CDK-6 receptors.

(Prakriti Monga) (Vikas Jaitak) (Prof. (Dr.) P.Ramarao)

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LIST OF ABBREVIATIONS

S.No	Full Form	Abbreviation
1.	Cyclin Dependent Kinase	CDK
2.	Phenylalanine Ammonia-lyase	PAL
3.	Cinnamate-4-hydroxylase	C4H
4.	Chalcone synthase	CHS
5.	Chalcone isomerase	CHI
6.	Flavone synthase	FSI
7.	Cytochrome P450 flavone synthase	FSII
8.	UDPG-flavonoid 3-*O*-glucosyl transferase.	3GT
9.	Cytochrome P450 isoflavone synthase	IFS
10.	Flavonone-3-β-hydroxylase	FHT
11.	dihydroflavonol 4-reductase	DFR
12.	Thin Layer Chromatography	TLC
13.	Retention Factor	R_f
14.	Nuclear Magnetic Resonance	NMR
15.	Leucine	LEU
16.	Valine	VAL
17.	Phenylalanine	PHE
18.	Tyrosine	TYR
19.	Alanine	ALA
20.	Tryptophan	TRP
21.	Aspartine	ASP
22.	Glutamate	GLU
23.	Arginine	ARG
24.	Deoxyribo nucleic acid	DNA
25.	4-nitro-*O*-phenylenediamine	NPD
26.	Cyclin Dependent Kinase-2	CDK-2
27.	Cyclin Dependent Kinase-6	CDK-6
28.	Institute of Microbial Technology	IMTech
29.	High-range statistical domain	HSD
30.	Standard Error	SE
31.	Ethylacetate	EtOAc
32.	Ultra-Violet	UV
33.	Absorption, Distribution, Metabolism, Excretion	ADME
34.	Area Under Curve	AUC
35.	Distortionless Enhancement by Polarization Transfer	DEPT

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Plants have been used in traditional medicine since ages. They have stood up to the test of time and contributed several novel compounds for the prevention and cure of many lethal diseases. Throughout the human history people have relied on medicinal plants for maintaining good health and to fight sickness, pain and other diseases (Bhatia *et al*., 2011). Many naturally occurring compounds are used in the treatment of various diseases like cancer, diabetes, rheumatoid arthritis etc (Nirmala *et al*., 2011). Himalayan plants play a major role and contribution to the herbal pharmaceutical industry both of India and other countries. Plants that grow at higher altitudes are subjected to an assault of diverse testing situations including higher doses of mutagenic UV-radiation, physiological drought, desiccation and strong winds. Plants interact with stressful environments by physiological adaptation and altering the biochemical profile of plant tissues thus producing a spectrum of secondary metabolites. Secondary metabolites like polyphenols, terpenes and alkaloids have been reported to possess anti-mutagenic and anticancer properties (Bhatia *et al*., 2011). Plant secondary metabolites have proved to be an excellent reservoir of new bioactive compounds. Many anticancer agents have been isolated from various plant sources like *Catharanthus roseus, Podophyllum* species*, Taxus brevifolia, Camptotheca acuminate, Betula alba, Cephalotaxus species, Erythroxylum pervillei, Curcuma longa, Ipomoeca batatas, Centaurea schischkinii* and many others (Karpagam *et al*., 2013). Scientists are still attempting to explore the bioavailability of anti-cancerous compounds in unexplored plant species (Nirmala *et al*., 2011). Hartwell, in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used in the treatment of cancer (Cragg *et al*., 2005). It is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms. Indeed, molecules derived from natural sources including plants, marine organisms and micro-organisms have played and continue to play a dominant

role in the discovery of leads in the development of conventional drugs for the treatment of most human diseases (Kaur *et al.*, 2011).

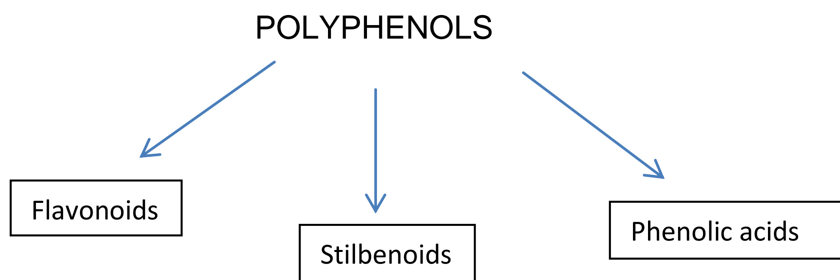
It has been stated that major constituents of more than 50% of all the drugs in clinical use are natural products and their derivatives and one of the potential uses of plant-derived compounds are as anti-mutagenic agents (Ammar *et al.*, 2007; Kaur *et al.*, 2010). Mutation is an important factor in carcinogenesis, so the occurrence of cancer may be reduced by decreasing the rate of mutation (Issazadeh *et al.*, 2012). A mutagen is an agent that is capable of destroying the integrity of a hereditary mechanism of the cell or organism. Any substance causing increased mutation can also increase the probability of cancer. The majority of cancers are initiated by genetic changes and a carcinogen is a chemical that is capable of increasing the incidence of cancer. Therefore carcinogenesis appears to be linked with mutagenesis (Zaveri *et al.*, 2011). Mutations in cell cycle may also lead to cancer (Lodish *et al.*, 2000). Cyclin-dependent kinases (CDKs) are considered as a probable target for anti-cancer medication trials (Rossi *et al.*, 2006). As a result of human development and global environmental pollution, the rate of mutations has increased and one of the ways to counteract the effect of such mutagenic agents is to identify those substances that can antagonize their effect (Aydin *et al.*, 2004). Plants are the promising source of anti-mutagens which are present in them as secondary metabolites such as flavonoids, alkaloids, terpenoids etc. These secondary metabolites help in strengthening the cell defenses against environmental mutagens/stress. Currently, there is an increasing interest in natural compounds that can act as protectors against diseases (Loeb *et al.*, 2003; Kaur *et al.*, 2010). Flavonoids are natural anti-mutagens. Choi *et al* (1994) reported that flavonoids like chrysin, apigenin, luteolin and its glucoside, kaempferol, fisetin, morin, naringenin, hesperetin, persicogenin, (+)-catechin and (−)-epicatechin showed anti-mutagenic effect against aflatoxin B1(AFB1) with more than 70% inhibition rate (Choi *et al.*, 1994). Major mechanism by which flavonoids act as anti-mutagenic agents is free radical scavenging abilities of flavonoids (Edenharder and Grunhage, 2003). Reactive oxygen species and other free radicals cause oxidative stress which in turn causes DNA, protein, and lipid damage which leads to change in chromosome instability,

genetic mutation, and modulation of cell growth leads to cancer (Klaunig *et al.*, 2010). Flavonoids stabilize the reactive oxygen species by reacting with reactive compound of the radical. Because of high reactivity of hydroxyl group of the flavonoids, radicals become inactive. Flavonoids exhibit antioxidative properties by several different mechanisms, such as scavenging of free radicals, chelation of metal ions such as iron and copper which are of major importance for the initiation radical reactions and inhibition of enzymes responsible for free radical generation (Benavente-Garcia *et al.*, 1997; Van Acker *et al.*, 1996).

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages (Scalbert *et al.*, 2005). They are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (Beckman *et al.*, 2000). Naturally occurring polyphenols are known to have numerous biological activities. They are found to be potential candidates for use as drugs, in diseases like AIDS, heart ailments, ulcer formation, bacterial infection, mutagenesis bioactivity. Some of the polyphenols studied for their anti-carcinogenic potential are flavones, flavonols, isoflavones, and catechins. From a chemical point of view, polyphenols can react with one-electron oxidants, which prevents free radical formation in biological systems. Such single electron oxidation promedicinal cesses are considered to be the key steps of polyphenols while acting as drugs (Gianmaria *et al.*, 2011).

1.2 Polyphenolic compounds with their classification

Polyphenols can be subdivided in three main subclasses



(A) Flavonoids can be classified as

1. Flavones and flavonols

a) Flavones (apigenin, cosmosiin, vitexin, luteolin, orientin, baicalein and baicalin)

b) Flavonols (kaempferol, quercetin, quercitrin, rutin, myricetin and myricitrin)

2. Flavanones (hesperetin, naringenin, hesperidin, naringin, fustin and aromadendrin)

3. Flavanolol (silibin,silymarin,taxifolin)

4. Chalcones (butein, carthamin, dihydrochalcone, phloretin and phlorizin)

5. Isoflavones (3-phenylchromone, genistein, genistin, daidzein, daidzin and puerarin) (Luthar, 1992)

(B) Stilbenoids

- Polyhydroxylated stilbenoids
- Resveretarol analouges

(C) Phenolic acids are usually derived from cinnamic acid or benzoic acid.

- Caffeic acid
- Ferulic acid
- Chlorogenic acid
- p-Coumarinic acid (Handique, 2002)

1.3 Flavonoids

Flavonoids are a diverse group of polyphenolic compounds widely distributed in the plant kingdom and over 4000 structurally unique flavonoids have been identified in plant sources. These are primarily recognized as the pigments responsible for the many shades of yellow, orange, and red of flowers, fruit, and leaves. They have been classified according to their molecular structure that consists of two benzene rings joined by a linear three-carbon chain and forms an oxygenated heterocycle (C6-C3-

C6) and their large number arises from the various combinations of multiple hydroxyl, methoxyl, and *O*-glycoside group substituents on the basic benzo-pyrone (C6-C3-C6) moiety (Holiman *et al.*,1996; Harborne and Williams, 2000). They are one of the common components in the human diet. They are present in foods generally as *O*-glycosides with sugars bound at C3 position. Average intake of all flavonoids is estimated to be 1 g/day (Hollman and Katan, 1999).

1.4 Core structure and nomenclature of flavonoids

Flavonoids are benzo-pyrone derivatives consisting of phenolic and pyrane rings. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A and B rings.The activity of flavonoids and their metabolites depends upon the arrangement of functional groups about the nuclear structure (Harborne and Williams, 2000). Figure 1 shows basic structural moiety of flavonoid.

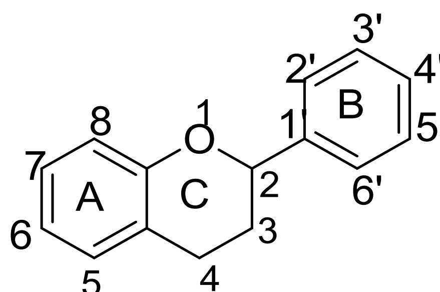


Fig 1. Basic moiety of flavonoid

The nomenclature of flavonoids is straight-forward with the aromatic ring A condensed to the heterocyclic ring C and the aromatic ring B most often attached at the C2 position. The various substituents are listed first for the A and C ring and - as primed numbers - for the B ring (Harborne and Williams, 2000). General classes of flavonoids include flavone, flavanol, flavonone, flavanol, isoflavone. Figure 2 represents structure of general classes of flavonoids.

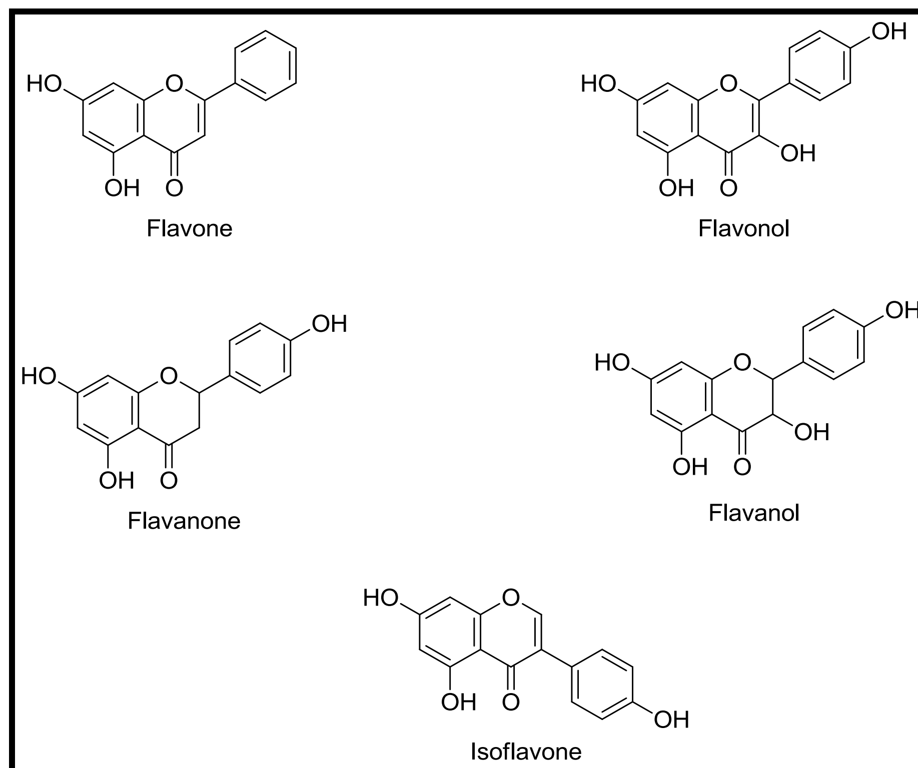


Fig 2. General classes of flavonoids

1.5 Biosynthetic pathway of flavonoids

The health-protecting effects of flavonoids have stimulated significant research towards the elucidation of their biosynthetic networks. Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which amino acid phenylalanine is used. With the help of enzyme Phenylalanine ammonia-lyase (PAL), phenyl alanine gets converted to cinnamic acid which further gets converted to *p*-coumaric acid with help of enzyme coumarin-4-hydroxylase(C4H). The metabolic pathway continues through a series of enzymatic modifications to yield chalcones→flavonones→flavones→anthocyanin glycosides. Along this pathway, many products are formed, including the flavonols, flavan-3-ols, proanthocyanidins (tannins) and a host of other various polyphenolics (Chemler *et al.*, 2006).

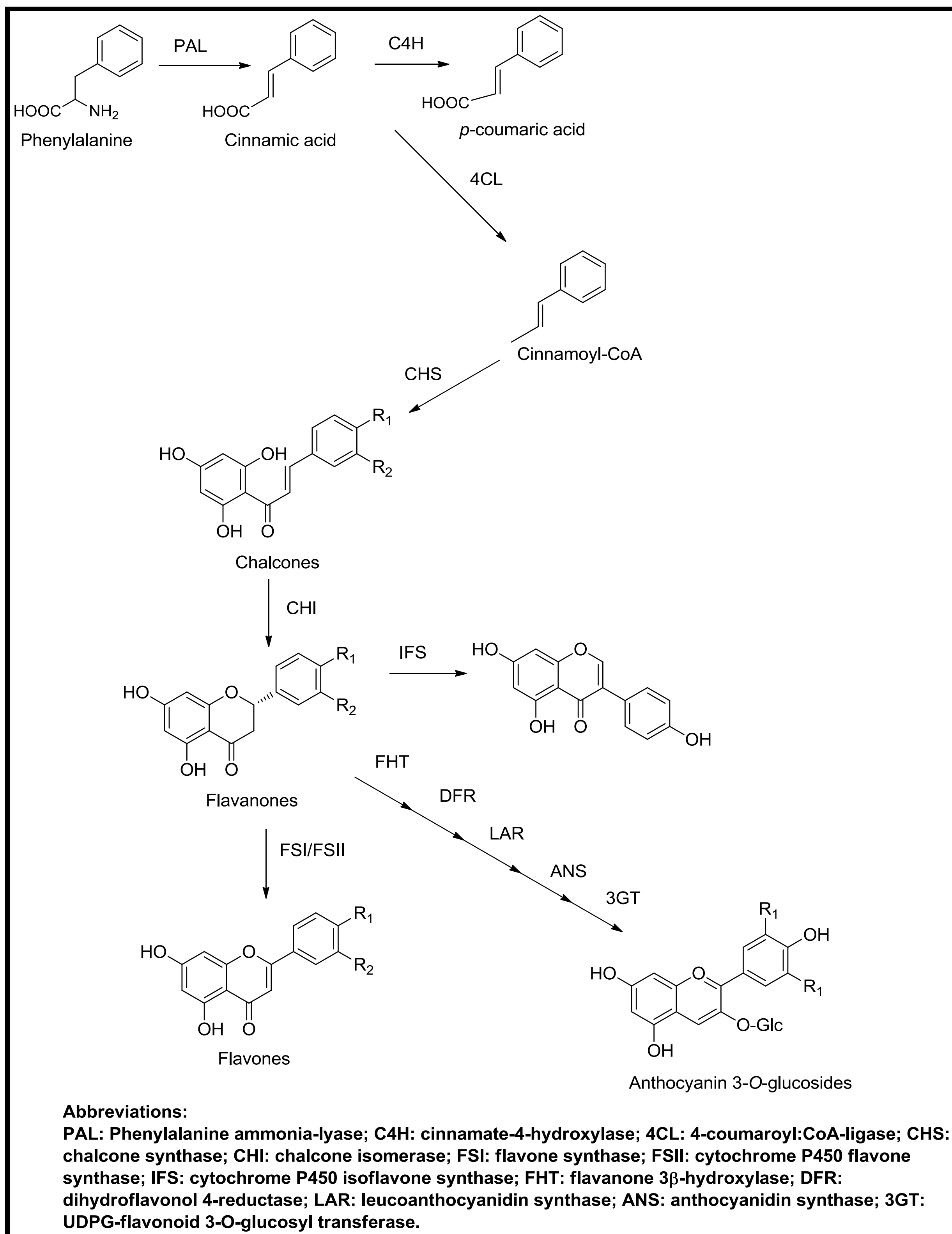


Fig 3. Biosynthetic pathway of flavonoids

1.6 Flavonoids and Cancer

Cancer is one of the most serious diseases in the world due to its high mortality rate (Center for Disease Control and Prevention., 2002). Cancer chemoprevention by use of natural or synthetic substances and its prevention through dietary intervention has become an important issue (Middleton *et al*., 2000; Galati *et al*., 2000; Yang *et al*., 2001). It may be controlled by various means, including suppression, blockage, and transformation. Suppressing agents prevent the formation of new cancers from procarcinogens, blocking agents prevent carcinogenic compounds from reaching critical initiation sites, and transformation agents facilitate the metabolism of carcinogenic components into less toxic materials or prevent their biological actions (Karikas, 2011). Flavonoids can act in all the three ways. Flavanoids show a remarkable range of biological activities, including those that might influence the processes that are dysregulated during cancer development. These includes antiallergic, anti-inflammatory, antioxidant, anti-mutagenic, anticarcinogenic, and modulation of enzymatic activities (Kandaswami *et al*., 2005; Fresco *et al*., 2006; Petti *et al*., 2009).

Therefore flavanoids have beneficial health effects and can be considered as chemopreventive or therapeutic agents against cancer.

1.7 Major Anticancer mechanisms of flavonoids

- Antioxidant and Antiaging activity (Chen *et al*., 2009; Sun *et al*., 2009; Haleagrahara *et al*., 2009)
- Inhibition of signal transduction pathways (Katula *et al*., 2005; Chung *et al*., 2009; Amado *et al*., 2011)
- Induction of cell cycle arrest (Choi *et al*., 2001; Zhang *et al*., 2009; Chahar *et al*., 2011)
- Antiangiogenesis and Anti-mutagenic activity (Fang *et al*., 2005; Jackson *et al*., 2006; Zhang *et al*., 2007)
- Enhancement of immune functions and surveillance (Havsteen *et al*., 2001; Pal *et al*., 2009)
- Induction of apoptosis (Zhang *et al*., 2009; Chahar *et al*., 2011)

- Enzyme inhibition (Nassani *et al.*, 2003; Lee *et al.*, 2006)
- Enzyme induction and enhancing detoxification (Dehn *et al.*, 2004; Yee *et al.*, 2005)

Currently, Phase I and II clinical trials are being conducted to investigate the anticancer effects of flavonoids in humans and it was observed that combination of quercitin and genistein (Quergen) can be used for treatment of prostate cancer (Zhu *et al.*, 2011). Moreover flavonoids diadzenin, flavopiridol are under Phase II clinical trials for prostate cancer while Protoapigenone analog is under Phase II clinical trials for breast cancer (Qianying *et al.*, 2012).

Potentilla is a Genus whose various species are used since ancient times for treatment of various diseases like cancer, diarrhea, ulcers, inflammation, viral and fungal infections, diabetes mellitus and other ailments (Bos *et al.*,1996; Syiem *et al.*, 2003; Yesilada and Kustal, 2005). *Potentilla fulgens* (Rosaceae) is an important traditional and medicinal plant of higher Himalaya that is mainly known for its medicinal and therapeutic importance (Delgado *et al.*, 2000). A number of antioxidant constituents have been reported from the plant which mainly consist of polyphenolic compounds. As the plant consist of polyphenolic compounds such as flavonoids which are potent bioactive molecules which possess anticarcinogenic effects as they can interfere with the initiation, development and progression of cancer by the modulation of cell cycle, apoptosis and angiogenesis.

Keeping in view the above facts about secondary metabolites like flavonoids in eradication of cancer, the present work is concentrated to meet the following objectives-

- Anti-mutagenic activity of different fractions of *Potentilla fulgens* Lodd.
- Isolation and characterization of secondary metabolites from *Potentilla fulgens* roots
- *In-silico* study of reported flavonoids with CDK-2, CDK-6 receptors

CHAPTER TWO

REVIEW OF THE LITERATURE

2.1 *Potentilla* species

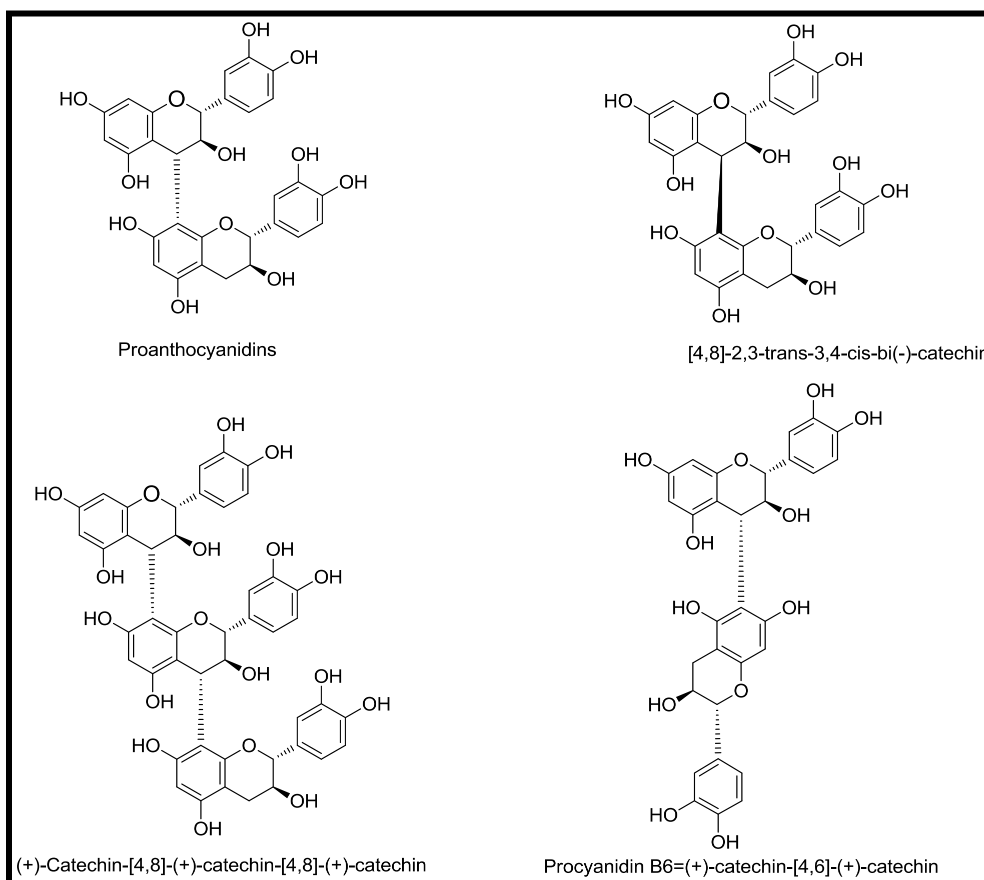
The genus *Potentilla* is a member of family rosaceae which is mainly found in Himalaya region. Various species of *Potentilla* are used since ancient times for treatment of various diseases like cancer, diarrhea, ulcers, inflammation, viral and fungal infections, diabetes mellitus and other ailments (Bos *et al*., 1996; Syiem *et al*., 2003) .

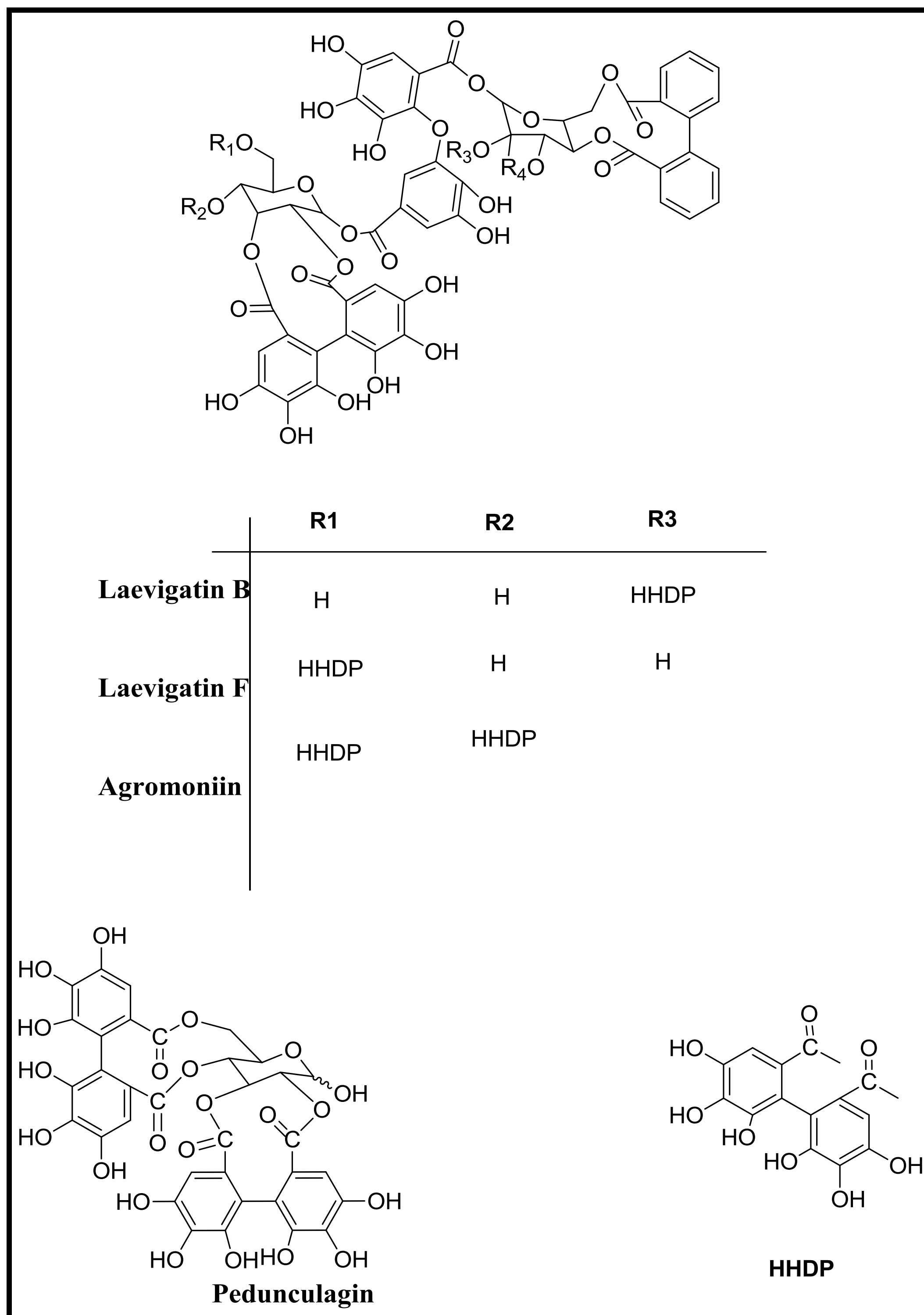
Potentilla species have been used for a long time in traditional medicine. In Greek and Latin language *Potentilla* species have been known under the names “Heptaphyllon” or “Pentaphyllon” and “Septifolium” sometimes as “Quinquefolium”. *Potentilla* is the genus of typical cinquefoils, containing about 500 species of annual, biennial and perennial herbs in the rose family Rosaceae. They are generally Holarctic in distribution, though some may even be found in montane biomes of the New Guinea Highlands (Tomczyk *et al*., 2009). They are generally found in regions of higher Himalaya and are known for their therapeutic and commercial importance. More than 500 species of *Potentilla* are used in ayurvedic, unani, siddha, chinese and tibetian systems of medicine due to high content of polyphenols in their aerial and underground parts (Haslam *et al*., 1996). Plants rich in polyphenols are gaining importance in maintaining good health due to their antioxidant properties. Polyphenolic compounds form stable complex with metal ions, proteins and polysaccharides thus helping in healing of wounds, burns and inflammations (Kirakosyan *et al*., 2003).

The genus name *Potentilla* comes from the Latin diminutive of **potens** meaning “powerful” in reference to the medicinal properties of some species. The genus *Potentilla* includes about 500 species of perennial, rarely biennial, and annual herbs and small shrubs with rhizomes. In their natural habitat they commonly occur in temperate, arctic and Alpine zones of the Northern hemisphere (Guillen *et al*., 2005).

2.1.1 Constituents of the roots and rhizomes

Various polyphenolic compounds are found from root and rhizomes of many *Potentilla* species. For *Potentilla erecta* 43 compounds have been identified and structurally elucidated. Due to the high amount of of tannins in the rhizomes of *Potentilla erecta* (i.e. 15–20% condensed tannins, 3.5% hydrolysable tannins), this group of natural compounds has been in the focus of many phytochemical studies. The condensed tannins of *Potentilla erecta* consist of dimeric and trimeric type B proanthocyanidin (Schleep *et al*., 1986). Several precursors for condensed tannins were identified for this plant source including (+)-catechin, (−)-epicatechin, (+)- gallocatechin and (−)-epigallocatechin (Gao *et al*., 2007). Also, sterols, amino acids and fatty acids were detected in a few *Potentilla* species (Tomczyka *et al*., 2009). Antioxidants have been isolated from dried root powder methanolic extract of *Potentilla fulgens* which are named as potifulgens (Jaitak *et al*., 2010c).



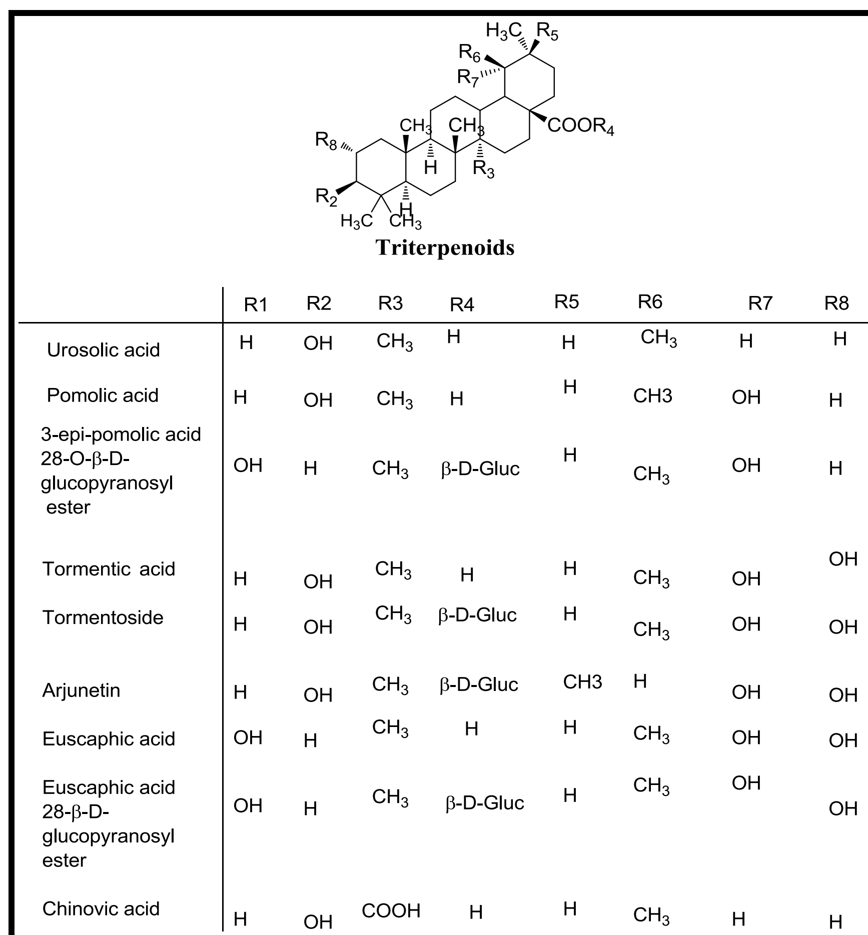


	R1	R2	R3
Laevigatin B	H	H	HHDP
Laevigatin F	HHDP	H	H
Agromoniin	HHDP	HHDP	

Fig 4. Constituents isolated from roots and rhizomes of *Potentilla* species

2.1.2 Constituents of the aerial parts

A number of compounds are elucidated from aerial parts of *Potentilla* species out of which main are *Potentilla anserina* (26 compounds) and *Potentilla erecta* (25 compounds), followed by *Potentilla fruticosa* (22compounds), *Potentilla chinensis* (19 compounds), *Potentilla discolor* (14 compounds), *Potentilla palustris* (12 compounds) and *Potentilla reptans* (8 compounds). A high number of triperpenoid compounds (27 structures) and hydrolysable tannins have been elucidated from aerial parts of *Potentilla* species (Okuda *et al*., 1984). Also several other constituents were described for *Potentilla* species which comprises coumarins,sterols, essential oils, polyphenols and 2-pyrone-4,6-dicorboxylic acid (Tomczyka *et al*., 2009). Two new triterpenes have also been isolated from aerial part of *P.fulgens* named Potentene A and B (Jaitak *et al*., 2010a,c)



	R1	R2	R3	R4	R5	R6	R7	R8
Urosolic acid	H	OH	CH_3	H	H	CH_3	H	H
Pomolic acid	H	OH	CH_3	H	H	CH3	OH	H
3-epi-pomolic acid 28-O-β-D-glucopyranosyl ester	OH	H	CH_3	β-D-Gluc	H	CH_3	OH	H
Tormentic acid	H	OH	CH_3	H	H	CH_3	OH	OH
Tormentoside	H	OH	CH_3	β-D-Gluc	H	CH_3	OH	OH
Arjunetin	H	OH	CH_3	β-D-Gluc	CH3	H	OH	OH
Euscaphic acid	OH	H	CH_3	H	H	CH_3	OH	OH
Euscaphic acid 28-β-D-glucopyranosyl ester	OH	H	CH_3	β-D-Gluc	H	CH_3	OH	OH
Chinovic acid	H	OH	COOH	H	H	CH_3	H	H

Fig 5. Constituents isolated from aerial parts of *Potentilla* species

2.1.3 Pharmacological profile of *Potentilla* species

Potentilla species (Rosaceae) and their extracts have been highly valued in many different ethnic cultures for hundreds of years throughout the world. Recent pharmacological studies have shown the traditional and medicinal uses of various *Potentilla* species and their extracts from roots, rhizomes and aerial parts. Medicinal and therapeutic applications of various *Potentilla* species are due to high content of polyphenolic compounds mainly condensed and hydrolysable tannins in roots, rhizomes and aerial parts (Fabricant and Farnsworth, 2001).

Various pharmacological actions of different *Potentilla* species are (Tomczyk *et al*., 2009)-

- Anti-neoplastic activity
- Antidiarrhoic activity
- Antihyperglycemic activity
- Anti-ulcerogenic activity
- Anti-inflammatory activity
- Spasmolytic activity
- Hepatoprotective activity
- Anti-ulcerogenic activity
- Antiviral activity
- Antimicrobial activity

2.2 *Potentilla fulgens*

Potentilla fulgens (Rosaceae) is an important traditional and medicinal plant of higher Himalaya that is mainly known for its medicinal and therapeutic importance. *Potentilla fulgens* is mainly used in ayurvedic, unani, siddha, chinese systems of medicine due to high content of polyphenolic compounds in their roots, rhizomes and aerial parts (Delgado *et al.*, 2000).

Common name-Cinquefoil in English and Bajradanti in Hindi

Family- Rosaceae

Duration- Perennial

Growth Habit- Herb

Fig 6. *Potentilla fulgens*

(Image adapted from uirig.altervista.org)

Commonly found in Jammu and Kashmir, Himachal Pradesh, Uttrakhand, Meghalaya, West Bengal, Manipur and higher Himalaya up to an altitude of 1800-4350m (Vashishtha *et al.*, 2009; Xue *et al.*, 2005; Zhao *et al.*, 2008)

2.2.1 Chemical constituents

Potentilla fulgens mainly consist of polyphenolic compounds. Aerial parts of the plant reported to contains triterpenes like Potentene-A, Potentene-B, flavonoids such as Afzelchin-4α→8”-catechin, Epiafzelchin and Rutin while root part of plant contain bioflavonoid potifulgene and epicatechin (Jaitak *et al* ., 2010a,c).

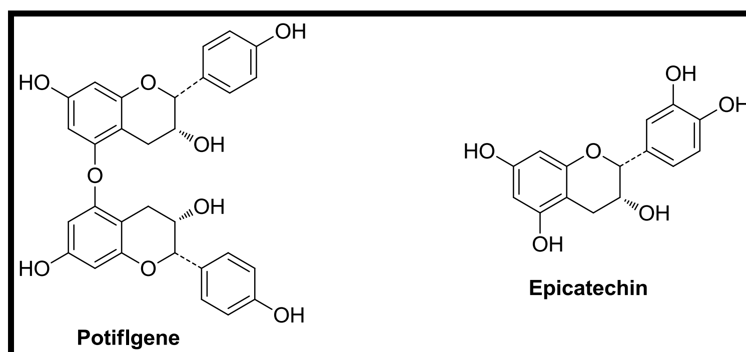


Fig 7. Constituents from root part of *P.fulgens*

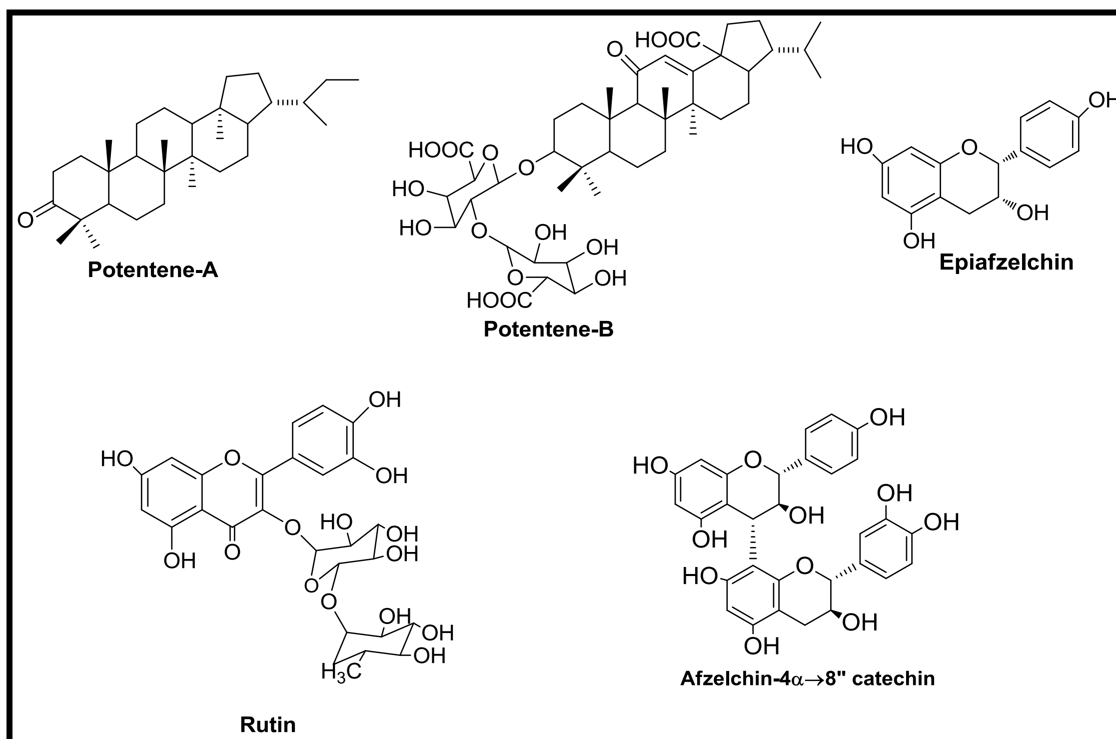


Fig 8. Constituents from aerial part of *P.fulgens*

2.2.2 Pharmacological activity

Pharmacological studies report that *P.fulgens* possesses anti-hyperglycemic, hypoglycemic, anti-hyperlipidemic, anti-tumor, anti-oxidant, anti-inflammatory and anti-ulcerogenic properties. Recently Gastroprotective activity of ethanolic root extract of *P. fulgens* has also been reported.

2.2.2.1 Anti-neoplastic activity

A preliminary study revealed that the methanolic extract of the roots of *Potentilla fulgens* L. was found to be active against certain tumors in a dose-dependent manner. The herb and the underground parts of the same plant were also used to treat various ailments, including neoplastic diseases (Syiem *et al*., 2003). It has also been reported that the aqueous root extracts of the herb of *Potentilla fulgens* are active against neoplastic tumours murine ascites Dalton's lyphoma (DL), depending on the method of administration (Rosangkima *et al*., 2004).

2.2.2.2 Antihyperglycemic activity

Diabetological studies showed the hypoglycemic activity of pure methanol *Potentilla fulgens* root extracts (Jang *et al*., 2007).

2.2.2.3 Antioxidative activity

Free radicals play an important role in pathogenesis of serious diseases such as diabetes, inflammation, cataracts, neurogenerative disorder. *Potentilla fulgens* act as antioxidant agent. Antioxidants play an important role in prevention of chronic diseases by reducing oxidative damage to cellular components that is caused due to reactive oxygen species (Choi *et al*., 1998; Miliaukas *et al*., 2004).

2.2.2.4 Gastroprotective activity

Ethanolic root extract of the plant possess potent gastroprotective and antisecretory effects for the treatment of gastric ulcers (Laloo *et al*., 2013).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Collection of plant material

P.fulgens was collected from Bharmour region of Chamba district during September 2012 at an altitude of 2000m. The roots and aerial parts of the plant (*P. fulgens*) were separated, cleaned and dried in oven for 4-5 days at about 40°C and subjected to chopping and grinding. Powder obtained was stored.

3.2 Chemicals

Salmonella typhimurium strains TA98 (frameshift mutationtest) and TA100 (base pair substitution test), were procured from IMTech (Institute of Microbial Technology), Chandigarh. Sodium azide for TA100, 4-nitro-*O*-phenylenediamine (NPD) for TA98, were procured from M/S Sigma Chemicals Co. (St Louis, MO, USA). Petroleum ether (batch), chloroform (batch), ethyl acetate(batch), methanol(batch) were procured from SDFCL. Laboratory grade Reverse osmosis water from ions R.O system was used. Silica gel 60/120 for column chromatography was procured from SDFCL. Glassware of fine quality were used and procured from Borosil, Perfit, JSGW. Readymade TLC plates F_{254} from Merck were used.

3.3 Extraction and isolation of dried root of *P.fulgens*

Dried root powder of *P. fulgens* was taken and extracted by the process of cold aqueous methanolic percolation (20:80, v/v). The above procedure was repeated three times. The 80% methanol obtained was further fractionated using different solvents i.e. Petroleum ether, Ethyl acetate, *n*-Butanol and Water. Solvents of different polarities were used so that the separation of different metabolites of methanol extract takes place as per their solubility. All the fractions were distilled using rota vapour under reduced pressure yielding aqueous methanolic (221gm) (AVP), petroleum ether (20.01gm), ethyl acetate (20.83gm)(EVAP), *n*-butanol (60.83gm)(BVP) and water (15.89gm) (WVP) respectively.

In order to determine the constituents from ethyl acetate fraction column chromatography was performed. For this column was packed with silica gel (60-120) mesh size. The column was eluted with gradient elution of petroleum ether: EtOAc, with an increasing proportion of EtOAc (5% and 10%) to give a total of 450 fractions. Fractions 65-90 were combined and dried on a rotavapor on the basis of a single spot on precoated silica gel TLC plate yielding **1** (4mg) as greenish-yellowish powder. Again fractions 110-140 were combined on the basis of a single spot on precoated silica gel TLC plate yielding **2** (4.1mg) as greenish semi-solid. Further combination of 150-275 fractions yielded 424 mg of a yellowish solid mixture, which was further chromatographed over silica gel (60-120 mesh, 750 mg). Sub-fractions 25-85 collected by eluting with 80% ethylacetate in petroleum ether were combined and dried on a rotavapor yielding **3** (125 mg) as a yellowish-brown powder.

The *n*-butanol extract (35 g) was subjected to column chromatography on silica gel (60-120 mesh) using gradient elution of 10%, 20% and 30% MeOH in EtOAc to give 620 fractions. Fractions 20-55 were combined and dried on a rotavapor yielding 100 mg of a yellowish solid mixture (2 compounds). The mixture was re-chromatographed over silica gel (60-120 mesh). By eluting with (solvent system), sub-fractions15-34 were combined together on the basis of a single spot on pre-coated silica gel TLC plates. The combined fractions were dried in vacuo yielding **4** (17 mg) as yellowish powder. Fractions 102-194 were combined and dried on a rotavapor yielding 102 mg of a brown colored solid mixture. This was recrystallized in methanol to yield **5** (16mg) of pure compound. Fractions 256-528 were combined and dried on a rotavapor yielding 230 mg of a yellow solid mixture. This mixture was rechromatographed over silica gel (60-120 mesh). Sub-fractions 25-74 were combined together on the basis of a single spot on precoated silica gel TLC plate. The combined fractions were dried in vacuo yielding **6** (25 mg) as a yellow semi-solid.

3.3.1 Determination of R_f Value

The Retention Factor (R_f) values of the isolated compounds was determined by performing thin layer chromatography (TLC) and calculated using the formula:

$$R_f \text{ Value} = \text{Distance moved by spot/Distance moved by solvent front}$$

Merck plates were used to perform TLC. Petroleum ether, ethyl acetate and methanol were used as mobile phase to elute TLC plates and determine the R_f value. The isolated compounds were dissolved in appropriate solvents and were spotted on the plate with fine capillary tube at the height of 0.8- 1.0 cm from the base. The plates were placed in TLC chamber and nearly two-third of the plate was run in that solvent. The plates were air dried and checked in UV chamber and were also developed with iodine in iodine chamber in order to visualize isolated compound and to calculate R_f value.

Table 1. Description of isolated molecules

Compound	Amount	R_f value	Percentage
1	4mg	0.3cm	30% ethylacetate:petroleum ether
2	4.1mg	0.5cm	50% ethyl acetate:petroleum ether
3	125mg	0.47cm	80% ethyl acetate:petroleum ether
4	17mg	0.43cm	75% ethyl acetate:petroleum ether
5	16mg	0.39cm	20% methanol:ethylacetate
6	25mg	0.53cm	5% methanol:ethyl acetate

3.3.2 Spectroscopic data

Catechin (3)

Melting point:175-178°C

^{13}C NMR (400MHz, $CDCl_3$): 80.99(C-2), 66.34(C-3), 27.72(C-4),155.37(C-5), 93.81(C-6),156.08(C-7),95.07(C-8),155.27(C-9), 98.99(C-10), 130.54(C-1') 114.97(C-2'), 144.75(C-3', C-4'), 114.38(C-5'), 118.30(C-6').

^{1}H NMR(400MHz, $CDCl_3$): 1.17-1.24 (m, 1H), 1.98 (s, 1H), 2.34-2.40 (dd, 1Ha), 2.65-2.71(dd, 1Hb), 3.80-3.86 (m,1H), 4.48-4.50(d, 1H, *J*=7.44), 5.70-5.71 (d,1H, *J*=2.24), 5.89-5.90(d,1H, *J*=2.28), 6.592-6.596 (m,1H,), 6.612-6.617(d,1H), 6.68-6.70(d, 1H, *J*=8.24), 6.743-6.748(d, 1H, *J*=2.08), 8.73-8.85 (m, 3H)

ESI-MS: *m/z* $[M-H]^-$ calculated for $(C_{15}H_{14}O-H)^-$ 289.08; found: 289.0 corresponding to molecular formula $C_{15}H_{14}O_6$.

3.4 Anti-mutagenic studies

Anti-mutagenic studies for different fractions of *P.fulgens* were carried out by employing Ames assay as propsed by Maron and Ames (1983) with minor variations as recommended by Bala and Grover (1989). TA98 and 100 tester strains of *Salmonella typhimurium* were used for the present study.

The Ames assay or *Salmonella typhimurium* microsome assay detect the mutagenicity of chemicals in bacterial tester strains carrying different mutations (frameshift or base pair substitutions) in various genes of histidine operon. These strains are auxotrophic in nature and are incapable to synthesize histidine (His), an amino acid required for their growth, and thus unable to form colonies in its absence. The principle of Ames assay lies in the use of these tester strains to test the mutagenicity of chemicals, as mutations in these strains act as hot spots for mutagens that causes DNA damage via different mechanisms.

3.4.1 *In vitro* Anti-mutagenic activity

The fresh cultures of tester strains, having density of 1-2×10^9 CFU/ml were used to test the anti-mutagenic activity of different fractions of *P.fulgens*. The minimal agar plates were prepared one day before use. Top agar was autoclaved and stored at 4˚C. Before the initiation of experiment, it was melted and kept at 45˚C. Two sets of experiments i.e. co-incubation and pre-incubation were carried out.

3.4.2 Prepration of samples

The different concentrations of fractions of *P.fulgens* were used. The concentrations used were: 100µg/0.1ml, 250 µg/0.1ml, 500 µg/0.1ml, 1000 µg/0.1ml and 2500 µg/0.1ml. All these concentrations were prepared in DMSO in sterile conditions.

3.4.3 Toxicity of different fractions (Negative Control)

The negative control was run with different concentrations of fractions to verify the toxicity of test sample. The concentrations were considered non-toxic if the number and size of revertant colonies in negative control were equivalent to that of spontaneous revertant colonies. Also for non-toxic effect of fractions, the intensity of background lawn should be equivalent to the control having only bacterial culture. For determining the toxicity of test sample, 0.1ml of extract or fractions of different concentrations along with 0.1ml of freshly grown culture was added to top agar maintained at 45˚C. The mixture was then plated on the minimal glucose agar plates which were then incubated at 37˚C for 48 hours.

3.4.4 Positive Control

The anti-mutagenicity of different fractions was determined against the known mutagens that are characteristic for each strain depending on the reversion event. Before checking the anti-mutagenicity, the effect of mutagen of known concentration was also checked on the tester strains. This was done in order to ensure responsiveness of tester strains to mutagens as well as the efficacy of promutagen. To confirm the effect of mutagen on tester strains, 0.1ml of freshly grown culture along with 0.1ml of mutagen of known concentration was added to soft agar. The

contents were then poured on to the minimal glucose agar plates after thorough mixing.The plates were then incubated at 37˚C for 48 hours and effect of mutagen was determined by counting the revertant colonies.

3.4.5 Co-incubation

In order to determine the anti-mutagenicity potential of different fractions, co-incubation mode of experiment was designed which involves the addition of 0.1ml of bacterial cultures, 0.1ml of direct-acting mutagen (NPD and sodium azide) and 0.1ml of non-toxic concentrations of extract and fractions into the 2ml of soft agar. The soft agar was then poured onto minimal agar plates. The plates were then incubated at 37˚C for 48 hours and efficacy of extracts was determined by counting the revertant colonies.

3.4.6 Pre-incubation

The mutagenicity potential of fractions was determined in pre-incubation mode of experiment. It involves the mixing of 0.1ml of mutagen (NPD or Sodium azide), 0.1ml of non-toxic concentrations of extract and fractions in sterile test tubes. The mixture was then incubated at 37˚C for 30 minutes. After incubation, the mixture along with 0.1ml of freshly grown culture was added to top agar which was then poured onto the minimal plates and incubated at at 37˚C for 48 hours.

3.4.7 Number of replicates and repeat experiments

Each sample (different fractions of *P.fulgens*) was assayed using triplicate plates per run and each experiment was conducted twice in order to make estimation of variation.

3.4.8 Calculations of Anti-mutagenic activity

3.4.8.1 Percent Inhibition

The anti-mutagenic activity of each fraction was expressed as percent decrease of reverse mutations as follows:

$$\text{Inhibition(\%)} = (a-b/a-c) \times 100$$

where

a =Number of histidine revertants induced by mutagen (sodium azide, NPD)

b =Number of histidine revertants induced by mutagen in the presence of extract

c =Number of histidine revertants induced in the presence of extract alone and solvent (negative control)

3.4.8.2 Percent of Control

$$\text{Percent of control} = (b/a) \times 100$$

where, a =number of histidine revertants induced by mutagen alone and b =number of mutagens in the presence of extract/fractions.

3.4.8.3 Statistical Analysis

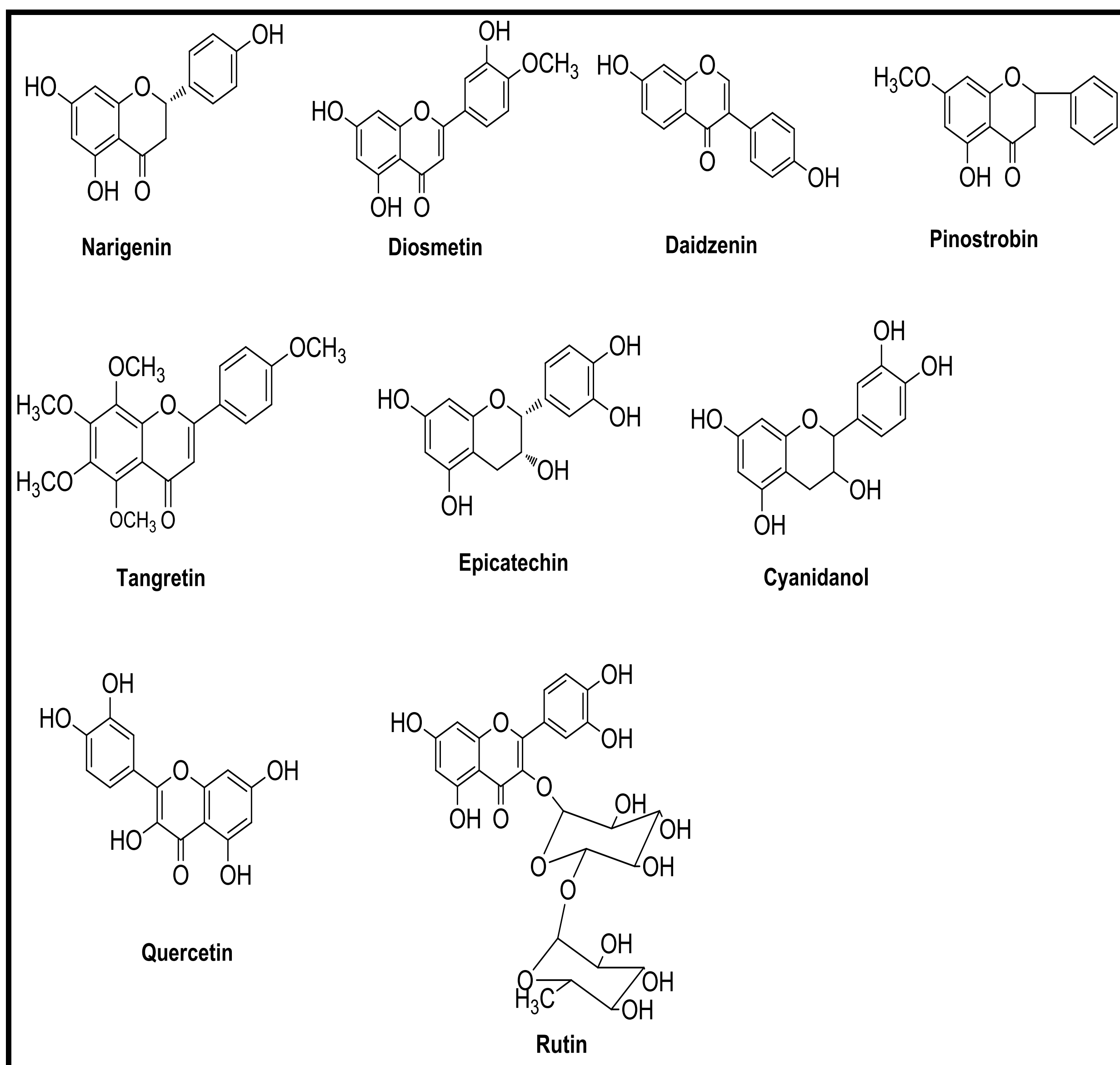
Results are presented as the Mean ± S.E. of two independent experiments with triplicate plates/dose/experiment. The data were analysed for statistical significance using analysis of variance (one-way and two-way ANOVA) and the difference among means was compared by high-range statistical domain (HSD) using Tukey's test (Meyers and Grosson, 1974).

3.5 *In-silico* study of reported flavonoids

3.5.1 Ligand preparation

The ligands used were sketched using ChemBioDraw ultra 12.0 and saved in .sdf format. The structure of ligands are shown in Figure 9. The ligands and the inhibitor were prepared by using the 'Ligprep'application of the maestro 9.3 and force field used was OPLS-2005. LigPrep application of Schrodinger suite involved addition of hydrogen atom; removal of unwanted molecules such as water and small ions; neutralization of charged groups, then generate ionization and tautomeric states with Epik; generation of stereoisomers, particularly if stereo chemical information is

missing; generation of low-energy ring conformations; removal of any badly prepared structures and optimization of the geometries.



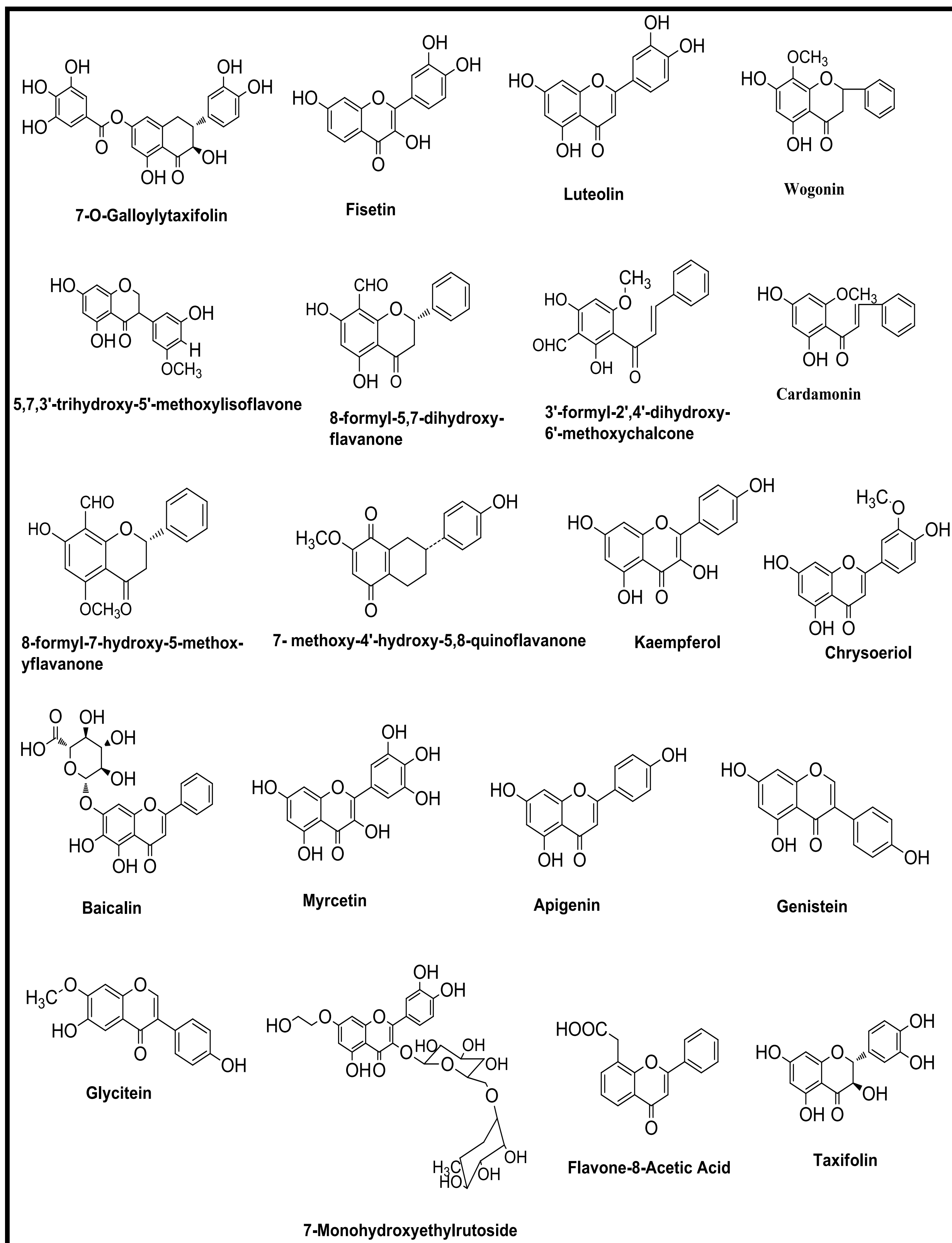


Fig 9. Structure of reported flavonoids for *in-silico* study

3.5.2 Protein preparation

Crystal structures of proteins for the CDK-2 and CDK-6 were retrieved from the RCSB (http://www.rcsb.org/pdb) with the PDB ID 2M1L and 1XO2 respectively. The protein was prepared as per the guidelines of protein preparation wizard application of the Maestro 9.3. Protein preparation involved two major function i.e. preparation and refinement. The preparation component neutralizes side chains that are not close to the binding cavity and do not participate in salt bridges while the refinement component performs a restrained impact minimization of the target which reorients the side chains and relieves steric clashes to perform docking studies.

3.5.2 Receptor grid generation

Grid of all prepared proteins were generated using 'Receptor Grid Generation' application of Maestro 9.3 (2012). Receptor grid defines the active site of receptor on the basis of already co-crystalized ligand present in the protein. Sometimes co-crystallized ligands are not present in the protein in that case 'Site Map' programme is run to identify best active site.

3.5.4 Docking

Docking of flavonoid molecules and the inhibitors was carried out using 'Glide docking' application of Maestro 9.3. There are three docking precision namely high throughput virtual screening (HTVS), standard precision (SP) and extra precision (XP) available in the Glide docking programme. Among these we have choosen XP tool because it gives highly accurate docked poses and suitable for docking of < 100 ligands. Ligand was taken as flexible. Vander waals scaling was adjusted to 0.8 and partial charge cutoff to 0.15 to soften the non-polar parts of the ligand. 10000 poses per docking run were allowed to run and 1 pose per ligand was allowed to be written. In the post docking minimization number of poses per ligand to be included was taken to be 10. The threshold energy below which the pose to be rejected was 0.5 Kcal/mol. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each ligand was selected.

3.5.5 QikProp

It is an application of Schrodinger suite 2012 which is used for fast and precise prediction of absorption, distribution, metabolism and excretion (ADME) of chemical compounds. It calculates physically important descriptions and pharmaceutically relevant properties of organic molecules. This application was used for *in-silico* ADME prediction of flavonoid molecules for which docking studies were carried out.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Extraction and isolation from dried root of *P.fulgens*

The different extracts and fractions of *P.fulgens* root powder, were prepared using cold extraction method.

4.1.1 Preliminary analysis

Preliminary analyses of different fractions of *Potentilla fulgens* were performed to identify and confirm presence of flavonoids in plant by using following standard methods-:

1. **Shinoda test**

About 5 mg of the extract was dissolved in ethanol. 3 mg magnesium powder was then added followed by few drops of conc. HCl. A red-crimson coloration indicated the presence of flavonoids.

2. **Sodium hydroxide test**

About 5 mg of the extract was dissolved in water, warmed, and filtered; to this solution (2 ml), 10% aqueous NaOH was added. This produced a yellow coloration. A change in color from yellow to colorless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids.

Inferences for different fractions are recorded in Table 2. The flavonoids being tested in the fraction contain polyphenolic molecules which are weakly acidic in nature. In the first general test for flavonoids, the extract was first treated with a small amount of a weak concentration of NaOH (10%). NaOH is completely ionic, containing sodium cations and hydroxide anions. It is a sufficiently strong base which deprotonates phenol entirely. The purpose of 10% NaOH in this test was to deprotonate the polyphenolic molecules cointained in flavonoids. NaOH turns phenols into phenoxides which are much more soluble in water than phenols. Fractions containing flavonoids showed yellow coloration. The objective of this experiment was to decolorize the yellow color of the fractions containing flavonoids. This was done through the addition of HCl to the fraction. Only a minimal amount of NaOH was added to avoid the

tendency of HCl to neutralize with the mixture. The addition of sufficient amounts of hydrochloric acid prevents the development of colored complexes of many phenols and all enols. This is the reason why upon addition of HCl to the mixture, the solution slowly decolorizes (Ayoola *et al*., 2008).

Table 2. Preliminary test on different fractions of *P.fulgens*

Fraction	Test	Observation	Inference
Aqueous methanolic	Shinoda test	Red –crimson color	Flavonoids confirmed
	Sodium hydroxide test	Yellow color changes to colorless on addition of acid	Polyphenolics confirmed
Ethyl acetate	Shinoda test	Red –crimson color	Flavonoids confirmed
	Sodium hydroxide test	Yellow color changes to colorless on addition of acid	Polyphenolics confirmed
n-butanol	Shinoda test	Red –crimson color	Flavonoids confirmed
	Sodium hydroxide test	Yellow color changes to colorless on addition of acid	Polyphenolics confirmed
Water	Shinoda test	Red –crimson color	Flavonoids confirmed
	Sodium hydroxide test	Yellow color changes to colorless on addition of acid	Polyphenolics confirmed

4.2 Anti-mutagenic activity

The anti-mutagenic activity of different fractions of *P.fulgens* was carried out by using Ames assay given by Maron and Ames (1983) with modifications as suggested by Bala and Grover (1989). The strains used to detect anti-mutagenicity was TA98 and TA100 of *Salmonella typhimurium*.

Different fractions of *P.fulgens* i.e. Aqueous methanolic(AVP), Ethylacetate(EVAP), *n*-Butanol(BVP) and Water(WVP) were taken and anti-mutagenic assay was performed.

4.2.1 Aqueous methanolic (AVP)

It was observed that aqueous methanolic fraction of plant exhibited 46.8% and 41.8% inhibitory activity at the maximum dose tested (2500µg/0.1ml) in co-incubation and pre incubation mode of treatment respectively against NPD a direct acting mutagen in TA98 strain of *S. typhimurium* whereas it reduced his^+ revertants induced by sodium azide in TA100 strain by 50.8% and 35.8% in co-incubation and pre-incubation mode of treatment respectively as shown in table 3. These results showed that AVP showed good inhibitory activity against NPD as well as sodium azide.

Figure 10 depicts graphic relationship of percent of control with dose and effect of aqueous methanolic fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S.typhimurium*. Aqueous methanolic fraction shows highest inhibitory activity at dose of 1000µg/0.1ml in co-incubation i.e 53.8% while for pre-incubation method highest inhibitory activity was observed at dose of 2500µg/0.1ml i.e. 50.8%. Aqueous methanolic fraction was quite effective against the mutagens and showed strong anti-mutagenic activity.

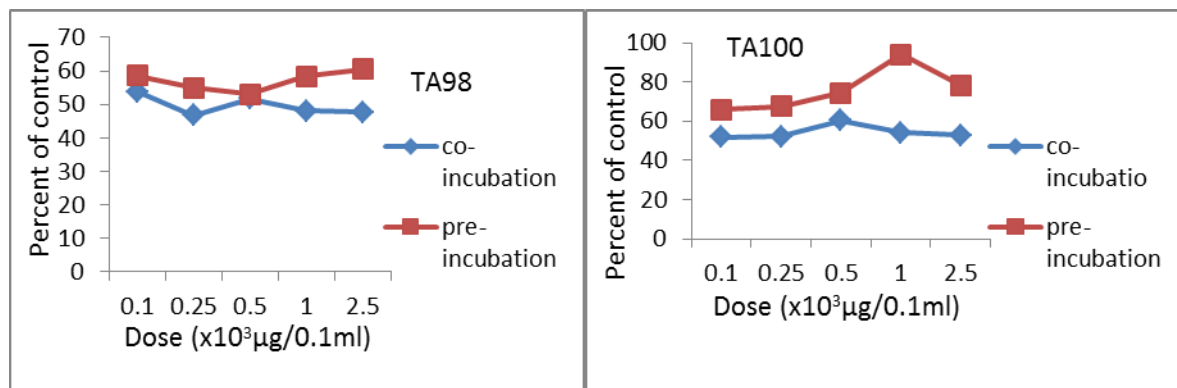


Fig 10. Effect of aqueous methanolic fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S.typhimurium.*

The inhibitory effect of different fractions is categorized mainly into four groups depending upon their activity. These groups are 'weak' (<25%), 'moderate' (25%-50%), 'strong' (50%-75%) and 'very strong' (>75%). AVP shows moderate to strong activity against NPD as well as sodium azide.

Table 3. Effect of aqueous methanolic fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium*

Treatment	Dose(µg/0.1ml)	TA98		TA100	
		(Mean±SE)	% inhibition	(Mean±SE)	% inhibition
Spontaneous	_	24.33±6.50	_	63.66±17.01	_
Positive control NPD Sodium azide	20 2.5	983±262.71	_	1028.66±274.92	_
Negative control	2500	13±3.47	_	52.66±14.07	_
	1000	11.33±3.02	_	57±15.23	_
	500	18±4.81	_	59.66±15.94	_
	250	23±6.14	_	49.66±13.27	_
	100	24.33±6.50	_	64±17.01	_
Co-incubation	2500	528.33±141.20	46.8	532.66±142.36	50.8
	1000	460±122.94	53.8	537.66±143.69	50.5
	500	508.66±135.94	49.1	622.33±166.32	41.9
	250	472.20±126.20	53.2	556.70±148.78	48.2
	100	468.53±125.22	53.6	541.85±144.81	50.4
Pre-incubation	2500	576.66±154.12	41.8	678.33±181.29	35.8
	1000	539.66±144.23	45.6	695±185.74	34.3
	500	521±139.24	47.8	764.66±204.36	27.2
	250	573.66±153.31	42.6	966.66±258.35	6.3
	100	595±159.02	40.4	804.66±215.05	23.2

One way ANOVA

	TA98	TA100
Positive control and coincubation	$F(5,12)= 28.69^{*}$ HSD=215.26	$F(5,12)= 3333.87^{*}$ HSD= 19.33
Positive control and preincubation	$F(5,12)=30.91^{*}$ HSD=188.06	$F(5,12)=1719.9^{*}$ HSD=20.86

Two way ANOVA
Coincubation and Preincubation

	TA98	TA100
Treatment	$F(1, 20)= 3.91$	$F(1,20)=7.29^{*}$
Dose	$F(4,20)=2.16$	$F(4,20)=1.89$
Treatment x Dose	$F(4, 20) = 0.32$	$F(4,20)=1.85$

NPD: 4-nitro-*O*-phenylenediamine. $^{*}P \leq 0.05$

4.2.2 Ethylacetate (EAVP)

It was observed that ethylacetate fraction exhibited 49.5% and 49.2% inhibitory activity at the maximum dose tested (2500µg/0.1ml) in co-incubation and pre-incubation mode of treatment respectively against NPD in TA98 strain of *S. typhimurium* whereas it inhibited his^+ revertants induced by sodium azide in TA100 strain by 9.5% and 51.9% in co-incubation and pre-incubation mode of treatment respectively (Figure 11).

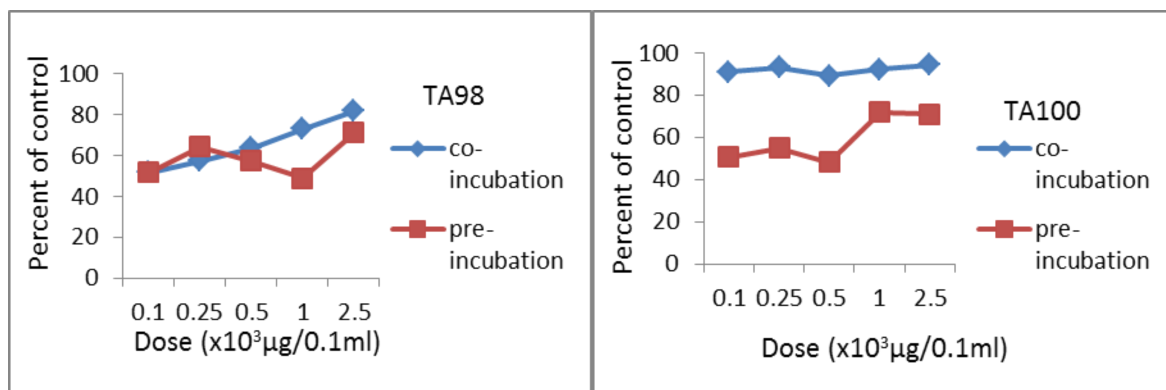


Fig 11. Effect of ethyl acetate fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S.typhimurium.*

As shown in table 4 EVAP fraction showed weak inhibitory avtivity at every dose of treatment for co-incubation in TA100 strain while it showed good inhibitory activity for TA98 strain with maximum inhibition activity of 49.5% at dose of 2500µg/0.1ml. This shows that co-incubation method was ineffective in TA100 strain at all doses for EVAP fraction while for TA98 strain it was quite effective. Good inhibitory activity was observed in both co-incubation and pre-incubation methods of treatment at a dose of 1000µg/0.1ml in TA98 strains i.e. 43.5% and 36.6% respectively while for TA100 strain inhibitory activity for co-incubation treatment was less i.e. 7.2%, but for pre-incubation treatment good inhibitory activity was observed i.e 47.6%. From these results it can be predicted that good inhibitory activity was observed only for pre-incubation method of treatment while for co-incubation method percentage of inhibition was not satisfactory.

Table 4. Effect of ethyl acetate fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium.*

Treatment	Dose(µg/0.1ml)	TA98		TA100	
		(Mean±SE)	% inhibition	(Mean±SE)	% inhibition
Spontaneous	_	21.33±5.70	_	63.66±17.01	_
Positive control NPD Sodium azide	20 2.5	682.66±182.45	_	1028.66±274.92	
Negative control	2500	16±4.27	_	51.33±13.71	_
	1000	14±3.74	_	54.33±14.52	_
	500	11.33±3.02	_	63.66±17.01	_
	250	15.33±4.09	_	54.33±14.52	_
	100	19±5.07	_	49.33±13.18	_
Co-incubation	2500	352.66±94.25	49.5	935±249.88	9.5
	1000	391.66±104.67	43.5	957.66±255.94	7.2
	500	434.33±116.08	36.9	915.66±244.72	11.7
	250	498.33±133.18	27.6	946.66±253.00	8.4
	100	558.66±149.30	18.6	969±258.97	6.0
Pre-incubation	2500	352.66±94.25	49.2	520.66±139.15	51.9
	1000	437.66±116.97	36.6	564.33±150.82	47.6
	500	390.33±104.32	43.5	497.33±132.91	55.0
	250	333.66±89.17	52.2	739.33±197.59	29.6
	100	485.66±129.79	29.6	729.33±194.92	30.5

One way ANOVA

Positive control and coincubation $F(5,12)=11.83^{*}$ HSD=211.40 $F(5,12)=6.36^{*}$ HSD=92.48

Positive control and preincubation $F(5,12)=18.81^{*}$ HSD=177.24 $F(5,12)=49.02^{*}$ HSD=171.24

Two way ANOVA
Coincubation and Preincubation

Treatment	$F(1, 20)= 7.18^{*}$	$F(1,20)=451.44^{*}$
Dose	$F(4,20)=9.68^{*}$	$F(4,20)=13.87^{*}$
Treatment x Dose	$F(4, 20) = 4.08^{*}$	$F(4,20)=8.47^{*}$

NPD: 4-nitro-*O*-phenylenediamine. $^{*}P\leq0.05$

4.2.3 *n*-Butanol (BVP)

BVP showed strong inhibitory activity in both tester strains in co-incubation as well as pre-incubation method. Inhibitory activity was 60.4% and 35.4% in co-incubation and pre-incubation respectively in TA 98 tester strains while 55.6% and 62.0% inhibition was observed in TA100 tester strains respectively (table 5).

Table 5. Effect of *n*-butanol fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium*

Treatment	Dose(µg/0.1 ml)	TA98		TA100	
		(Mean±SE)	% inhibition	(Mean±SE)	% inhibition
Spontaneous	_	22±5.87	_	50.33±13.45	_
Positive control NPD Sodium azide	 20 2.5	 902.33±241.15	_	 869±232.25	_
Negative control	1000	20±5.34	_	159±42.49	_
	500	11.33±3.02	_	155.66±41.60	_
	250	14.66±3.91	_	188.66±50.42	_
	100	17.33±4.62	_	179±47.83	_
Co-incubation	1000	369±98.61	60.4	474±126.68	55.6
	500	508.33±135.85	44.2	479.66±128.19	54.5
	250	391.66±104.67	57.5	562.33±150.28	45.0
	100	572.33±152.96	37.2	735±196.43	19.4
Pre-incubation	1000	589.66±157.59	35.4	428.33±114.47	62.0
	500	555.33±148.41	38.9	522.33±139.59	48.5
	250	641±171.31	29.4	555±148.33	46.1
	100	635±169.71	30.2	675±180.40	28.1

One way ANOVA

Positive control and coincubation $F(5,12)=65.07^{*}$ HSD= 219.40 $F(5,12)=363.26^{*}$ HSD=93.80

Positive control and preincubation $F(5,12)=85.21^{*}$ HSD=193.60 $F(5,12)=1135.38^{*}$ HSD= 51.91

Two way ANOVA

Coincubation and Preincubation

Treatment	$F(1, 20)= 30.48^{*}$	$F(1,20)=3.10$
Dose	$F(4,20)=106.72^{*}$	$F(4,20)=885.23^{*}$
Treatment x Dose	$F(4, 20) = 5.64^{*}$	$F(4,20)=5.16^{*}$

NPD: 4-nitro-*O*-phenylenediamine. *P≤0.05

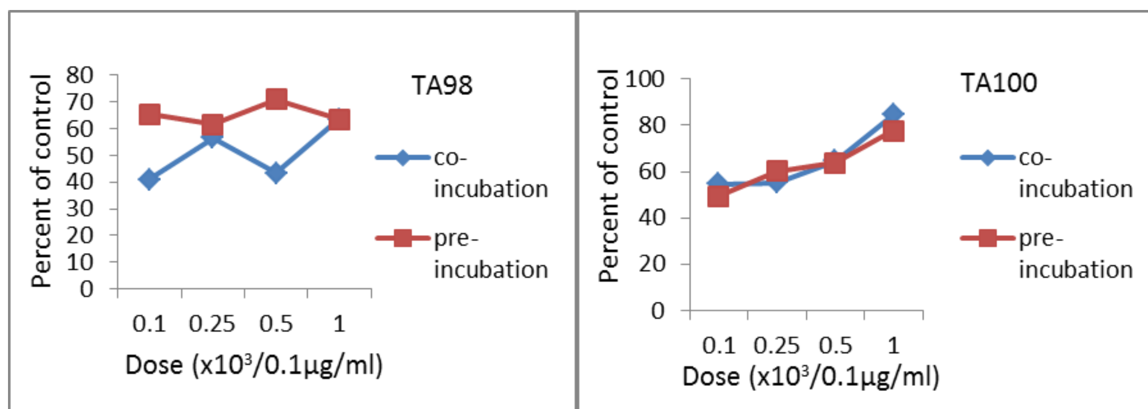


Fig 12. Effect of *n*-butanol fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S.typhimurium*

Graph between percent of control and dose was plotted (Figure 12) and it depicted that at highest dose of 2500µg/0.1ml, *n*-butanol fraction exhibited high inhibition of mutagens in co-incubation as well as pre-incubation method while as the concentration was decreased to 100µg/0.1ml, there was decrease in the inhibition of the mutagens. Inhibitory activity was 37.2% and 30.2% in co-incubation and pre-incubation respectively for TA98 strain while 19.4% and 28.1% inhibition was observed in TA100 tester strains respectively. This shows that with the decrease in concentration the effect of *n*-butanol fraction on mutagens decreased. At dose of 500µg/0.1ml inhibitory activity for co-incubation and pre-incubation treatments were satisfactory. At this dose inhibitory activity was 44.2% and 38.9% in co-incubation and pre-incubation respectively in TA 98 tester strains while 54.5% and 48.5% inhibition was observed in TA100 tester strains respectively. *n*-Butanol fraction showed strong inhibition activity againt the mutagens. The anti-mutagenic activity of the fractions may be due to their free radical scavenging abilities. Reactive oxygen species and other free radicals cause oxidative stress which in turn causes DNA, protein, and lipid damage which leads to change in chromosome instability, genetic mutation, and modulation of cell growth that result in cancer (Klaunig *et al.*, 2010).

4.2.4 Water (WVP)

Water fraction also showed good inhibitory activity as shown against both tester strains. It was observed that water fraction exhibited 56.6% and 60.7% inhibitory activity in co-incubation and pre-incubation mode of treatment respectively against NPD a direct acting mutagen in TA98 strain of *S. typhimurium* whereas it reduced his$^+$ revertants induced by sodium azide in TA100 strain by 34.5% and 50.6% in co-incubation and pre-incubation treatment respectively (Table 6). Figure 13 depicts graph between percent of control and dose, water fraction shows strong inhibitory activity at all concentrations in co-incubation as well as pre-incubation treatments.

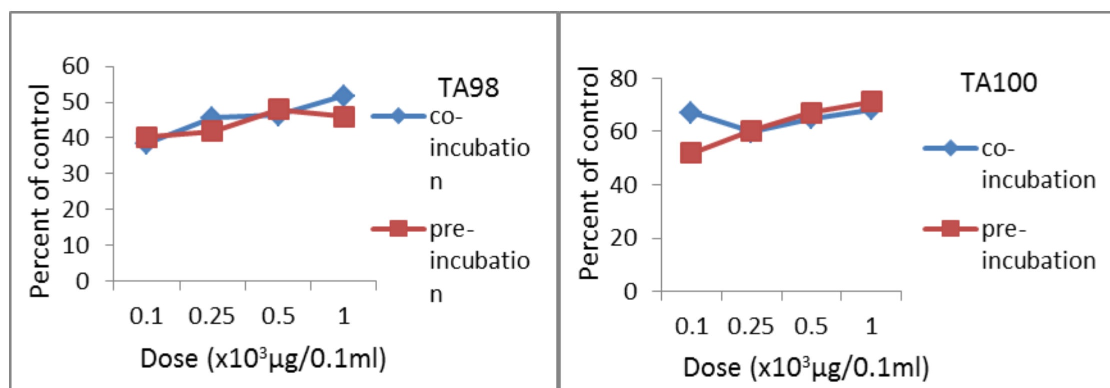


Fig 13. Effect of water fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S.typhimurium*

Inhibitory activity was good even when the concentration of dose was decreased to 100µg/0.1ml. It was observed that water fraction exhibited 49.1% and 54.9% inhibitory activity in co-incubation and pre-incubation mode of treatment respectively against NPD a direct acting mutagen in TA98 strain of *S. typhimurium* whereas it reduced his$^+$ revertants induced by sodium azide in TA100 strain by 33.4% and 30.4% in co-incubation and pre-incubation treatment respectively. These results indicate that good inhibitory activity was observed for pre-incubation treatment.

Table 6. Effect of water fraction on the mutagenicity of NPD in TA98 and sodium azide in TA100 tester strains of *S. typhimurium*

Treatment	Dose(µg/0.1ml)	TA98		TA100	
		(Mean±SE)	% inhibition	(Mean±SE)	% inhibition
Spontaneous	-	21.66±5.79	_	70±18.70	_
Positive control NPD Sodium azide	 20 2.5	 927.66±247.92	 _	 1051±280.89	 _
Negative control	1000	15.66±4.18	_	51.66±13.80	_
	500	8.66±2.31	_	52±13.89	_
	250	11±2.93	_	51.33±13.71	_
	100	16.33±4.36	_	56.33±15.05	_
Co-incubation	1000	356.66±95.32	56.6	705.66±188.59	34.5
	500	422.66±112.96	54.9	628.66±168.01	42.2
	250	430±114.92	54.2	681.66±182.18	36.9
	100	479.66±128.19	49.1	718±191.89	33.4
Pre-incubation	1000	373.33±99.77	60.7	545.33±145.74	50.6
	500	388±103.69	58.7	633.66±169.35	41.7
	250	445±118.93	52.9	704±188.15	34.7
	100	427±114.12	54.9	748.33±200.00	30.4

One way ANOVA

Positive control and coincubation $F(5,12)=267.61^*$ HSD=108.83 $F(5,12)=167.52^*$ HSD=159.27
Positive control and preincubation $F(5,12)=142.83^*$ HSD=148.54 $F(5,12)=224.82^*$ HSD=138.52

Two way ANOVA
Coincubation and Preincubation

Treatment	F(1, 20)= 1.57	F(1,20)=3.61
Dose	$F(4,20)=361.51^*$	$F(4,20)=361.51^*$
Treatment x Dose	F(4, 20) = 2.44	F(4,20)=2.44

NPD: 4-nitro-*O*-phenylenediamine. $^*P \le 0.05$

The anti-mutagenic activity of extracts appears to be linked to their scavenging property as well as due to their ability to affect the process of enzymatic activation, on the other hand there are many ways in which environmental factors, such as polyphenols, can inhibit or alter the sequence of events leading to mutations. Thus the modifying action of extracts may not be limited to interaction at the level of cytochrome P450-mediated metabolism of promutagen/ carcinogens. The mechanism of protection of these structurally very diverse compounds may be multifactorial (Newmark, 1996).

The consumption of the drugs, fruits and vegetables, however, often results in enhancement of enzymes involved in the metabolism of xenobiotic and carcinogens thereby accelerating their metabolic disposal. Of particular interest, in this respect, are the glutathione S-transferases (GST), a family of phase II transformation enzymes (Gradelet *et al*., 1998).

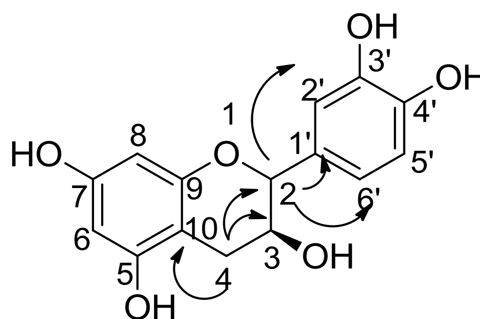
Owing to the varied nature of the compounds, a combination of anti-mutagens will probably be necessary and, moreover, it is essential to confirm putative anti-mutagenic activity observed *in vitro* through the use of animal models. The net effect is that we currently know very little about what may well prove to be an interesting and exciting area of anti-mutagenic and anti-carcinogenic research.

This study supports the argument that traditional medicinal plants like *P.fulgens* are valuable sources that are effective in fighting against variety of ailments including cancer.

4.3 Isolation and characterization of molecules

Compound **3**, melting point 175-178°C, was isolated as a yellowish-brown amorphous powder from ethylacetate fraction. The compound showed positive response to $FeCl_3$. A negative ESI-MS showed an ion peak at m/z 289.0$[M-H]^-$ (calculated for $[C_{15}H_{14}O$-$H]^-$,289.08) corresponding to molecular formula $C_{15}H_{14}O_6$, with six degrees of unsaturation. In the ^1H NMR spectrum, δ_H between 5.70-6.74 indicated six aromatic protons, possibly arranged in two benzene moieties. The DEPT spectrum showed eight well resolved peaks corresponding to 1-CH_2, 7-CH signals. Seven signals were

assigned to quaternary carbons. In ^{13}C NMR spectrum values at δ_C 80.9 and 66.34 were assigned to C-2 and C-3 respectively. In ^1H NMR δ_H at 4.48 showed HMBC correlation with 130.54 and 66.34 while δ_H 5.70 showed HMBC correlation with 155.27 and 95.07. δ_H at 2.34 (H-4) showed correlation with 80.9, 66.34 and 98.99. ^{13}C NMR signals showed chemical shift values indicative of either catechin or epicatechin. Examination of coupling constant (*J*=7.4) between H-2 and H-3, clearly indicated that present molecule is catechin. NMR and Mass spectra of compound 3 are attached in appendix-A.



Catechin (3)

From previous studies it was reported that *Potentilla* species consists of large polyphenolic contents in aerial as well as root parts (Jaitak *et al* ., 2010a,c). Although, flavonoids act as anticancer agents by participating in cell cycle (Choi *et al*., 1994; Edenharder and Grunhage, 2003), their molecular interaction with different CDK's (Cyclic dependent kinase) are unknown. Thus, we carried out moleculer docking studies of the already reported flavonoid molecules with CDK-2 and CDK-6 receptors.

4.4 *In-silico* study

Neoplastic diseases are characterized by uncoordinated cell growth and cellular proliferation which follows an orderly progression through the cell cycle, governed by protein complexes composed of cyclins and cyclin-dependent kinases (CDK's). Also mutations and overexpression of cyclins and cyclin-dependent kinases have been reported and proposed to be oncogenic events (Cordon-Cardo, 1995). *In-sillico* study of already reported thirty natural and semisynthetic flavonoid molecules were

performed with CDK-2, CDK-6 receptors. The reason for preferring different flavonoid molecules was the significant *in-vitro* activities exhibited by selected molecules against different cancer cell lines (Scholar and Toewa, 1994; Van Acker *et al.*, 1997, 2000; Kandaswami *et al.*, 2005; Fresco *et al.*, 2006; Vrba *et al.*, 2013). Docking results of flavonoid molecules were compared with the standard inhibitors of the receptors and it was found that 7-*O*-galloyl-taxifolin which is a semisynthetic molecule has strong affinity with both receptors as compared to their selective inhibitor.

4.4.1 Molecular interaction of flavonoids with CDK-2 receptor

7-*O*-galloyl-taxofolin showed highest binding affinity for CDK-2 receptor with glide score of -6.83kcal/mol followed by 7-Monohydroxyethylrutoside with glide score of -6.36 kcal/mol, having greater binding affinity than inhibitor of CDK-2 i.e. SU9156 with glide score of -4.24kcal/mol.

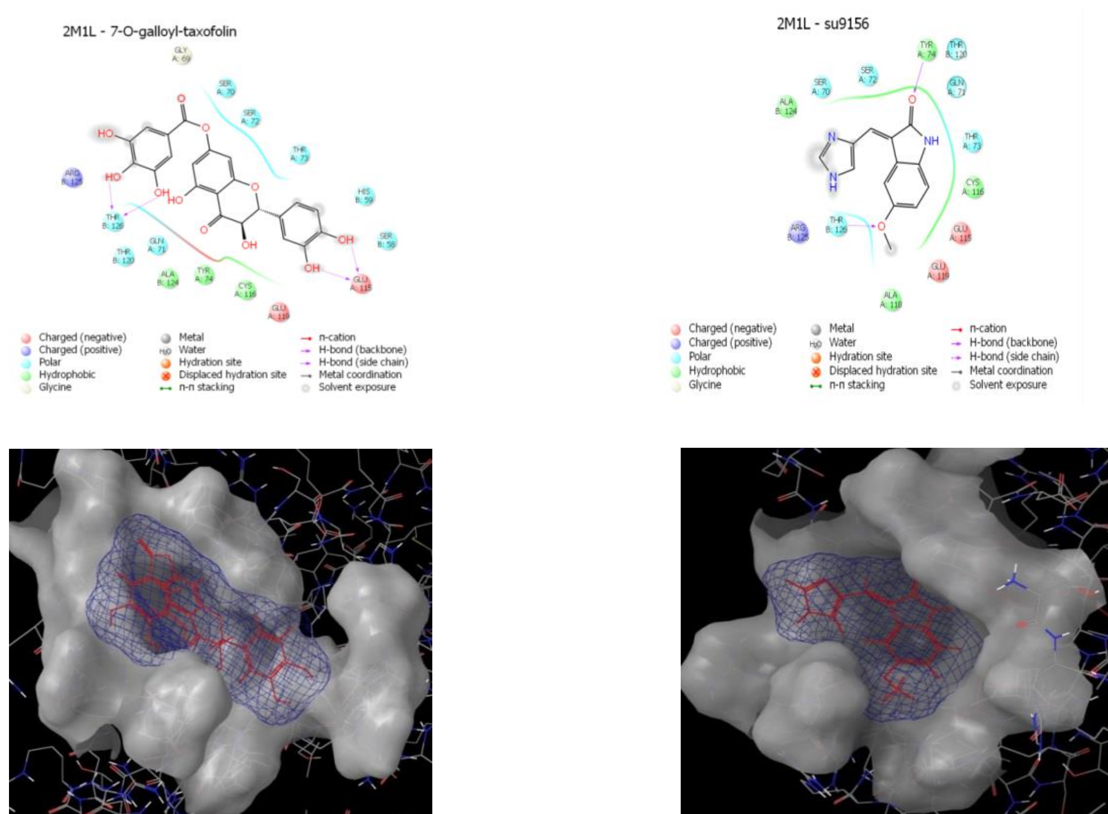


Fig 14. Interaction profile and molecular docking poses of (a)7-*O*-galloyl-taxifolin and (b)SU9156 with receptor CDK-2.

Table 7. Glide score, Lipophilicity, Hydrogen bond and electrostatic energies of flavonoids docked with CDK-2

Ligand	GScore(Kcal/mole)	LipophilicEvdW	HBond	Electro
7-*O*-galloyl-taxofolin	-6.83	-2.29	-3.84	-1.12
7-Monohydroxyethylrutoside	-6.36	-2.75	-2.77	-1.13
Myrcetin	-6.29	-2.4	-2.88	-0.91
Taxifolin	-5.95	-2.29	-2.4	-0.85
Quercetin	-5.78	-2.35	-2.4	-0.89
Diosmetin	-5.74	-3.14	-1.69	-0.5
Baicalin	-5.53	-1.98	-2.4	-1.09
Luteolin	-5.5	-2.42	-1.92	-0.82
Catechin	-5.23	-1.8	-1.92	-1.35
Epicatechin	-5.06	-2.99	-1.54	-0.53
Fiestin	-5.05	-1.97	-2.05	-0.89
Flavone-8-acetic acid	-5.02	-3.01	-1.81	-0.76
Glycetin	-4.9	-2.34	-0.96	-1.23
Rutin	-4.83	-2.42	-1.44	-0.83
Cyanidanol	-4.77	-1.5	-1.89	-1.16
5,7,3'-trihydroxy-5'-methoxyl-isoflavone	-4.64	-2.41	-1.29	-0.53
3'-formyl-2',4'-dihydroxy-6'-methoxychalcone	-4.53	-4.53	0	-1.18
Apigenin	-4.48	-2.4	-1.29	-0.47
Kaempferol	-4.36	-1.61	-2.55	-0.94
Tangretin	-4.3	-3.15	-1.01	-0.37
SU9156	**-4.24**	**-2.23**	**-0.91**	**-0.44**
Narigenin	-4.22	-1.79	-1.48	-0.71
Genistein	-4.2	-2.72	-1.18	-0.3
8-formyl-7-hydroxy-5-methoxy-flavanone	-4.1	-2.05	-1.85	-0.46
Pinostrobin	-4.01	-3.4	0	-0.22
Chyrseriol	-4	-3.51	-1.39	-0.5
Wogonin	-3.8	-2.63	-1.44	-0.28
Cardamonin	-3.8	-2.63	-1.44	-0.28
8-formyl-5,7-dihydroxy -flavanone	-3.6	-1.87	-0.96	-0.47
Daidzenin	-3.5	-2.6	-0.77	-0.35
7-methoxy-4'-hydroxy-5,8-quinoflavanone	-3.08	-2.66	-0.35	-0.09

7-*O*-galloyl-taxofolin is semisynthetic flavonoid that up-regulates Heme Oxygenase-1 in RAW264.7 Cells via MAPK/Nrf2 Pathway (Vrba *et al.*, 2013). It shows polar

interactions with THR 126, GLN 71, THR 120, THR 73, SER 70, SER 72, SER 58 and HIS 59 amino acid residues while it forms hydrophobic interactions with ALA 124, CYS 116 and TYR 74. This indicates that polar and hydrophobic interactions of 7-*O*-galloyl-taxofolin are responsible for strong binding affinity of the molecule with receptor. Also pre-clinical experiments in mice have shown that 7-Monohydroxyethylrutoside administration before doxorubicin effectively protects against doxorubicin induced cardiotoxicity, without interfering with its antitumor activity (Van Acker *et al*., 2000). 7-Monohydroxyethylrutoside has glide score of -6.36 kcal/mol which is slightly less than 7-*O*-galloyl-taxofolin. Also myrcetin has dockscore of -6.29kcal/mol and it has been reported that myrcetin induces pancreatic cancer cell death via the induction of apoptosis and inhibition of the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Phillips *et al*., 2011). Glide score of other flavonoid molecules with CDK-2 are shown in table-7 and molecular docking pose of 7-*O*-galloyl-taxofolin with CDK- 2 is shown in figure 14(a) while figure 14(b) shows molecular docking pose of SU9156 (inhibitor of CDK-2).

4.4.2 Molecular interaction of flavonoids with CDK-6 receptor

7-*O*-galloyl-taxofolin has strong binding affinity with receptor (CDK-6) as compared to its selective inhibitor.7-*O*-galloyl-taxofolin has glide score of-11.66 kcal/mol while that of inhibitor PD0332991 is -7.71kcal/mol, which illustrates that 7-*O*-galloyl-taxofolinhas highest binding affinity for the receptor site than the selective inhibitor PD0332991. 7-O-galloyl-taxofolinis semisynthetic flavonoid that upregulates Heme Oxygenase-1 in RAW264.7 Cells via MAPK/Nrf2 Pathway. It increases both mRNA and protein levels of HO-1 at concentrations of 25 μM and above (Vrba *et al*., 2013). 7-O-galloyl-taxofolin forms H-bond (back bone) with GLU 21, GLU 99 and ILE 19 while it forms side chain H-bond with GLN 149 and ASP 163. The inhibitor PD0332991 forms side chain H-bond with LYS-29. It shows hydrophobic interactions with PHE 98, VAL 77, PHE 164, ALA 162, LEU 65, LEU 152, VAL 101, VAL 27, ALA 41 amino acid residues which is responsible for its strong binding interactions with the receptor. As discussed above semisynthetic flavonoid 7-Monohydroxyethylrutoside is a constituent of the registered drug Venoruton, which is used in the treatment of chronic venous

insufficiency (Petruzzellis *et al.*, 2002) and is a potent antioxidant. Preclinical experiments in mice have shown that 7-Monohydroxyethylrutoside administration before doxorubicin effectively protects against doxorubicin induced cardiotoxicity, without interfering with its antitumor activity (Van Acker *et al.*, 1997, 2000). 7-Monohydroxyethylrutoside has glide score of -11.58 kcal/mol, slightly less than 7-*O*-galloyl-taxofolin. This indicates that strong hydrophobic interactions of 7-*O*-galloyl-taxofolin are responsible for its higher docking score. Glide score of other flavonoid molecules with CDK-6 are shown in table-8 and molcular docking pose of 7-*O*-galloyl-taxofolin with CDK-6 is shown in figure 15(c) while molecular docking pose of PDO332991(inhibitor of CDK-6) is shown in figure 15(d).

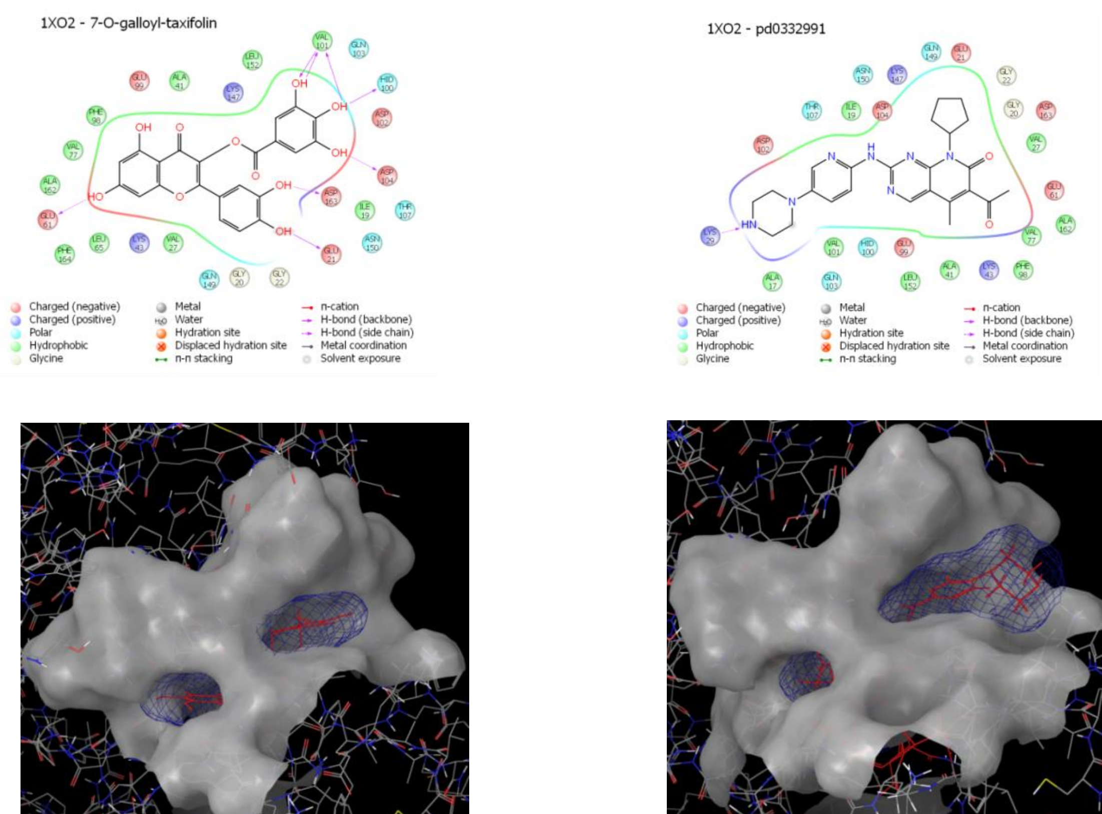


Fig 15. Interaction profile and molecular docking poses of 7-*O*-galloyl-taxifolin (c) and PD0332991 (d) with receptor CDK-6

Table 8. Glide score, Lipophilicity, Hydrogen bond and electrostatic energies of flavonoids docked with CDK-6

Ligand	GScore(Kcal/mole)	LipophilicEvdW	HBond	Electro
7-*O*-galloyl-taxofolin	-11.66	-6.31	-4.99	-1.6
7-Monohydroxyethylrutoside	-11.58	-4.39	-4.42	-1.9
Fisetin	-11.24	-5.17	-3.69	-1.29
Rutin	-10.88	-5.26	-2.82	-0.56
Luteolin	-10.84	-4.98	-2.87	-0.78
Kaempferol	-10.35	-5.24	-2.47	-0.49
Chyrseriol	-10.29	-5.36	-2.34	-0.71
Baicalin	-10.21	-5.23	-2.5	-0.66
Myrcetin	-10	-4.82	-3.32	-1
Apigenin	-9.85	-5.12	-1.89	-0.57
8-formyl-5,7-dihydroxy -flavanone	-9.71	-4.85	-2.15	-0.59
Flavone-8-acetic acid	-9.58	-5.08	-1.74	-0.53
Taxifolin	-8.82	-4.41	-2.3	-1.07
Quercetin	-8.7	-4.85	-2.29	-0.95
3’-formyl-2’,4’-dihydroxy-6’-methoxychalcone	-8.29	-8.29	0	-1.66
Cyanidanol	-8.24	-4.49	-2.51	-1.36
Epicatechin	-8.22	-4.49	-2.52	-1.3
Genistein	-8.01	-4.69	-1.59	-0.57
Pinostrobin	-7.94	-4.34	-1.55	-0.44
8-formyl-7-hydroxy-5-methoxy-flavanone	-7.9	-5.24	-0.96	-0.4
Wogonin	-7.89	-5.23	-1.44	-0.49
Cardamonin	-7.89	-5.23	-1.44	-0.49
Narigenin	-7.87	-4.24	-1.69	-0.62
5,7,3’-trihydroxy-5’-methoxyl-isoflavone	-7.86	-4.6	-1.53	-0.67
PD0332991	**-7.71**	**-6.11**	**-0.35**	**-0.44**
Glycetin	-7.69	-4.29	-1.7	-0.84
Catechin	-7.46	-3.99	-1.89	-1.09
Diosmetin	-7.35	-4.25	-1.66	-0.68
Daidzenin	-7.11	-4.41	-1.04	-0.6
7-methoxy-4’-hydroxy-5,8-quinoflavanone	-6.27	-3.82	-0.7	-0.45
Tangretin	-4.8	-4.62	0	0.02

4.4.3 *In-silico* ADME profile

The *in-silico* ADME studies of selected flavonoid molecules were studied and a comparision was drawn with their *in vitro* oral absorption studies for few flavonoids. It was found that for *in-silico* study pinostrobin and tangretin showed 100 percent oral absorption while semisynthetic molecule like 7-*O*-galloyl-taxifolin which has highest binding affinity for CDK-2 and CDK-6 receptors have oral absorption of only 1.37 percent. It was reported by Kwon *et al* (2011) that in vitro absorption of genistein after oral administration with various doses (4, 20, 40 mg/kg), the bioavailability of genistein was 38.58, 24.34 and 30.75%, respectively (Kwon *et al.*, 2011) while in silico study shows that absorption of genistein has oral absorption of 76.48%. Bioavailability can be measured by determining the area under the plasma concentration–time curve AUC (Area Under Curve). So *in vitro* AUC calculations for some flavonoids were studied. In silico study showed that luteolin, kaempferol, fiestin, chyrseriol, cyanidanol and 7-Monohydroxyethylrutoside have their oral absorption between 60-70%. *In vitro* AUC calculation for luteolin was studied by Zhou *et al* (2008) and it was found that the peak concentration of luteolin in plasma (Cmax) and the area under the concentration curve (AUC) for pure luteolin were 1.97±0.15 μg/mL and 10.7±2.2μg/mL.h, respectively (Zhou *et al.*, 2008). According to an *in vitro* study conducted by Barve *et al*, Kaempferol undergoes low to moderate absorption. The oral bioavalibility of Kaempferol is very low (~2%) and this atleast in part is due to extensive first-pass metabolism by phase I oxidative metabolism and phase II glucuronidation in the intestine as well as in the liver (Barve *et al.*, 2009).Abou *et al* in 2003 conducted a study on bioavailability of 7-Monohydroxyethylrutoside and it was found that intraperitoneal and subcutaneous bioavailabilities were about 30% and 40%, respectively. After oral administration, 7-Monohydroxyethylrutosidecould not be detected in plasma, indicating that monoHER had a very poor oral bioavailability (Abou *et al.*, 2003).

In-silico study shows that 7-*O*-galloyl-taxifolin has very less oral absorption of 1.37% while no in vitro data for this molecule is reported so far. So methods should be

employed to improve or increase its bioavailibilty without being harmful or producing adverse effect. Table-9 shows *in-silico* ADME profile of other flavonoid molecules.

Table 9. Qikprop results of flavonoid molecules

Molecule	MW	Donor HB	Accpt HB	QPPCao	QPlogB	QPPMDCK	QPlogKp	Percent Human Oral Absorption
Quercetin	302.24	4	5.25	20.82	-2.31	7.53	-5.436	52.745
Pinostrobin	270.284	0	3.25	1423.353	-0.36	724.541	-1.969	100
Narigenin	272.257	2	4	130.853	-1.405	54.916	-3.989	74.456
Myrcetin	318.239	5	6	7.528	-2.823	2.508	-6.325	27.995
Luteolin	286.24	3	4.5	42.115	-1.946	16.126	-4.852	61.622
Kaempferol	286.24	3	4.5	57.804	-1.894	22.708	-4.475	65.46
Glycetin	300.267	2	4.5	217.007	-1.308	94.876	-3.391	79.769
Genistein	270.241	2	3.75	166.681	-1.314	71.335	-3.552	76.48
Flavone-8-acetic acid	296.279	1	4.25	45.048	-1.252	22.058	-3.567	72.775
Fiestin	286.24	4	5.5	48.9	-1.884	18.952	-4.704	60.083
Epicatechin	274.273	4	3.75	122.938	-1.495	51.334	-4.077	72.312
Diosmetin	300.267	2	4.5	174.708	-1.377	75.055	-3.651	78.122
Rutin	286.24	3	4.5	42.115	-1.946	16.126	-4.852	61.622
Tangretin	372.374	0	6.25	3562.623	-0.164	1953.206	-1.26	100
Taxifolin	304.256	4	6.45	23.22	-2.257	8.473	-5.399	52.046
Wogonin	286.284	1	4	475.759	-0.826	221.638	-2.911	89.361
Daidzenin	254.242	2	4	391.184	-0.903	179.375	-2.823	83.693
Cyanidanol	290.272	5	5.45	53.177	-1.91	20.75	-4.698	60.587
Chyrseriol	316.267	2	6.2	69.045	-1.93	27.516	-4.374	67.038
Cardamonin	286.284	1	4	475.759	-0.826	221.638	-2.911	89.361
Baicalin	286.24	3	4.5	50.401	-1.86	19.581	-4.679	63.322
Apigenin	270.241	2	3.75	116.373	-1.443	48.378	-3.966	73.498
8-formyl-7-hydroxy-5-methoxy-flavanone	298.295	0	5.25	345.738	-1.017	156.96	-3.24	84.421
8-formyl-5,7-dihydroxy -flavanone	284.268	0	4.25	135.155	-1.348	56.87	-3.976	75.961
7-*O*-galloyl-taxofolin	456.362	6	10.45	1.294	-4.453	0.374	-7.191	1.377
7-Monohydroxyethylrutoside	330.293	3	6.2	41.093	-2.348	15.704	-4.637	62.831
7-methoxy-4’-hydroxy-5,8-quinoflavanone	284.311	1	5.5	347.01	-0.95	157.585	-3.535	83.632
5,7,3’-trihydroxy-5’-methoxyl-isoflavone	302.283	2	4.75	179.146	-1.329	77.118	-3.849	77.546
3'-formyl-2',4'-dihydroxy-6'-methoxychal	298.295	0	4.25	224.615	-1.522	98.476	-3.08	83.92

CHAPTER FIVE

SUMMARY

Natural products specifically from plants have a long history in treatment of various diseases including cancer. Secondary metabolites isolated from plants are excellent source of new medicinal compounds. Many chemical companies with a conferred interest in finding new drugs have thoroughly searched the world for seeking new sources of plant material used by native people for the treatment of cancer. There are hundreds of traditional plants that are used in the treatment of cancer throughout the world over past centuries. *Potentilla fulgens* is an important medicinal plant of higher Himalayas that is known globally for its therapeutic importance. Antioxidant constituents isolated from this plant are mainly polyphenolic compounds such as flavonoids that are potent bioactive molecues which are used in treatment of cancer. In this study *in-vitro* anti-mutagenic activity of different fractions of *P.fulgens* was carried out which shows that *n*-butanol and water fractions are strongly anti-mutagenic. For *n*-butanol fraction, inhibitory activity was 60.4% and 35.4% in co-incubation and pre-incubation respectively in TA98 tester strains while 55.6% and 62.0% inhibition was observed in TA100 tester strains respectively. For water fraction 56.6% and 60.7% inhibitory activity in co-incubation and pre-incubation mode of treatment respectively against TA98 strain and 34.5% and 50.6% in TA100 strain for co-incubation and pre-incubation treatment respectively. Results clearly indicate that *P. fulgens* is a new source of anti-mutagenic agents. Isolation of molecules from ethyl acetate and *n*-butanol fractions led to the characterisation of one molecule namely catechin out of total six isolated molecules. Further *in-silico* study of reported natural and semisynthetic flavonoids were done on CDK-2 and CDK-6 receptor which specifies that these flavonoids can be used in future for treatment of cancer by inhibition of CDK-2 and CDK-6 receptors.

REFERENCES

Abou El Hassan, M. A., Kedde, M. A., Zwiers, U. T., Tourn, E., Haenen, G. R., Bast, A. and Van-der, V. W. J. (2003). Bioavailability and pharmacokinetics of the cardioprotecting flavonoid 7-monohydroxyethylrutoside in mice. *Cancer Chemotherapy and Pharmacology* ***52***(5), 371-376.

Amado, N. G., Fonseca, B. F., Cerqueira, D. M., Neto, V. M. and Abreu, J.G. (2011). Flavonoids: Potential Wnt/beta-catenin signaling modulators in cancer. *Life Science* ***89***(15-16), 545-554.

Ammar, R. B., Bouhlel, I., Valenti, K., Ben Sghaier, M., Kilani, S., Mariotte, A. M., Dijoux-Franca, M. G., Laporte, F., Ghedira, G. and Chekir-Ghedira, L. (2007). Transcriptional response of genes involved in cell defense system in human cells stressed by H_2O_2 and pre-treated with (Tunisian) *Rhamnus alaternus* extracts: combination with polyphenolic compounds and classic *in vitro* assays. *Chemico-Biological Interactions* ***168***(3), 171-183.

Aydin, S., Basaran, A. A. and Basaran, N. (2004). The protective effects of some phenylethanoid glycosides on the mitomycin c induced DNA strand breakage. *Journal of Faculty of Pharmacy* ***24***(1), 1-11.

Ayoola, G. A., Coker, H. A. B., Adesegun, S. A., Adepoju-Bello, A. A., Obaweya, K., Ezennia, E. C. and Atangbayila, T. O. (2008). Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research* ***7***(3), 1019-1024.

Bala, S. and Grover, I. S. (1989). Anti-mutagenicity of some citrus fruits. *Mutation Research* ***222***(3), 141-48.

Barve, A., Chen, C., Hebbar, V., Desiderio, J., Sawb, C. L. and Kong, A. N. (2009) Metabolism, oral bioavailability and pharmacokinetics of chemopreventive kaempferol in rats. *Biopharmaceutics & Drug Disposition* ***30***(7), 356–365.

Beckman C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants. *Physiological and Molecular Plant Pathology* ***57***(3),101-110.

Benavente-Garcia, O., Castillo, J., Marin, F. R., Ortuno, A. and Del Rio J. A. (1997). Uses and properties of Citrus flavonoids. *Journal of Agricultural and Food Chemistry* ***45***(12), 4505–4515.

Bhatia, A., Arora, S., Singh, B., Kaur, G. and Nagpal, A. (2011). Anticancer potential of Himalayan plants. *Phytochemistry Review* ***10***(3), 309–323.

Birt, D. F., Hendrich, S. and Wang, W. (2001). Dietary agents in cancer prevention Flavonoids and isoflavonoids. *Pharmacology Therapy* ***90***(2-3),157–77.

Bos, M. A., Vennat, B., Meunier, M. T., Pouget, M. P., Pourrat, A. and Fialip, J. (1996). Procyanidins from tormentil: antioxidant properties towards lipoperoxidation and anti-elastase activity. *Biological & Pharmaceutical Bulletin* ***19***(1), 146.

Centers for Disease Control and Prevention (CDC). (2002) Program Fact Sheet. ComprehensiveCancer Control.<http://www.cdc.gov/cancer/ncccp/about.htm>

Chahar, M. K., Sharma, N. and Dobhal, M. P. (2011). Flavonoids: A versatile source of anticancer drugs. *Pharmacognosy Review* ***5***(9), 1-12.

Chemler, J. A., Yan, Y. and Koffas, M. A. G. (2006). Biosynthesis of isoprenoids, polyunsaturated fatty acids and flavonoids in *Saccharomyces cerevisiae*. *Microbial Cell Factories* ***5***(20), 1-9.

Chen, H. M., Wu, Y. C., Chia, Y. C., Chang, F. R., Hsu, H. K., Hsieh, Y. C., Chen, C. C. and Yuan, S. S. (2009). Gallic acid, a major component of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. *Cancer Letters* ***286***(2), 161–171.

Choi, J. A., Kim, J. Y., Lee, J. Y., Kang, C. M., Kwon, H. J., Yoo, Y. D., Kim, T. W., Lee, Y. S. and Lee, S. J. (2001). Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *International Journal of Oncology* ***19***(4), 837–844.

Choi, J. S., Park, K. Y., Moon, S. H., Rhee, S. H. and Young, H. S. (1994). Anti-mutagenic effect of plant flavonoids in the *Salmonella* assay system. *Archives of Pharmacal Research* ***17***(2), 71-75.

Choi, Y. H., Kim, M. J., Yun, B. S. and Kwak, S. S. (1998). Antioxidant compounds in *Potentilla fragarioides*. *Korean Journal of Pharmacognosy* **29:** 79-85.

Chung, W. G., Miranda, C. L., Stevens, J. F. and Maier, C. S. (2009). Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species. *Food and Chemical Toxicology* ***47***(4), 827–836.

Cordon-Cardo, C. (1995). Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *American Journal of Pathology* ***147***(3), 545-560.

Cragg, G. M. and Newmann, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology* ***100***(1-2), 72-79.

Dehn, D. L., Winski, S. L. and Ross, D. (2004). Development of a new isogenic cell-xenograft system for evaluation of NAD(P)H:quinone oxidoreductase- directed antitumor quinones: evaluation of the activity of RH1. *Clinical Cancer Research* ***10***(9), 3147–3155.

Delgado, L., Gallego, F. and Rico, E. (2000). Karyosystematic study of Potentilla L. subgenes *Potentilla* (Rosaeace) in the Iberian Peninsula. *Botanical Journal of the Linnean Society* ***132***(2), 263-280

Edenharder, R. and Grunhage, D. (2003) Free radical scavenging abilities of flavonoids as mechanism of protection against mutagenicity induced by tert-

butyl hydroperoxide or cumene hydroperoxide in *Salmonella typhimurium* TA102. *Mutation Research* ***540***(1),1–18.

Fabricant, D. S. and Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives*, ***109***(1), 69-75

Fang, J., Xia, C., Cao, Z., Zheng, J. Z. and Reed, E. (2005). Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. *FASEB Journal* ***19***(3), 342–353.

Ferrazzano, G. F., Amato, I., Ingenito, A., Zarrelli, A., Pinto, G. and Pollio, A. (2011). Plant Polyphenols and Their Anti-Cariogenic Properties: A Review. *Molecules* ***16***(2), 1486-1507.

Forkmann, G. and Martens, S. (2001). Metabolic engineering and applications of flavonoids. *Current Opinion in Biotechnology* ***12***(2),155-160.

Fresco, P., Borges, F., Diniz, C. and Marques, M. P. (2006). New insights on the anticancer properties of dietary polyphenols. *Medicinal Research Reviews* ***26***(6), 747-766.

Galati, G., Teng, S., Moridani, M. Y., Chan, T. S. and O'Brien, P. J. (2000). Cancer chemoprevention and apoptosis mechanisms induced by dietary polyphenolics. *Drug Metabolism and Drug Interactions* ***17***(1-4), 311–349.

Gao, W., Shen, Y., Zhang, H., Tang, H., Lin, H. and Qiu, F. (2007). The chemical constituents of *Potentilla chinensis. Pharmaceutical Care and Research* ***7***(4), 262-269.

Gradelet, S., Le Bon, A. M., Berge, R., Suschetet, M. and Astrog, P. (1998). Dietary carotenoids inhibit aflatoxin B1-induced liver preneoplastic loci and DNA damage in the rat: Role of the modulation of aflatoxin B1 metabolism. *Carcinogenesis* **19:** 403–411.

Guillen, A., Rico, E. and Castroviejo, S. (2005). Reproductive biology of the Iberian species of *Potentilla* L. (Rosaceae). *Anales del Jardin Botánico de Madrid* ***62***(1), 9-21.

Haleagrahara, N., Radhakrishnan, A., Lee, N. and Kumar, P. (2009). Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats. *European Journal of Pharmacology* ***621***(1-3), 46–52.

Handique, J. G. and Baruah, J. B. (2002). Polyphenolic compounds: an overview. *Reactive and Functional Polymers* ***52***(3), 163-188.

Harborne, J. B. and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry* ***55***(6), 481-504.

Haslam, E. (1996). Natural polyphenols(vegetable tannins) as Drugs: Possible mode of action. *Journal of Natural Products* ***59***(2), 205-215.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology Therapy* ***96***(2-3), 67-202.

Holiman, P. C. H., Hertog, M. G. L. and Katan, M. B. (1996). Analysis and health effects of flavonoids. *Food Chemistry* ***57***(1), 43-46

Hollman, P. and Katan, M. (1999). Dietary flavonoids: intake, health effects and bioavailability. *Food and Chemical Toxicology* ***37***(9-10), 937-942.

Issazadeh, K., Aliabadi, M. A., Darsanaki, R. K. and Pahlaviani, R. M. K. (2012). Anti-mutagenic Activity of Olive Leaf Aqueous Extract by Ames Test. *Advanced Studies in Biology* ***4***(9), 397 – 405.

Jackson, S. J. and Venema, R. C. (2006). Quercetin inhibits NOS, microtubule polymerization, and mitotic progression in bovine aortic endothelial cells. *Journal of Nutrition* ***136***(5), 1178–1184.

Jaitak, V., Kaul, K. and Kaul, V. K. (2010a). Review on pharmaceutical properties and conservation mesures *of P.fulgens Wall.*ex Hook.- A medicinal endangered herb of higher Himalya. *Indian Journal of Natural Products and Resources* ***2***(3), 298-306.

Jaitak, V., Kaul, V. K., Kumar, N., Singh, B., Dhar, J. and Sharma, O. P. (2010b). New hopane triterpenes and antioxidant contituents from *Potentilla fulgens*. *Natural Product Communication* ***5***(10),1561-1566.

Jaitak, V., Sharma, K., Kalia, K., Kumar, N., Singh, H., Kaul, V. and Singh, B. (2010c). Antioxidant activity of Potentilla fulgens: An alpine plant of western Himalaya. *Journal of Food Composition and Analysis* ***23***(2), 142-147.

Jang, D. S., Yoo, N. H., Kim, J. M., Lee, Y. M., Yoo, J. L., Kim, Y. S. and Kim, J. S. (2007). An ellagic acid rhamnoside from the roots of *Potentilla discolor* with protein glycation and rat lens aldose reductase inhibitory activity*. Natural Product Sciences* ***13***(2), 160-163.

Kandaswami, C., Lee, L. T., Lee, P. P. H., Hwang, J. J., Ke, F. C., Huang, Y. T. and Lee, M. T. (2005) The Antitumor Activities of Flavonoids. *In Vivo* ***19***(5), 895-910.

Karikas, G. A. (2011). Chemoprevention molecular and biochemical mechanisms involved in cancer control and management. *Health Science Journal* ***5***(2), 149-156.

Karpagam, N. and Kumar, H. P. (2013). Study of Anticancer agents from Medicinal Plants. *World Journal of Pharmacy and Pharmaceutical Sciences* ***2***(4),1673-1680.

Katula, K. S., McCain, J. A. and Radewicz, A. T. (2005). Relative ability of dietary compounds to modulate nuclear factor-kappaB activity as assessed in a cell-based reporter system. *Journal of Medicinal Food* ***8***(2), 269–274.

Kaur, R., Kapoor, K. and Kaur, J. (2011). Plants as a source of anticancer agents *Journal of Natural Product and Plant Resources* ***1***(1), 119-124.

Kaur, S., Kumar, S., Kaur, P. and Chandel, M. (2010). Study of Anti-mutagenic Potential of Phytoconstituents Isolated from *Terminalia arjuna* in the Salmonella/Microsome Assay. *American Journal of Biomedical Sciences*. ***2***(2), 164-177.

Kirakosyan, A., Seymour, E., Kaufman, O. B., Warber, S., Bolling, S.and Chang, S. C. (2003). Antioxidant activity of polyphenolic extracts from leaves of *Crataegus laevigata* and *Crataegus Monogyna (Hawthorn)* subjected to drought and cold stress. *Journal of the Science of Food and Agriculture* ***51***(14), 3973-3976.

Klaunig, J. E., Kamendulis, L. M. and Hocevar, B. A. (2010). Oxidative Stress and Oxidative Damage in Carcinogenesis. *Toxicologic Pathology* ***38***(1), 96-109.

Kwon, S. H., Kang, M. J., Huh, J. S., Ha, K. W., Lee, J. R., Lee, S. K., Lee, B. S., Han, I. H., Lee, M. S., Lee, M. W., Lee, J. and Choi, Y. W. (2007). Comparison of oral bioavailability of genistein and genistin in rats. *International Journal of Pharmaceutics* ***337***(1-2),148-154.

Laloo, D., Prasad, S. K., Krishnamurthy, S. and Hemalatha, S. (2013). Gastroprotective activity of ethanolic root extract of *Potentilla fulgens* Wall. ex Hook. *Journal of Ethnopharmacology* ***146***(2), 505-514.

Lee, W. J. and Zhu, B. T. (2006). Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. *Carcinogenesis* ***27***(2), 269-277.

Lee, Y. Y., Westphal, A. H., de Haan, L. H., Aarts, J. M., Rietjens, I. M. and Van Berkel, W. J. (2005). Human NAD(P)H:Quinone oxido reductase inhibition by flavonoids in living cells. *Free Radical Biology and Medicine* ***39***(2), 257 – 265.

Lodish, H., Berk, A. and Zipursky, S. L. (2000). Molecular Cell Biology. 4th edition New York

Loeb, L. A., Loeb, K. R. and Anderson, J. P. (2003). Multiple mutations and cancer. *Proceedings of the National Academy of Sciences of United States of America* ***100***(3), 776-781.

Luthar, Z. (1992). Polyphenol classification and tannin content of buckwheat seeds (*Fagopyrum esculentum Moench*) *Fagopyrum* **12:** 36 – 42.

Maron, D. M. and Ames, B. N. (1983). Revised method for Salmonella mutagenicity test. *Mutation Research* ***113***(3-4),173-215.

Meyers, L. S. and Grossen, N. E. (1974). Analysis of independent group designs. In: Behavioural Research, Theory, Procedure and Design, pp. 237–252. W.H. Freeman and Co., San Francisco

Middleton, E. J., Kandaswami, C. and Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. *Pharmacology Review* ***52***(4), 673–751.

Miliauskas, G., VanBeek, T. A., Venskutonis, P. R., Linssen, J. P. H., DeWaard, P. and Sudholter, E. J. (2004). Antioxidant activity of Potentilla fruticosa. *Journal of the Science of Food and Agriculture* ***84***(15), 1997-200

Naasani, I., Oh-Hashi, F., Oh-Hara, T., Feng, W. Y,, Johnston, J., Chan, K. and Tsuruo, T. (2003). Blocking Telomerase by Dietary Polyphenols Is a Major Mechanism for Limiting the Growth of Human Cancer Cells *in Vitro* and *in Vivo. Cancer Research* ***63***(4), 824-830.

Newmark, H. L. (1996). Plant phenolics as potential cancer prevention agents. *Advances in Experimental Medicine and Biology* **401:** 25–34.

Nirmala, M. J., Samundeeswari, A. and Sankar, P. D. (2011). Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology* ***1***(3), 1-14.

Okuda, T., Yoshida, T., Kuwahara, M.,Memon, M. U. and Shingu, T. (1984). Tannins of rosaceous medicinal plants. I. Structures of potentillin, agrimonic acids A and B, and arimoniin, a dimeric ellagitannin. *Chemical and Pharmaceutical*

Bulletin **32:** 2165-2173.

Pal, R. S., Ariharasivakumar, G., Girhepunjhe, K. and Upadhay, A. (2009). *In-vitro* antioxidative activity of phenolic and flavonoids compounds extracted from seeds of *Abrus precatorius*. *International Journal of Pharmacy and Pharmaceutical Sciences* ***1***(2), 136-140.

Petruzzellis, V., Troccoli, T., Candiani, C., Guarisco, R., Lospalluti, M., Belcaro, G. and Dugall, M. (2002). Oxerutins (Venoruton): efficacy in chronic venous insufficiency—a double-blind, randomized, controlled study. *Angiology* ***53***(3), 257–263.

Petti, S. and Scully, C. (2009). Polyphenols oral health and disease: A review. *Journal of Dentistry* ***37***(6),413–423.

Phillips, P. A., Sangwan, V., Borja-Cacho, D., Dudeja, V., Vickers, S. M. and Saluja, A. K. (2011). Myricetin Induces Pancreatic Cancer Cell Death via the Induction of Apoptosis and Inhibition of the Phosphatidylinositol 3-Kinase (PI3K) Signaling Pathway. *Cancer Lett*ers ***308***(2), 181–188.

Qianying, Y., Shaoxin, C., Xuenong, Z., Ziwei, L., Zhaoming, L., Xuelai, L., Chaomei, X., Jianping, W., Junbo, H. and Jinlan, R. (2012) A new protoapigenone analog RY10-4 induces apoptosis and suppresses invasion through the PI3K/Akt pathway in human breast cancer. *Cancer Letters* ***324***(2), 210-220.

Rosangkima, G. and Prasad, S. B. (2004). Antitumor activity of some plants from meghalaya and against murine ascites Dalton's lymphoma Indian. *Journal of Experimental Biology* ***42***(10), 981-988.

Rossi, A. G., Sawatzky, D. A., Walker, A., Ward, C., Sheldrake, T. A., Riley, N. A., Caldicott, A., Martinez-Losa, M., Walker, T. R., Duffin, R., Gray, M., Crescenzi, E., Martin, M. C., Brady, H. J., Savill, J. S., Dransfield, I. and Haslett, C. (2006). Cyclin-dependent kinase inhibitors enhance the resolution of

inflammation by promoting inflammatory cell apoptosis. *Nature Medicine* ***12***(9), 1056 – 1064.

Scalbert, A., Manach, C., Morand, C., Rémésy, C. and Jimenez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition* ***45***(4), 287-306.

Schleep, S., Friedrich, H. and Kolodziej, H. (1986). The first natural procyanidin with a 3,4-cis-configuration. *Journal of the Chemical Society, Chemical Communications* **5:** 392-396.

Scholar, E. M. and Toewa, M. L. (1994) Inhibtion of invasion murine mammary carcinoma cells by the tyrosine kinase inhibitor genistein. *Cancer letters* ***87***(2), 159-162.

Sun, Y., Chen, J. and Rigas, B. (2009). Chemopreventive agents induce oxidative stress in cancer cells leading to COX-2 overexpression and COX-2-independent cell death. *Carcinogenesis* ***30***(1), 93-100.

Syiem, D., Syngai, C., Kharbuli, B., Kayang, H. and Khongwir, B. (2003). Anti-tumor activity of crude root extract of *Potentilla fulgens*. *Indian drugs* ***40***(2), 124-125.

Syiem, D., Syngai, C., Khup, P. Z., Khongwir, B. S., Kharbuli, B. and Kayang, H. (2002). Hypoglycemic effects of *Potentilla fulgens* L. in normal and alloxan-induced diabetic mice. *Journal of Ethnopharmacology* ***83***(1-2), 55-61.

Tomczyk, M., Drozdowska, D., Bielawska, A., Bielawski, K. and Gudej, J. (2008). Human DNA topoisomerase inhibitors from *Potentilla argentea* and their cytotoxic effect against MCF-7. *Die Pharmazie-An International Journal of Pharmaceutical Sciences* ***63***(5), 389-393.

Van Acker, F. A., Van Acker, S. A., Kramer, K., Haenen, G. R., Bast, A. and Van der, V. W. J. (2000). 7-Monohydroxyethylrutoside protects against chronic doxorubicin-induced cardiotoxicity when administered only once per week. *Clinical Cancer Research* ***6***(4), 1337–1341

Van Acker, S. A., Boven, E., Kuiper, K., Berg, D. J., Grimbergen, J. A., Kramer, K., Bast, A. and Van der, V. W. J. (1997) Monohydroxyethylrutoside, a dose-dependent cardioprotectiveagent, does not affect the antitumor activity of doxorubicin. *Clinical Cancer Research* ***3***(10), 1747–1754

Van Acker, S. A., Van Balen, G. P., Berg, D. J., Bast, A. and Van der, V. W. J. Influence of iron chelation on the antioxidant activity of flavonoids. *Biochemical Pharmacology* ***56***(8), 935–943.

Vashishtha, R. K., Rawat, N., Nautiyal, B. P., Prasad, P. and Nautiyal, M. C. (2009). An exploration on the phenology of different growth forms of an alpine expense of North West Himalaya. *New york Science Journal* ***2***(6), 29-41.

Vrba, J., Gazak, R., Kuzma, M., Papouskova, B., Vacek, J., Weiszenstein, M., Kren, V. and Ulrichova, J. (2013). A Novel Semisynthetic Flavonoid 7-O-Galloyltaxifolin Upregulates Heme Oxygenase-1 in RAW264.7 Cells via MAPK/Nrf2 Pathway. *Journal of Medicinal Chemistry* ***56***(3), 856-866.

Xue, P. F., Luo, G., Zeng, W. Z., Zhao, Y. Y. and Liang, H. (2005). Secondary metabolites from *Potentialla multifida* (Rosaaceae). *Biochemical Systematics and Ecology* ***33***(7), 725-728.

Yang, C. S., Landau, J. M., Huang, M. T. and Newmark, H. L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *The Annual Review of Nutrition* **21:** 381–406.

Zaveri, M., Patel, P., Dhru, B. and Patel, S. (2011). Screening of *In-vitro* Anti-mutagenic Activity of Selected Plants. *American Journal of PharmTech Research* ***1***(4), 232-243.

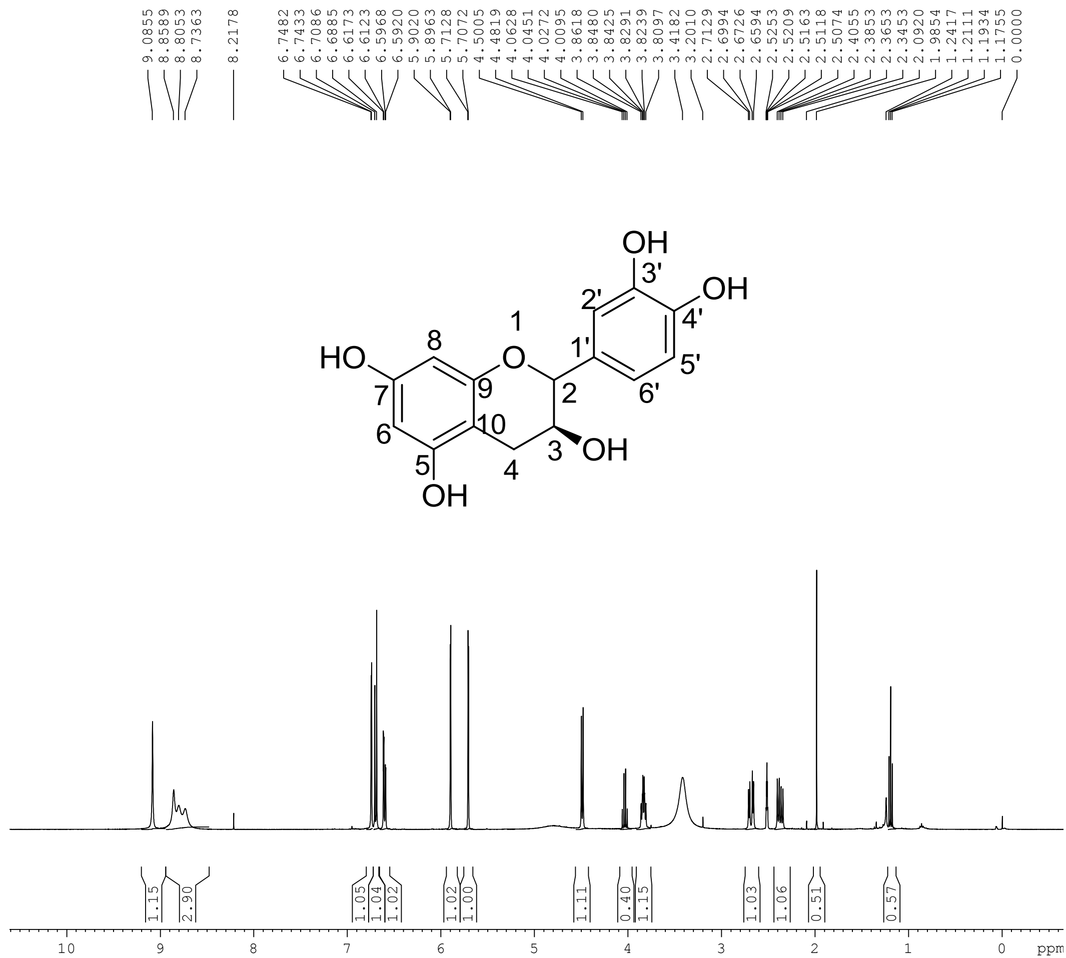
Zhang, Q., Zhao, X. H. and Wang, Z. J. (2009). Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G2/M arrest and apoptosis. *Toxicology In Vitro* ***23***(5), 797–807.

Zhang, Y. and Tang, L. (2007). Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. *Acta Pharmacologica Sinica* ***28***(9), 1343–1354.

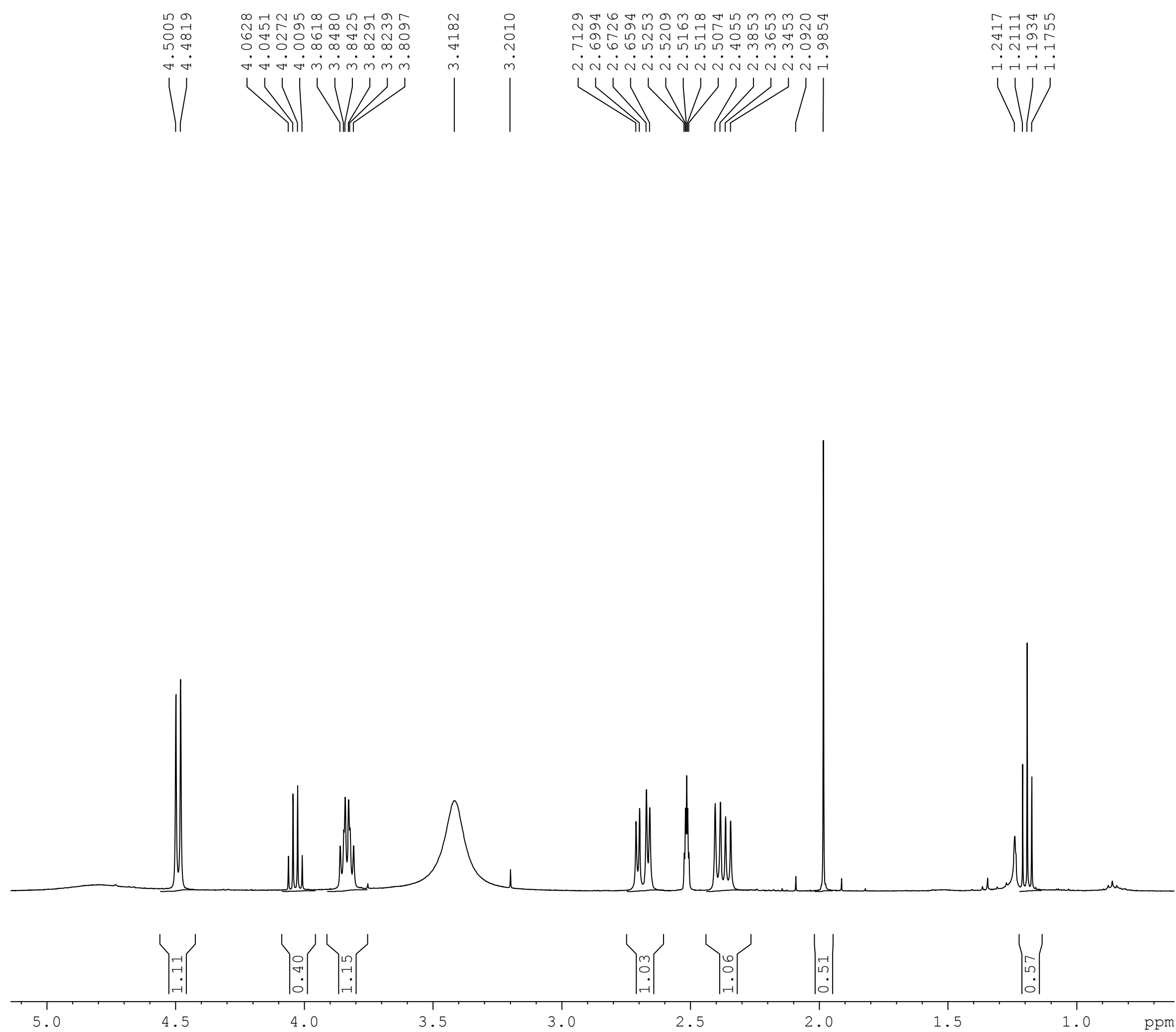
Zhao, Y. L., Cai, G. M., Hong, X., Shan, L. M. and Xiao, X. H. (2008). Anti-hepatitis B virus activities of triterpenoid saponin compound from *Potentialla anserina*. *Phytomedicine* ***15***(4), 253-258.

Zhou, P., Li, L. P., Luo, S. Q., Jiang, H. D. and Zeng, S. (2008). Intestinal Absorption of Luteolin from Peanut Hull Extract Is More Efficient than That from Individual Pure Luteolin. *Journal of Agricultural and Food Chemistry* ***56***(1), 296–300.

Zhu, F., Qin, C., Tao, L., Liu, X., Shi, Z., Ma, X., Jia, J., Tan, Y., Cui, C., Lin. J., Tan, C., Jiang, Y. and Chen, Y. (2011). Clustered patterns of species origins of nature-derived drugs and clues for future bioprospecting. *Proceedings of the National Academy of Sciences of United States of America* ***108***(31), 12943-129488.



Spectra 1: ^{1}H NMR spectra of compound 3



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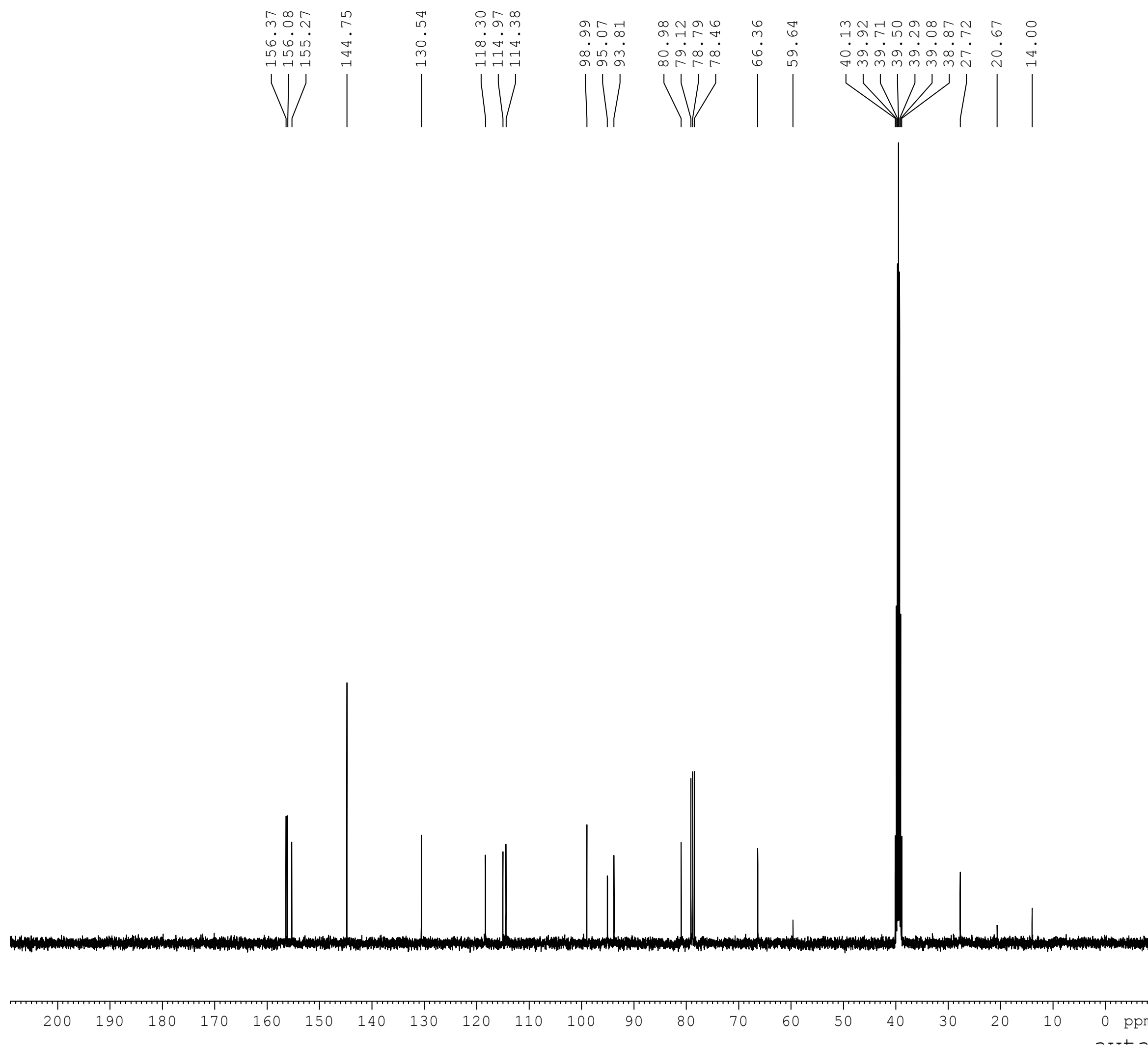
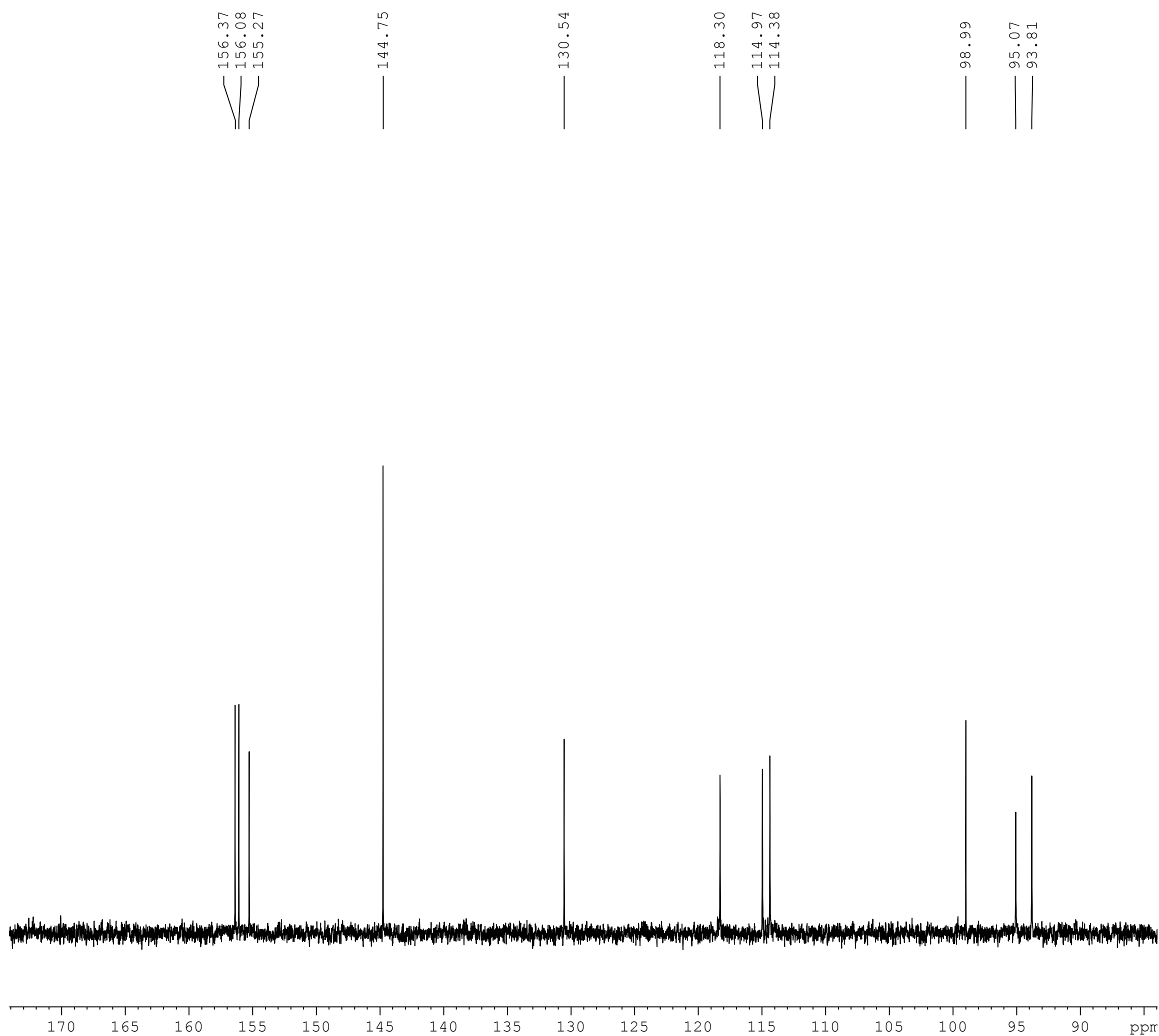


Spectra 3: ^{13}C NMR spectra of compound 3



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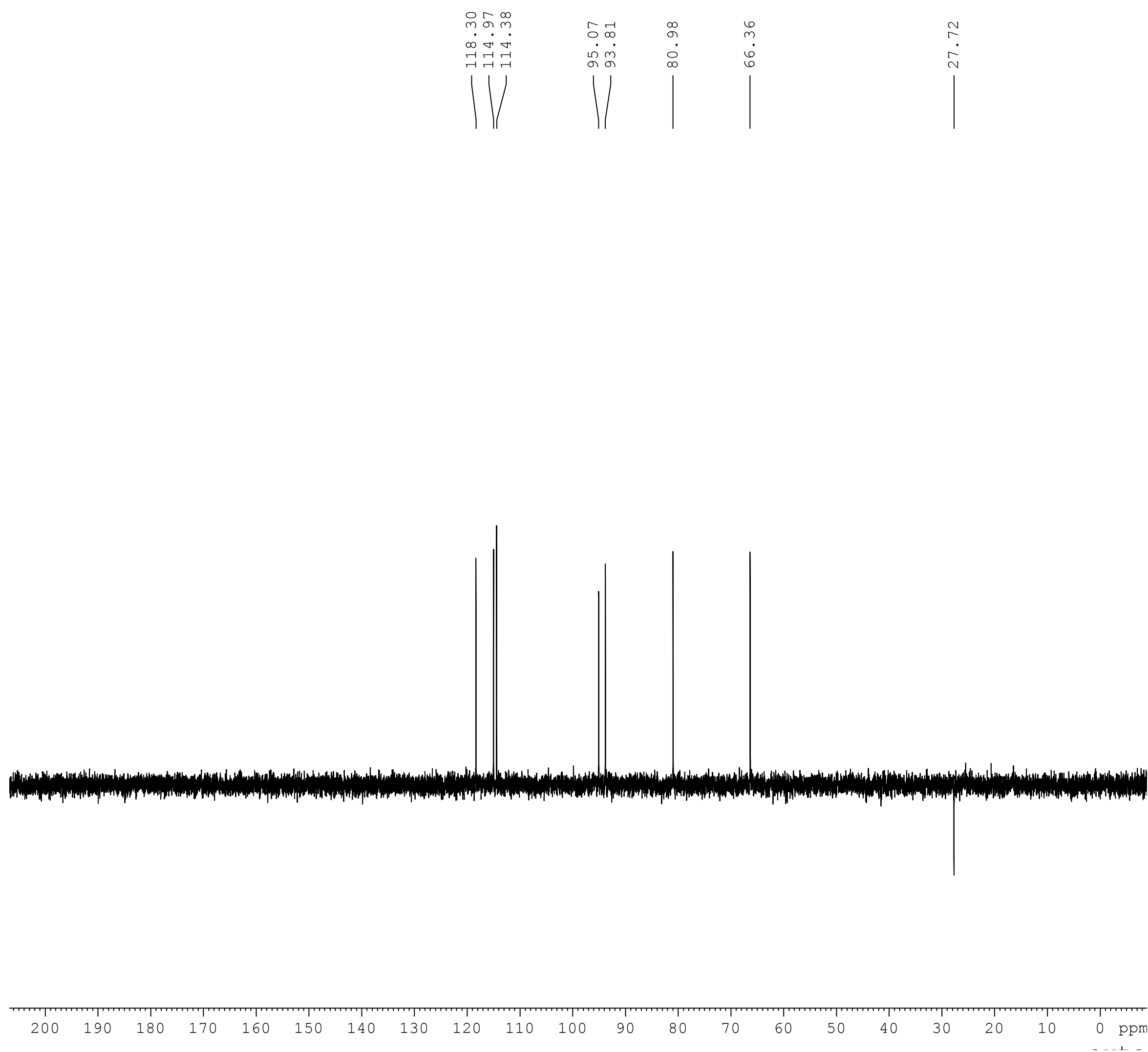
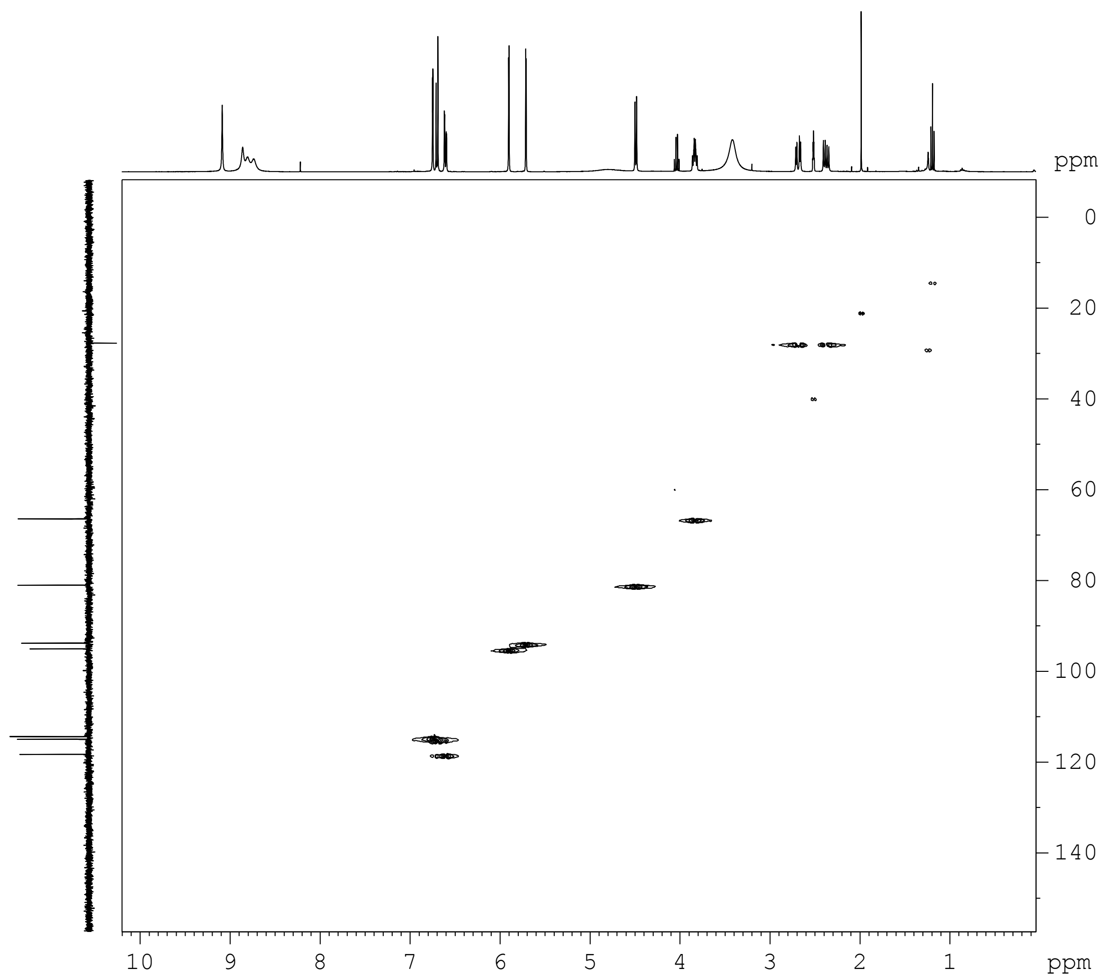
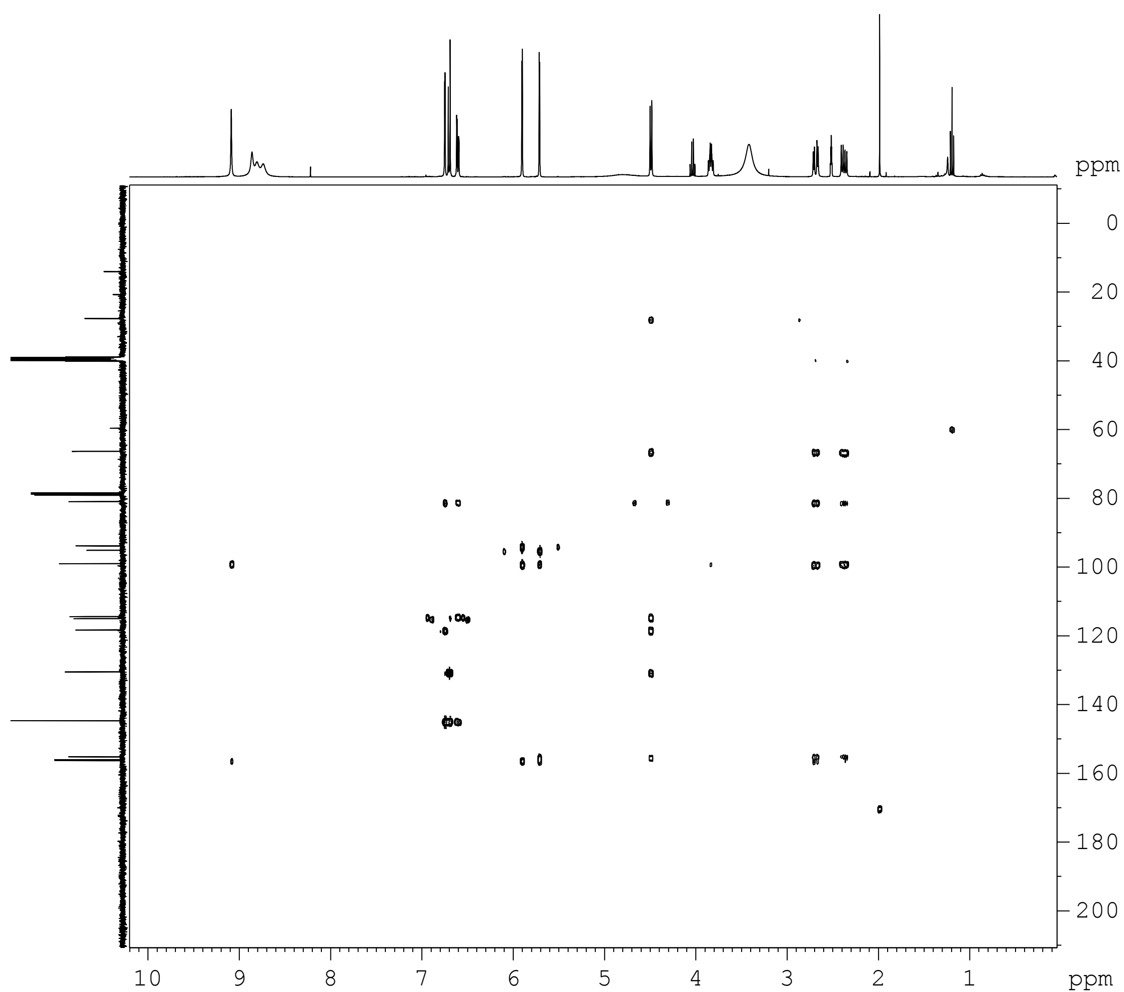


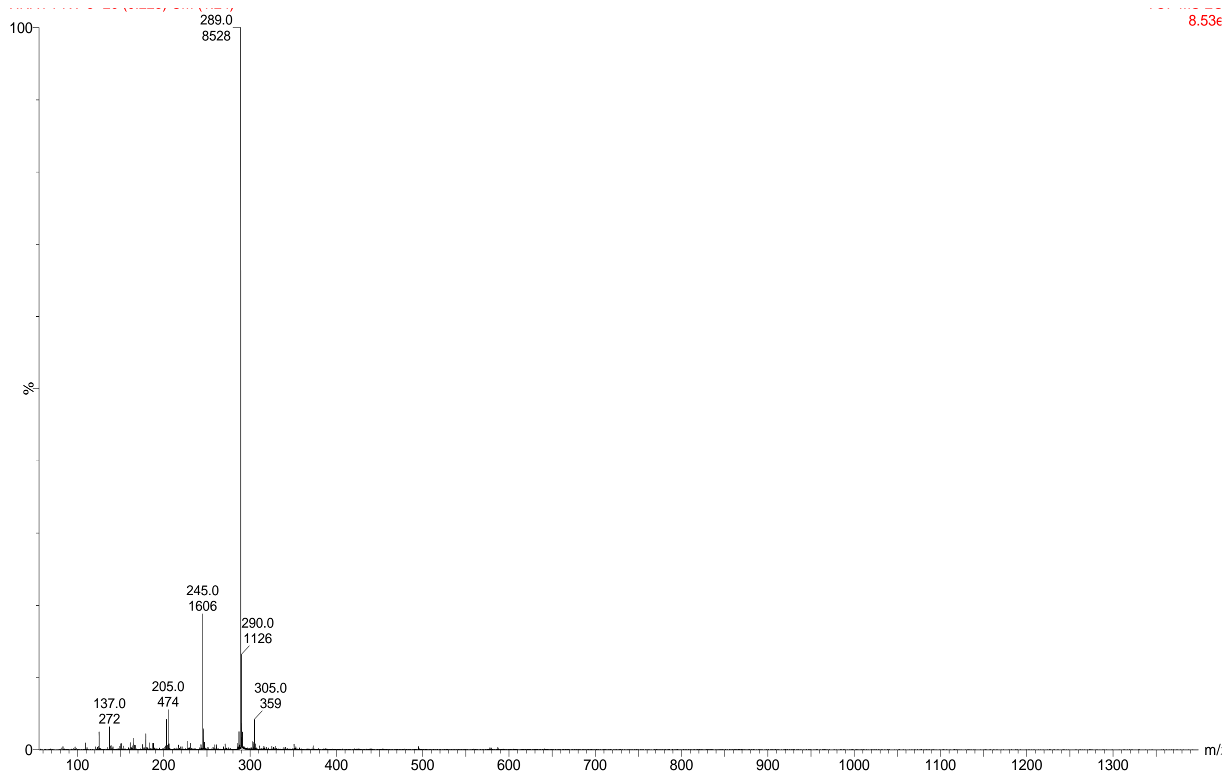
Spectra 5: DEPT spectra of compound 3



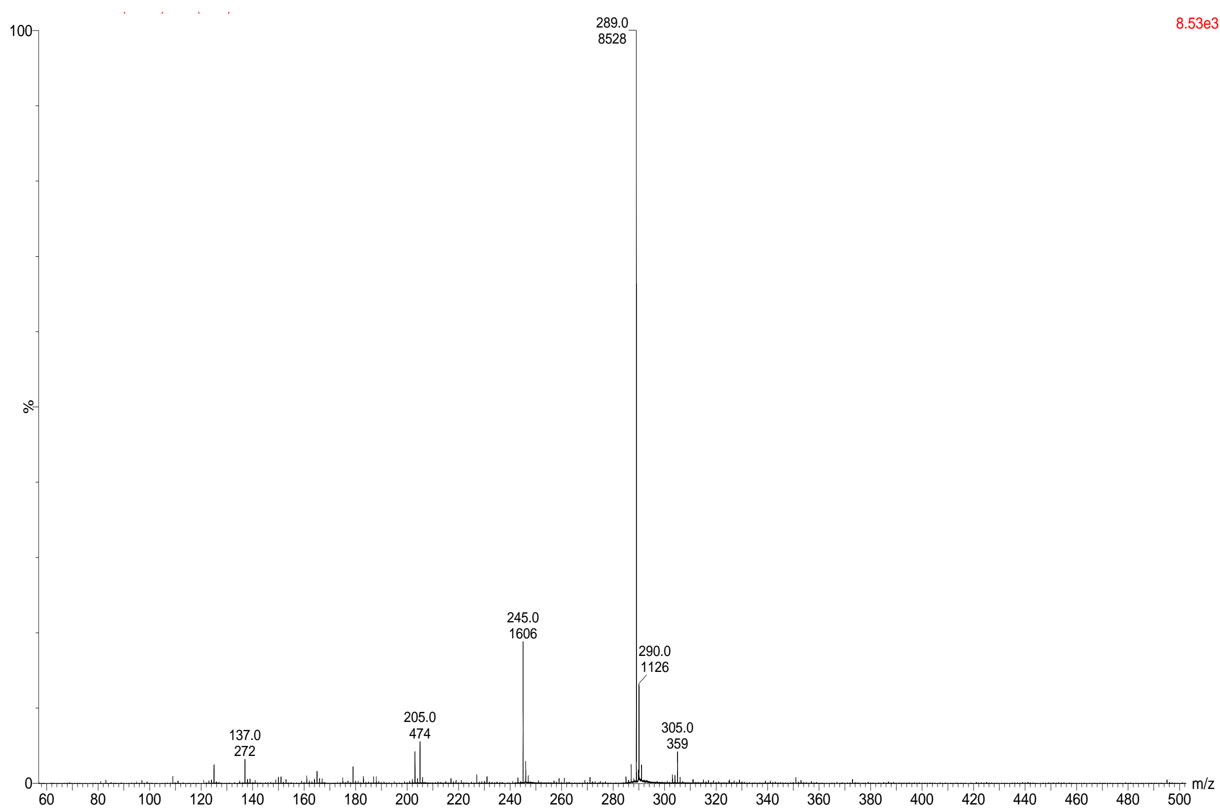
Spectra 6: HSQC Spectra of compound 3



Spectra 7: HMBC Spectra of compound 3



Spectra 8: Mass Spectra of compound 3



Spectra 9: Mass Spectra of compound 3

List of Publications

Monga, P., Jaitak, V. (2013). Flavonoids as anticancer agents: An update. *Pharmaceutical biology* (under review)

Monga, P., Kaur R., Arora S., Jaitak, V. (2013). *In-vitro* anti-mutagenic potential of *Potentilla fulgens*: A Western Himalayan Plant. *Journal of Herbs, Spices and Medicinal plants* (under review)

Monga, P., Gupta., V.K., Jaitak, V. (2013). *In-silico* study of flavonoids with VEGFR-2, CDK-2, and CDK-6 receptors as target for anticancer therapy. *Journal of Molecular modeling* (under review)