# *In-Vitro* Assay Guided Fractionation of Crude Root Extracts of *Potentilla atrosanguinea* Lodd. and *In-Silico* Study of Polyphenolic Compounds with MRP-1 and GSTP1-1 receptors in Cancer Chemotherapy

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**Medicinal Chemistry**

**BY**

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**Centre for Chemical & Pharmaceutical Sciences**

**School of Basic & Applied Sciences**

**Central University of Punjab, Bathinda**

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## DECLARATION

I declare that the dissertation entitled "***In-Vitro* Assay Guided Fractionation of Crude Root Extracts of *Potentilla atrosanguinea* Lodd. and *In-Silico* Study of Polyphenolic Compounds with MRP-1 and GSTP1-1 receptors in Cancer Chemotherapy**" 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.

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## CERTIFICATE

I certify that Vinay Kumar Gupta has prepared his dissertation entitled "***In-Vitro* Assay Guided Fractionation of Crude Root Extracts of *Potentilla atrosanguinea* Lodd. and *In-Silico* Study of Polyphenolic Compounds with MRP-1 and GSTP1-1 receptors in Cancer Chemotherapy**", for the award of M. Pharm. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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## ABSTRACT

### "*In-Vitro* Assay Guided Fractionation of Crude Root Extracts of *Potentilla atrosanguinea* Lodd. and *In-Silico* Study of Polyphenolic Compounds with MRP-1 and GSTP1-1 receptors in Cancer Chemotherapy"

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Modern therapeutic system is a kind of inspiration from traditional plant based medicine used for various diseases and ailments. Advancement in drug discovery technology including computational drug design and bioassay guided fractionation emboldens the interest of 'Medicinal Chemists' concerning to lead identification from medicinal plants for complicated diseases, in the last few years. There are so many traditional plants which are used in the treatment of various diseases in different parts of our country but scientific information is missing for the same. *Potentilla atrosanguinea* is a native to the western Himalaya region has been used traditionally for the treatment of wound-healing, diarrhea, influenza and bleeding but there is not even a single published evidence about its activity accept antioxidant activity of aerial part. In this context, the aim of the present study was to explore the roots of *P.atrosanguinea* in terms of its medicinal value for instances *in-vitro* photoprotective and antioxidant activity. The photoprotective activity was evaluated in the term of SPF (sun protection factor) by spectrophotometric method in the range of 290-320 nm (UVB region) whereas antioxidative activity was evaluated using a free radical scavenging assay (DPPH, superoxide anion scavenging and CUPRAC). Total phenolic contents of the extract/fractions were

determined by Folin Ciocalteu reagent. The ability of photo-protection of different fraction against UVB region followed the trend *Pa*-AcOEt > *Pa*- *n*-BuOH > *Pa*-$H_2O$-MeOH > *Pa*-$H_2O$. Ethyl acetate fraction of *Potentilla atrosanguinea* indicated the highest sun protection factor (SPF) (7.319 ± 0.353) at a concentration of 120 µg/ml. $IC_{50}$ values of aqueous methanolic (*Pa*-$H_2O$-MeOH) and ethyl acetate fraction (*Pa*-AcOEt) for DPPH assay was comparable as that of rutin (80 µg/ml). Superoxide anion scavenging activity of all fractions was found to be excellent than standard ($IC_{50}$ 150 µg/ml). Calculated $IC_{50}$ value for the aqueous-methanolic, ethyl acetate, *n*-butanol (*Pa*- *n*-BuOH) and aqueous fractions (*Pa*-$H_2O$) were 60, 70, 90 and 140 µg/ml respectively. In CUPRAC assay percentage reduction capacity of the aqueous methanolic crude extract was highest among all other fractions. Total phenol contents of aqueous methanol extract and ethyl acetate fraction were almost comparable and indicated high phenol content. Results indicated the importance of ethyl acetate extract of *P. atrosanguinea* as a photoprotective agent in sunscreen preparation in the pharmaceutical industry and natural antioxidants as well. Further isolation of molecules from ethyl acetate fraction was performed using column chromatography which led to the isolation of total seven molecules out of them two were characterized namely methyl pentatetraconta-30, 32, 34, 36, 38, 40, 42-heptaenoate (VVR-I) and pentadecyl butyrate (VVR-III). VVR-I is novel compound while VVR-II is already reported in literature. Moreover, *in-silico* study of already reported polyphenolic compounds which are considered to be anticancer agents were also carried out using Glide docking to investigate interaction pattern with MDR receptors (MRP1 and GSTP-1) involved in cancer chemotherapy. *In-silico* findings suggest that rutin may be used as dual modulator for MRP-1 and GSTP1-1 mediated multidrug resistance.

**(Vinay Kumar Gupta)** **(Dr. Vikas Jaitak)** **(Prof. (Dr.) P. Ramarao)**

DEDICATED

TO

MY LOVING

MUMMY AND PAPA

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I bow to the almighty for showering his blessings all time and giving me the wisdom, the strength and will power to complete my work successfully.

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(Vinay Kumar Gupta)

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## LIST OF ABBREVIATIONS

| Sr. No. | Full form | Abbreviation |
|---|---|---|
| 1 | Multidrug resistance | MDR |
| 2 | UV | Ultra-violet |
| 3 | 2, 2’ diphenyl-1- picryl hydrazyl | DPPH |
| 4 | Hexahydroxydiphenoyl | HDDP |
| 5 | Sun protection factor | SPF |
| 6 | Minimal erythema dose | MED |
| 7 | Erythema effect spectrum | EE |
| 8 | Solar intensity spectrum | I |
| 9 | Cupric ion reducing antioxidant assay | CUPRAC |
| 10 | Column Chromatography | CC |
| 11 | Nuclear magnetic resonance | NMR |
| 12 | Mass spectra | MS |
| 13 | Multidrug resistance protein-1 | MRP-1 |
| 14 | Glutathione s-transferase protein1-1 | GSTP1-1 |
| 15 | Absorption, Distribution, Metabolism, Excretion | ADME |

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1

## INTRODUCTION

Natural products comprising of plants, animals and marine sources play an important role in the treatment of diseases and ailments since ancient time (Strohl, 2000). Nature has provided a special kind of defense mechanism for each living species to cope up with any harsh conditions and disease (Devasagayam *et al*., 2004). Plants produce secondary metabolites via different biosynthetic pathway during harsh condition and disease state. These secondary metabolites are derived from primary metabolites like carbohydrates and include alkaloids, polyphenols (Flavonoids), resins, tannins, terpenoids and essential oils etc. (Harvey, 2000; Geldenhuys *et al.*, 2012). Natural products continue to provide greater structural diversity than standard combinatorial chemistry and so they offer major opportunities for finding novel low molecular weight lead structures that are active against a wide range of assay targets. The famous examples of these natural products include salicylic acid, which comes from willow bark leading to the discovery of aspirin (Patrono and Rocca, 2009), lovastatin, to treat high plasma cholesterol levels (Tobert, 2003), and paclitaxel, used in the treatment of cancer (Kingston and Newman, 2007). Despite of such achievements only 10% of the total world's biodiversity have been tested for biological activity, many more useful natural lead compounds are awaiting discovery (Harvey, 2000). Pharmaceutical companies may not certainly want a more potent compound, but one that can differentiate from a competitor's compound clinically as well as different enough structurally so that a patent would be granted (Geldenhuys *et al*., 2012). The challenge is how to access this natural chemical diversity (Harvey, 2000).

Skin is the largest organ of the body that engenders most versatile, self-repairing and protects the body from obnoxious physical, environmental and biological influences (Afaq, 2011). Oxidative stress and inflammatory response provoked by ultraviolet radiation (UV) can cause a variety of intolerable effects on the skin, including premature photo aging and skin carcinogenesis (De Oliveira Junior *et al*., 2013). As per an appraisal by the American Cancer Society, the prevalence of freshly diagnosed skin cancer in the United States alone is

estimated to exceed 1 million per year (Jemal *et al.*, 2010). The total radiant energy emitted from the sun consists of about 5% UV range which is divided into three regions, UVA (320-400), UVB (280-320) and UVC (200-280nm). Solar UV radiation is a potent mutagen and regarded as the main cause of cancer, is confirmed by both epidemiological and molecular evidence (Afaq, 2011). Among all regions of the UV, UVC is more dangerous and can induce genotoxic stress in humans being who are directly exposed to UVC radiation because of its low wavelength and high energy. UVC radiation is filtered by atmospheric ozone layer before reaching the earth. However, UV radiation, especially UVA and UVB, which reaches the earth and penetrates the skin, causes a variety of adverse effects (Afaq, 2011; Afaq and Mukhtar, 2006). Only about 4–5% of total UV radiation emitted from the sun is associated with the UVB region which is considered to be more genotoxic and capable of causing cell damage such as inflammation, DNA damage, oxidative stress, free radical production, immunosuppression, photo aging and skin cancer. However, UVB has not as much of penetrating power as that of UVA and acts predominantly on the epidermal basal layer of the skin (Afaq *et al.*, 2005; Bickers and Athar, 2006; Halliday and Lyons, 2008; Timares *et al.*, 2008). It has been reported in literature that natural agents can be utilized as a photoprotective agent as they possess different pharmacological activity including antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic and immunomodulatory activity thereby inhibits UV-induced skin damage (Afaq, 2011; Afaq *et al.*, 2005; Bowden, 2004). Moreover, several skin care product have been marketed as having these natural agents as the principal active ingredient (Afaq, 2011; Afaq *et al.*, 2005; Bowden, 2004; Nichols and Katiyar, 2010). Role of natural product especially polyphenols in photoprotection of skin have been well known in literature at the cellular and molecular levels. (Afaq, 2011).

Changing lifestyle and dietary habits of human beings has led to the production of free radicals (Reactive oxygen species and Reactive Nitrogen Species) beyond the neutralizing limits (Fang *et al.*, 2002; Surh *et al.*, 2005). In a healthy human being, there is always balance between free radical generation and quenching phenomenon (Temple, 2000). Natural protective mechanisms against oxidative stress involve superoxide dismutase (SOD), glutathione peroxidase,

glutathione reductase, thioredoxin, thiols and disulfide bonding that can counteract the oxidative stress in each cell of the body (Devasagayam *et al.*, 2004). Long term accumulation of these free radicals can cause deterioration in the normal signalling pathway subsequently involved in the etiology and prognosis of various degenerative aging diseases such as cancer and atherosclerosis (Devasagayam *et al.*, 2004; Halliwell *et al.*, 1992). Free radical induced damage at molecular level includes lipid peroxidation, DNA damage and oxidation of proteins into protein hydroperoxides (Devasagayam *et al.*, 2004). Antioxidants may be used for inhibition and control of such kind of deterioration (Knight, 2000).

Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure (C6-C3-C6), and they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols and flavanones (Harborne and Williams, 2000; Holiman *et al.*, 1996) and represented in Figure 1.

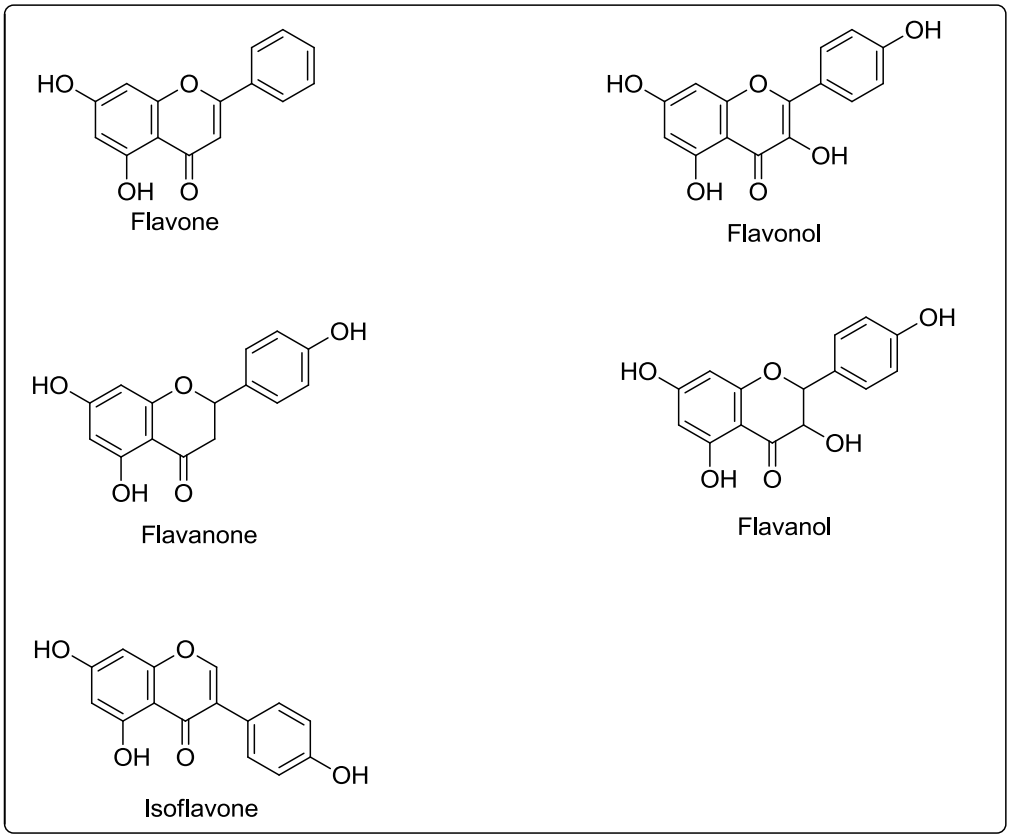



**Figure 1.** Major classes of flavonoids

Flavonoids have probably existed in the plant kingdom for over one billion years. They are present practically in all dietary plants, like fruits and vegetables (Table 1). Therefore, they are consumed in considerable amounts and are also heat stable. It is estimated that the human intake of all flavonoids is a few hundreds of milligrams per day (Hollman and Katan, 1999).

**Table 1**. Subclasses and dietary sources of flavonoids

| **Flavonoid subgroup** | **Representative flavonoids** | **Major food sources** | **References** |
|---|---|---|---|
| **Flavonols** | Kaempherol, myricetin, quercetin, rutin | Onions, cherries, apples, broccoli, kale, tomato, berries, tea, redwine, tartary buckwheat | (Hollman and Katan, 1999) |
| **Flavones** | Apigenin, chrysin, luteolin Daidzein, genistein, glycitein, | Parsley, thyme | (Hollman and Katan, 1999) |
| **Isoflavones** | formononetin | Soya beans, legumes | (Beecher, 2003) |
| **Flavanols** | Catechin, gallocatechin | Tea, grape and red wine | (Beecher, 2003) |
| **Flavanones** | Eriodictyol, hesperitin, naringenin | Oranges, grapefruit | (Hollman and Katan, 1999) |

A growing number of epidemiological studies suggest that high flavonoid intake may be correlated with a decreased risk of cancer (Le Marchand, 2002). In a population-based case-control study conducted in Shanghai from 1996–1998 which included 250 incident breast cancer cases and their individually matched controls (Dai *et al*., 2002). It has been reported that urinary excretion of total isoflavonoids and mammalian lignans was substantially lower in breast cancer cases than in controls. Knekt and co-workers (Knekt *et al*., 2002) also estimated flavonoid intakes of 10,054 men and women mainly on the basis of the flavonoid concentrations in finish foods with a dietary history method. They found that men with higher quercetin intakes had a lower lung cancer incidence, and men with

higher myricetin intakes had a lower prostate cancer risk. These data suggest a protective role of flavonoids against cancer. A population-based case-control study in Hawaii further investigated the association between intake of flavonoids-powerful diet and lung cancer risk. After adjusting for smoking and intake of saturated fat and beta-carotene, an inverse association was observed between lung cancer risk and the consumption of onions, apples, or white grapefruits as well as the calculated total intake of quercetin (Marchand *et al.*, 2000). Flavonoids have been screened *in-vitro* for their anticancer activity against different cell lines (Hirano *et al.*, 1994). Flavonoids have been also screened *in-vivo* for their anti-cancer activity. They may inhibit carcinogenesis by affecting molecular events in the initiation, promotion and progression stages. A study showed that fermented soy milk containing larger amounts of genistein and daidzein than unfermented one and isoflavone mixtures, given to rats starting at 7 weeks of age, inhibited mammary tumorigenesis induced by 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (Ohta *et al*., 2000).

Major molecular mechanisms of flavonoids involve anti-proliferative activity (Fotsis *et al.*, 1997; Lahiri-Chatterjee *et al.*, 1999), induction of apoptosis (Wang *et al.*, 1999), cell cycle arrest (Senderowicz, 1999), modulation of multidrug resistance (MDR) (Gupta *et al.*, 2013), inhibition of angiogenesis (Fotsis, *et al*., 1997) and carcinogen metabolic activation (Zhai *et al.*, 1998). Among all above activity of flavonoid, modulation of MDR is very important. The emergence of drug resistance in many cases makes the currently available chemotherapeutic agents ineffective (Fojo and Bates, 2003). MDR is the resistance of a tumor cell population against drugs differing in chemical structure and cellular target. MDR may very well be “intrinsic”, whenever illness is actually refractory to be able to chemotherapy with analysis or even “acquired”, once the pill gets to be insensitive right after the remedy (Krishna and Mayer, 2000). MDR is in part mediated by the over-expression of plasma membrane transporters, such as P-glycoprotein (P-gp, MDR1 or ABCB1), MDR-associated proteins (MRP1 or ABCC1, and MRP2 or ABCC2), or breast cancer resistant protein (BCRP or ABCG2) (Ishikawa, 1992). Other important enzymes responsible for clinical multidrug resistance are glutathione-s-transferase (GSTs), especially of the π class (Stavrovskaya, 2000).

Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in a multitude of disease states in most commonly cancer, cardiovascular disease, and neurodegenerative disorders (Havsteen, 2002; Rice-Evans, 2001; Spencer *et al.*, 2004). Many evidences indicated that flavonoids interact with ABC transporter and modulate MDR in tumors. The dual effects, i.e. modulation of multidrug resistance and antitumor activity of these herbal derivatives may synergistically act in cancer chemotherapy (Gupta *et al*., 2013).

*Potentilla* is a genus of about 500 species of annual, biennial and perennial herbs of the *Rosaceae* family, native to most of the Northern Hemisphere (Delgado, 2000). Pharmacological evaluations have shown that some extracts of different parts of plant from *Potentill*a species exhibit antioxidant, hypoglycemic, anti-inflammatory, antitumor and antiulcerogenic potential properties (Bos *et al.*, 1996; Gürbüz *et al.*, 2005; G. Miliauskas *et al.*, 2004; Syiem *et al.*, 2003). Phytochemical investigations of different *Potentilla* species have demonstrated the presence of proanthocyanidins, catechin polyphenols, flavonoids (quercetin and myricetin glycosides) and secondary metabolites like tannins of the ellagic-acid type, with monomeric and dimeric ellagitannins, similar to those found in green tea in various species of *Potentilla* (Oszmianski *et al.*, 2007; Zhao *et al.,* 2008).

Keeping in view the above mentioned facts about natural products and *Potentilla* species in the treatment of various diseases and ailments, the present study was planned to explore the medicinal value of root extracts of *Potentilla atrosanguinea* Lodd. To meet the following objectives:

- To extract and prepare different fractions of dried roots of *P. atrosanguinea*.
- Photoprotective and antioxidant activities of root extracts of *P. atrosanguinea*.
- Isolation and characterization of secondary metabolites from active part of extract.
- *In-silico* study of interaction pattern of polyphenolic compounds with MRP-1 and GSTP1-1 receptors.

# CHAPTER 2

# REVIEW OF LITERATURE

# CHAPTER 2

## REVIEW OF LITERATURE

The genus name *Potentilla* comes from the Latin diminutive of *potent* meaning “powerful” in reference to the medicinal properties of some species. *Potentilla* species and their extracts, respectively, have been widely used in different cultures of the Northern hemisphere; little has been known about the phytochemistry and pharmacology of this genus. Traditional use of *Potentilla* species have been summarized in Table 2. In 1811, C.H. Pfaff described for the first time the presence of tannins as main ingredients in *Potentilla erecta* rhizomes and concluded that these compounds are responsible for the astringent effects of this plant (Herrmann and Enge, 1957). In the 1960s, investigations on the phytochemistry mainly of *Potentilla erecta* rhizomes were performed and published. Starting from the 1980s many more papers dealing with *Potentilla* species which are in part only locally important were published. In parallel pharmacological (*in-vitro* and *in-vivo*) evaluations of *Potentilla* species and their extracts were put forward, especially since the 1980s. The first clinical studies were performed for *Potentilla erecta* rhizome extracts for the treatment of colitis ulcerosa (Huber et al., 2007) and also for the treatment of virus-induced diarrhoea in children (Subbotina *et al.*, 2003). The Greek physician Pedanius Dioscurides recommended a condensed decoction of the underground parts of *Potentilla erecta* (L.) (tormentil) to bathe a purulent facial eczema and to rinse oral cavity ulceration. During the medieval ages the European physicians and botanists H. Bock, L. Fuchs, Paracelsus, Tabernaemontanus, C. Bauhin and others described and depicted *Potentilla* species in their herbal books. Fuchs, for example, mentioned five *Potentilla* species in his “New Kreuterbuch”comprising *Potentilla alba* L., *Potentilla reptans* L. and *Potentilla neumanniana* (underground parts and leaves), *Potentilla anserina* L. (herbal part) and *Potentilla erecta* (undergroundparts and herbal parts). Extracts were prepared with water, milk, honey and alcoholic solutions and were applied for the treatment of toothache, inflammations of the throat, for wound-healing, jaundice, ulcers of the mouth, dysentery and as a homeostatic. In Chinese traditional medicine *Potentilla* extracts have been used to treat diarrhoea, hepatitis, rheuma and scabies and as a remedy

for detoxification (Xue *et al.*, 2007). In Tibetan traditional medicine *Potentilla anserina* root extracts have been applied for the treatment of certain viral infections (Zhao *et al.*, 2008). The therapeutic indications of the herbal parts of *Potentilla anserina* and the rhizomes of *Potentilla erecta* include according to the Commission E simple forms of dysmenorrhoa, the supporting therapy of simple forms of unspecific, acute diarrhoea and also simple forms of mucosal inflammations of throat and mouth. The gynecological indication for silverweed (*Potentilla anserina*) is based on pharmacological studies showing that the herb increases the tonus of the isolated uterus in various animal species.

**Table 2.** Traditional use of *Potentilla* species in different region of world

| Regions | Plant species | Part used | Traditional use | References |
|---|---|---|---|---|
| *Europe*, e.g. Central Europe, Italy, Sweden, Serbia and Montenegro, Russia, Bulgaria, Turkey | *Potentilla erecta* | Roots | Inflammations, treatment of wounds, bleeding, dysentery, diarrhea inflammatory bowel disease, bacterial, fungal and viral infections, certain forms of cancer,antiseptic for the mouth and throat. | (Latté, 2006; Palombo, 2006; Spiridonov *et al.*, 2005) |
| | *Potentilla fruticosa* | Aerial parts | Viral infections, impairment of immune system | (Tomczyk and Latté, 2009) |
| | *Potentilla anserina* | Aerial parts | Acute, nonspecific diarrhea with mild discomforts, mild inflammation of the oral and pharyngeal mucosa, tooth ache | (Tomczyk and Latté, 2009) |
| | *Potentilla spectosa* | Aerial parts, roots | Inflammations, anti-ulcer activity Microbial infections | (Kovzačević and Ristić, 2007) |
| | *Potentilla recta* | Aerial parts | Microbial infections | (Tosun *et al.*, 2006) |

| | | | | |
|---|---|---|---|---|
| | *Potentilla reptans* | Aerial parts, leaves | Tooth ache, ulcers, inflammations of the throat | (De Natale and Pollio, 2007) |
| | *Potentilla fulgens* | Roots | Stomach disorders, certain forms of cancer, diabetes mellitus | (Rosangkima and Prasad, 2004; Syiem *et al.*, 2003; Syiem *et al.*, 2002) |
| Asia, e.g. China, korea, japan, Nepal, india | *Potentilla chinensis*<br>*Potentill multicaulis* | Aerial parts<br>Roots | Certain form of cancer | (P. L. Li *et al.*, 2007; Tomczyk and Latté, 2009) |
| | ***Potentilla atrosanguinea*** | **Roots** | **Wound-healing, diarrhea, influenza, bleeding** | **(**Sharma *et al.*, 2004) |
| | *Potentilla kleintana* | Aerial parts | Cough, parotitis, lymphadenitis, hepatitis, scare, numbness of limbs, dysmenorrheal, ulcer | (Long and Li, 2004) |
| | *Potentilla peduncularis* | Leaves, buds | Fever ,influenza,cough | (Manandhar, 1995) |
| | *Potentilla freyniana* | Roots | Viral infections | (Chen *et al.*, 2005) |
| | *Potentilla discolor* | Aerial parts, roots | Diarrhea, hemorrhage,diabetes mellitus | (Jang *et al.*, 2006) |
| | *Potentilla multifida* | Aerial parts, roots | Hepatitis, enterobiasis, functional uterine hemorrhage, type 2 diabetes mellitus | (Xue *et al.*, 2005) |
| | *Potentilla arguta* | Roots | Viral infections | (McCutcheon *et al.*, 1995) |
| | *Potentilla simplex* | Leaves, stems | Fungal infections | (Webster *et al.*, 2008) |

## 2.1 Chemical constituents of *Potentilla* species

Both aerial and underground parts of *Potentilla* species have been investigated for bioactive molecules with great success.

### 2.1.1 Constituents of the roots and rhizomes

Due to the high amount of 17–22% of tannins in the rhizomes of *Potentilla erecta* (i.e. 15–20% condensed tannins, ca. 3.5% hydrolyzable 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 proanthocyanidins (Schleep, 1986) (Figure 2). Tannins have been recognized as important chemical constituents of *Potentilla* species. A series of compound have been investigated as precursors for condensed tannins e.g. (+) -catechin, (-) -epicatechin, (+) -galloctechin, (-) -epigallocatechin (Gao *et al*., 2007). Hydrolyzable tannins have been procured from only two species of *Potentilla* plants. g. *Potentilla erecta* and *Potentilla discolor*. Apart from pentadigalloylglucose, four monomeric and three dimeric ellagitannins were isolated with agrimoniin being the most complex structure with altogether four hexahydroxydiphenoyl residues in one molecule (Figure 2). Moreover the degradation product of ellagitannins, ellagic acid and two of its derivatives were found in phytochemical analysis of *Potentilla candicans* and *Potentilla discolosr.* A very low number of flavonoids have only been reported in underground parts of *Potentilla erecta*. Further constituents include number of organic acids and phenol carboxylic acids which were exclusively described for *Potentilla erecta.* Triterpenoids also have been isolated from the underground part of *the Potentilla* plant (Li *et al.*, 2007). These compounds are generally based on an olean or ursan skeleton. Some more antioxidants have been isolated from dried root powder methanolic extract of *Potentilla fulgens* named potifulgen and epicatechin (Jaitak *et al*., 2010) (Figure 2).

### 2.1.2 Chemical constituents of aerial parts of *Potentilla* species

In aerial parts of *Potentilla* species flavonoids are found in high concentration. Hydrolysable tannins around 5-10% have been reported in *Potentilla anserine*. Interestingly, no condensed tannins were elucidated, however their precursors

were isolated especially in *Potentilla erecta*. Aerial parts of *Potentilla* also have triterpenoid as like in underground parts.

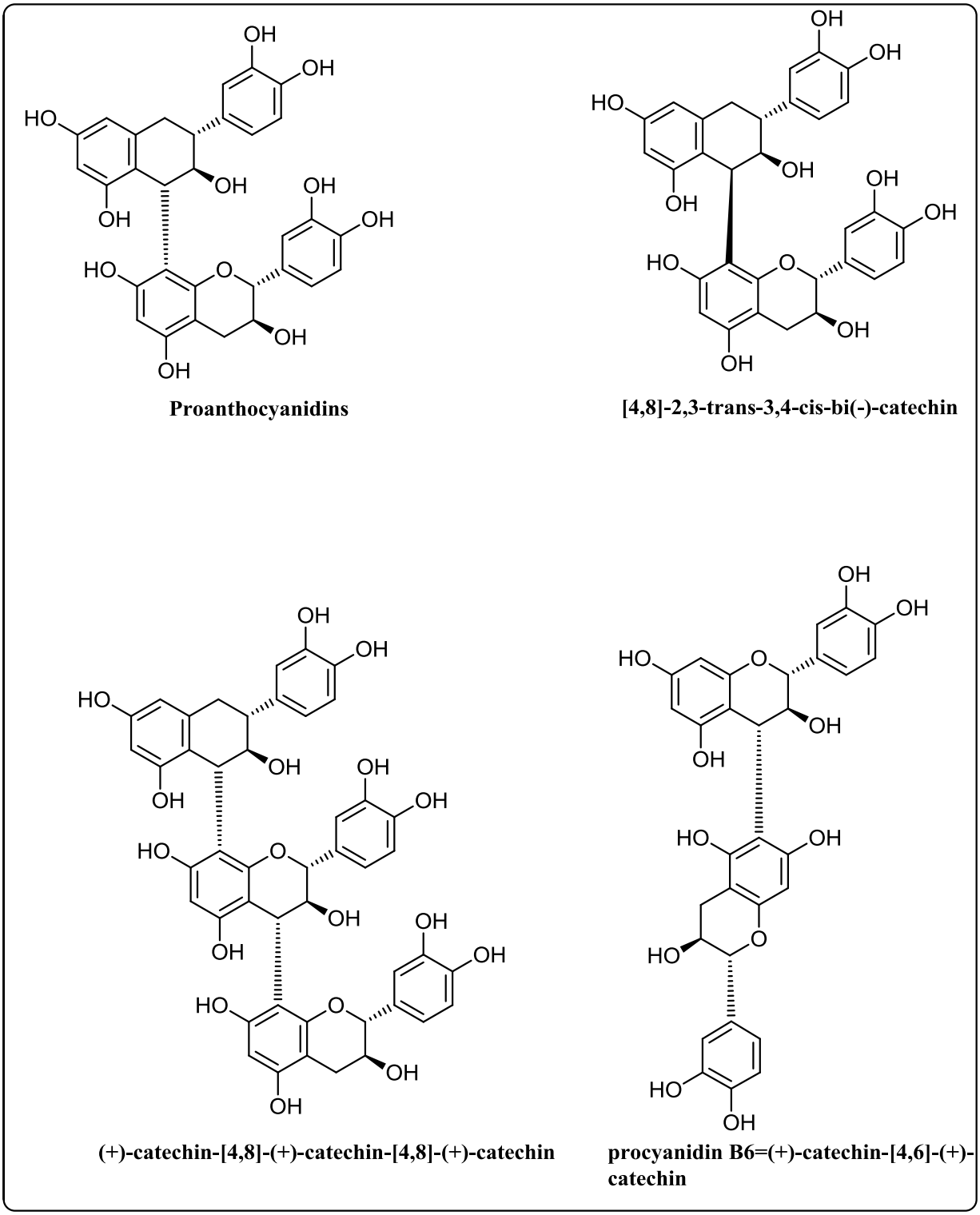
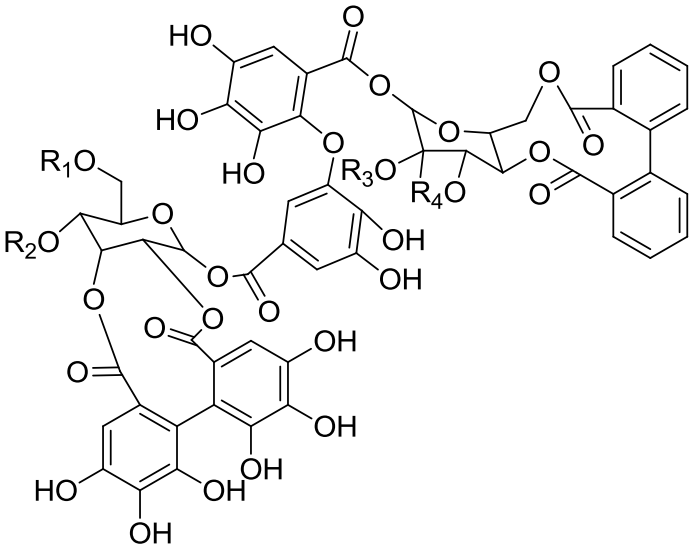
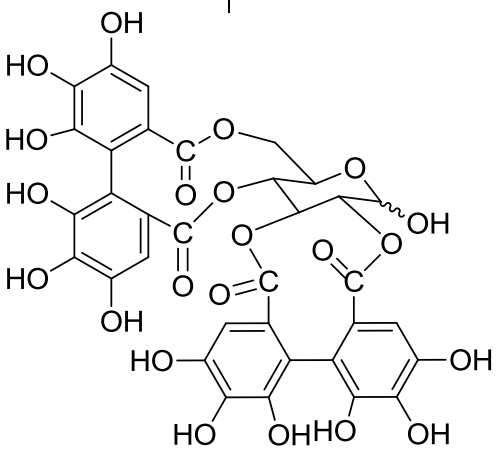


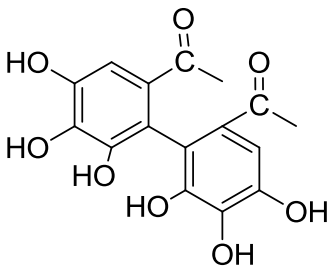

**Figure 2.** Structure of constituents from *Potentilla* species



| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| **Laevigatin B** | H | H | HHDP | |
| **Laevigatin F** | HHDP | | H | H |
| **Agromoniin** | HHDP | | HHDP | |

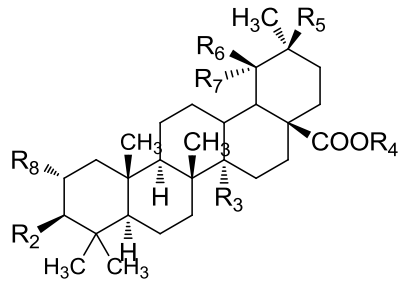


**Pedunculagin**



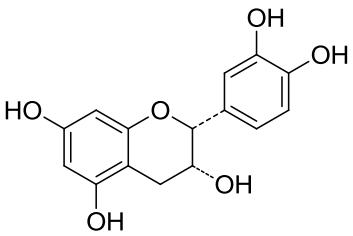
**HHDP**

**Figure 2.** Continued

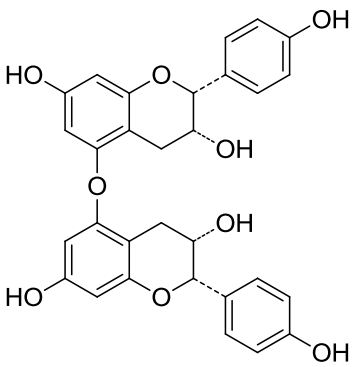



**Triterpenoids**

| | 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-b-D-glucopyranosyl ester | OH | H | $CH_3$ | b-D-Gluc | H | CH3 | OH | H |
| Tormentic acid | H | OH | $CH_3$ | H | H | CH3 | OH | OH |
| Tormentoside | H | OH | $CH_3$ | b-D-Gluc | H | CH3 | OH | OH |
| Arjunetin | H | OH | $CH_3$ | b-D-Gluc | CH3 | H | OH | OH |
| Euscaphic acid | OH | H | $CH_3$ | H | H | CH3 | OH | OH |
| Euscaphic acid 28-b-D-Glucopyranosyl ester | OH | H | $CH_3$ | b-D-Gluc | H | CH3 | OH | OH |
| Chinovic acid | H | OH | COOH | H | H | CH3 | H | H |




**Epicatechin**




**Potifulgene**

**Figure 2**. Continued

The common properties of these triterpenoid is again the olean or ursan skeleton (Li *et al*., 2007). Moreover, several other constituents were described for *Potentilla* species, comprising coumarrins, organic acids and phenolic carboxylic acids, sterols, megastigmanes, essential oils and a pectin along with polyphenols and 2-pyrone-4,6-dicorboxylic acid.

## 2.2 An Overview of pharmacological profile of *Potentilla* species

The scientific world's particular interest in the genus *Potentilla* and their curative properties originated from the realm of traditional medicine. Ethnic medicine has come to be an irreplaceable source of knowledge of medicinal plants and their curative qualities, as well as creating clues for scientific research, which usually confirms the legitimacy of their usage (Fabricant and Farnsworth, 2001).

### 2.2.1 Anti-ulcer activity

A variety of herbal products have been reported to possess antiulcer activity, but the documented literature has focused primarily on pharmacological effects in animals. It should be noted that especially polyphenolics such as tannins are of particular therapeutic importance as gastroprotective agents (Borrelli and Izzo, 2000).

### 2.2.2 Antidiarrhoic activity

The oligomeric and polymeric flavan-3-ols also known as condensed tannins or proanthocyanidins showed therapeutic properties to treat diarrhea (Palombo, 2006; Santos-Buelga and Scalbert, 2000). The antidiarrhoeal effect of tannins is commonly attributed to the unspecific complexation of mucosal proteins in the gut with formation of a protective layer (Subbotina *et al.*, 2003).

### 2.2.3 Antineoplastic activity

Chinese authors (Li *et al*., 2006) have attributed a significant anti-cancer activity to a number of triterpenoid compounds isolated from the aerial parts of *Potentilla chinensis* and the roots of *Potentilla multicaulis* which were evaluated for their *in-vitro* cytotoxic activities against SMMC-7221 (human hepatoma) and HL-60 (human promyelocytic leukemia) cells. *Potentilla erecta*, a traditional plant in

Russian medicine, was used to alleviate disease symptoms in patients with cancer. The ethanol extract of the rhizomes may inhibit the growth of lymphoma cells as concluded from *in vitro* data. The crude ethanol (40%) extract from *Potentilla erecta* displayed the highest cytotoxicity, completely suppressing the cell growth of the cells at concentrations of 10 and 50 µg/ml. Wall *et al.* found out that tannin-containing topoisomerase I and II inhibitory plant extracts which were subjected to tannin removal procedures lost their activity in this test assay. Thus tannins play an important role in inhibitory effects on topoisomerases I or II. Polyphenolics, i.e. kaempferol 3-*O*-β-d-(6"-*E*-p-coumaroyl) - glucopyranoside (tiliroside) and methyl brevifolincarboxylate isolated from the aerial parts of *Potentilla argentea* L., were evaluated for their cytotoxicities against human breast carcinoma cell line (MCF-7) and their DNA-binding ability. Methyl brevifolincarboxylate was much more active and showed higher cytotoxic potency than tiliroside. In DNA topoisomerase I and II inhibition assays *in vitro* both investigated compounds were more effective against topoisomerase II than I. The results of DNA binding studies revealed that methyl brevifolincarboxylate has a greater DNA-binding affinity than tiliroside, which correlates with its greater potency as a topoisomerase I/II inhibitor (Tomczyk *et al.*, 2008).

### 2.2.4 Antiviral and antimicrobial activity

May and Willuhn (1978) found out that extracts of *Potentilla erecta* rhizomes and also of the herbal part of *Potentilla anserina* had moderate antiviral effects against the *Herpes* virus *in vitro*. In addition this *Potentilla anserina* extract showed some activity against the *Vaccine* virus. Earlier, other authors had also shown the suppressive effect of a *Potentilla arguta* methanol root extract against bovine respiratory syncytial virus proliferation (McCutcheon *et al.*, 1995). On several occasions, the antiviral characteristics of isolated hydrolysable and condensed tannins from *Potentilla erecta* rhizome against *Herpes* virus types I and II have also been reported, as well as its cytotoxic activity against *Influenza* virus type A2 and *Cowpox*. It has been reported that 70% aqueous ethanol extract of underground parts of *Potentilla gracilis* showed more than 20% inhibition effects on HIV-1 reverse transcriptase at 0.5µg/ml concentration in this in vitro screening test (Nakanishi and Mizuo, 1993). The whole plant of *Potentilla sericea* L. (syn.

*Potentilla pensylvanica* L.) is also used in the general treatment of tuberculous lupus as an adjuvant drug, along with tuberculocidal and tuberculostatic agents (Gautam *et al.*, 2007).

### 2.2.5 Antihyperglycemic activity

Japanese authors noticed that an ellagic acid derivative isolated from *Potentilla candicans* inhibited aldose reductase activity—an enzyme catalyzing glucose transition into sorbitol. Blocking of this metabolic path prevents fructose and sorbitol accumulations in the organs, as well as in the eyes, therefore decreasing the likelihood of complications in the future (Terashima and Morita, 1990). Similarly, a methanolic extract of *Potentilla recta* (aerial parts) exhibited potent inhibitory activity exceeding 60% on human aldose reductase (h-AR) (Enomoto, 2004). Recently, 4-*O*-methylellagic acid 3-*O*-α-l-rhamnoside obtained from ethyl acetate fraction of an ethanolic extract from the roots of *Potentilla discolor* was subjected to *in vitro* bioassays to evaluate advanced glycation end products (AGEs) and rat lens aldose reductase (RLAR) inhibitory activities. Diabetological studies performed in India showed the hypoglycemic activity of pure methanol *Potentilla fulgens* root extracts. In both healthy and alloxan-induced diabetes mice, the blood glucose level was lowered. Alloxan-induced diabetic mice were administered intraperitoneally (i.p.) the extract at varying doses (150–450 mg/kg b.w.) and the blood glucose levels were measured at varying time intervals up to a period of 5 days. Toxicity studies carried out on mice up to a dose of 450 mg/kg b.w. did not show any adverse effects during the 4 weeks of observation (Syiem, *et al.*, 2002). An extract of *Potentilla erecta* rhizome showed similar effects, probably due to the presence of tormentoside. An increase of insulin secretion was also proven during oral administration of this compound preparation. In the initial diabetic stages, both extracts could be used as subsidiaries and the genus *Potentilla* may be helpful in diabetic complications such as neuropathy and retinopathy (Strzelecka, 2000).

### 2.2.6 Anti-inflammatory, spasmolytic and hepatoprotective activity

Based on the available literature and observation of traditional, native methods of inflammatory process prevention, Swedish authors selected 52 plant species and

studied their aqueous extracts *in vitro* with regard to prostaglandin and exocytosis biosynthesis inhibition induced by platelet activating factors (PAF). It was observed that some of them, including *Potentilla erecta* (rhizome extract), have very strong cyclooxygenase inhibiting properties, and therefore show anti-inflammatory activity (Tunon *et al.*, 1995). Externally, the extracts of *Potentilla* species are also used for bathing (dermatoses, mycoses, excessive perspiration), irrigation (white leucorrhea, vulvo-vaginitis conditions), compresses (burns, frostbite, skin injuries), enemas (large bowel and rectal ulcerative inflammation), and ointments (purulent and allergic dermatitis). Analogous activity has also been shown for the *Potentilla anserina* herb. Ethyl acetate extracts from the aerial part of *Potentilla anserina* have a significant spasmolytic activity in abnormal smooth muscle tones of the intestines and uterus. It appears then, that spasmolytic activity is comparable with papaverine hydrochloride. Moreover, it has been proven that spasmolytic properties do not include blood vessels and the urinary system, so this activity may be used in oligomenorrhea alleviation. Additionally, the *Potentilla anserina* herb has a good influence on liver functions and increases bile secretion. As a cataplasm, it is used in the alleviation of rheumatic and arthritic pains and neuralgia (Youngken *et al.*, 1949).

*In-vivo* local anti-inflammatory effects of rhizomes and roots of *Potentilla alba* and *Potentilla erecta* acetone and ethanol extracts have been observed in the test model of mouse ear. The local antiinflammatory effect of these extracts was tested on a modified model of a mouse ear. For the purpose of provoking inflammation, both mouse ears were applied 3% oleum crotonis acetone solution. The strongest pharmacological reaction was found with acetone extract of the rhizome of *Potentilla alba*, the pharmacological reaction of which was similar to a hydrocortisone ointment. An aqueous extract derived from flowers and young shoots of *Potentilla fruticosa* had a protective effect on the liver. Studies performed in rats with chronic and toxic hepatitis showed the hepatoprotective influence of that extract on the microsomal liver system, as well as a protective effect on the xenobiotic metabolism and plasma lipid peroxidation. Moreover, it normalized the alanine aminotransferase activity and bilirubin levels in plasma (Kolpakov *et al*., 2001).

### 2.2.7 Antioxidant activity

Currently, polyphenolic compounds are of great interest in nutrition and medicine for their potent antioxidant capacity and protective effects on human health and diseases with free radical ethiologies, including cardiovascular diseases, neoplastic diseases and blood clotting diseases. As a result, a number of works on antioxidative substances, isolated from aerial and underground parts of various genus *Potentilla* species, have been published. Methanolic root extracts of *Potentilla alba* and isolated polyphenolic compounds have been tested for antioxidant activity (Oszmianski *et al.*, 2007). In another study antioxidant activity of methanolic extracts, fractions and isolated compounds from *Potentilla fulgens* have been tested via three *in vitro* experiments, namely, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), 1,1-diphenyl-2- picrylhydrazyl (DPPH), and FRAP assays (Jaitak *et al*., 2010). Effects of different extraction method on total polyphenol content and antioxidant activity in the aerial part of *Potentilla atrosanguinea* have been studied (Kalia *et al.*, 2008). Antioxidant activity was reported on different extracts (methanol, acetone, ethyl acetate) and isolated compounds obtained from the flowers of *Potentilla fruticosa* (Miliauskas *et al.*, 2004).

## 2.3 *Potentilla atrosanguinea*

Other common names- Himalaya cinquefoil/ ruby cinquefoil

Group- Dicot

Family- Rosaceae

Duration- Perennial

Growth Habit- Herbs

**Figure 3.** *Potentilla atrosanguinea*

*P. atrosanguinea* Lodd. (Rosaceae); commonly known as Himalaya cinquefoil is a perennial herb native to western Himalayas (Kalia *et al.*, 2008; Sahoo *et al.*, 2001). Traditionally *P. atrosanguinea* plants have been used for wound-healing, diarrhea, influenza, and bleeding (Sharma *et al.*, 2004; Tomczyk and Latté, 2009). The

leaves of *P. atrosanguinea* are consumed as a healthful tea while its starchy roots have taste like parsnips, sweet potatoes, or chestnuts have served as a human food (Ballabh and Chaurasia, 2007; Kalia *et al.*, 2008). Aerial parts of *P. atrosanguinea* consist of the high concentration of polyphenolic compounds (Flavonoids) (Kalia, *et al.*, 2008). Polyphenolic compounds have shown a series of biological activity in literature, i.e. anti-inflammatory, anticarcinogenic, anti-oxidative, and anti-mutagenic and modulation of enzymatic activity etc. There are very few literatures regarding medicinal value of *P. atrosanguinea.* As per our knowledge there is only one article stating that total polyphenolic content and antioxidant activity in the aerial parts of *P. atrosanguinea* (Kalia *et al.*, 2008). Recently an anti-aging enzyme gene has been isolated from this plant by the scientists at the CSIR Institute of Himalayan Bioresource Technology (IHBT), Palampur which produces superoxide dismutase, an enzyme required to scavenge the superoxide radical in plant and animal cells (Gill *et al.*, 2012; Gill *et al.*, 2010). Keeping in mind the traditional uses of *P. atrosanguenia*; one can explore its medicinal value for the benefit of human being as well as molecules responsible for that.

# CHAPTER 3

# MATERIAL AND METHODS

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## 3.1 Plant material

Whole plants *P. atrosanguinea* were collected from Kunzum Pass (light intensity, 2500 µEinstein/$m^2$/s; daytime air temperature, 3-10 °C; altitude, 4517 m; 32° 24′ 20″ N; 077° 38′ 40″ E) in Lahaul and Spiti district of Himachal Pradesh in Western Himalayas of India in the month of September 2012.

## 3.2 Chemicals

Methanol, ethyl acetate and *n*-butanol used for extraction were purchased from Sisco research laboratory, Mumbai, India. Water used for the experiments was of free from ions and double distilled. 2,2´-Diphenyl-1-picryl hydrazyl (DPPH), 2-thiobarbituric acid (TBA), ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bromophenol blue, EDTA, L-ascorbic acid, Tris (hydroxymethyl) aminomethane, Folin–Ciocalteu reagent, trichloroacetic acid (TCA) and gallic acid were of analytical grade.

## 3.3 Instruments

The UV spectra were recorded on a Shimadzu (2450) double-beam UV-Visible spectrophotometer. $^{1}H$ and $^{13}C$ Nuclear magnetic resonance (NMR) spectra was obtained in $CDCl_3$/d6-DMSO on a Bruker Avance II (400 MHz) NMR spectrometer using TMS (δ = 0) as internal standard.

## 3.4 Extraction of plant material

The roots of plants were separated using knife and then thoroughly washed with water followed by drying at room temperature. After complete drying it was grounded to fine powder. Air dried root powder (3.8 kg) of *P. atrosanguinea* was extracted with MeOH:H2O (80:20, v/v) for 12 h at room temperature. The percolation was repeated three times. The combined percolations were evaporated to dryness and fractionated with petroleum ether, ethyl acetate and *n*-butanol. All fractions were treated with anhydrous $Na_2SO_4$ and concentrated under

reduced pressure at 50±5 ˚C yielding petroleum ether (7 g), ethyl acetate (21.5 g), *n*-butanol (141.4 g) and aqueous extracts (52 g), respectively (Figure 4).

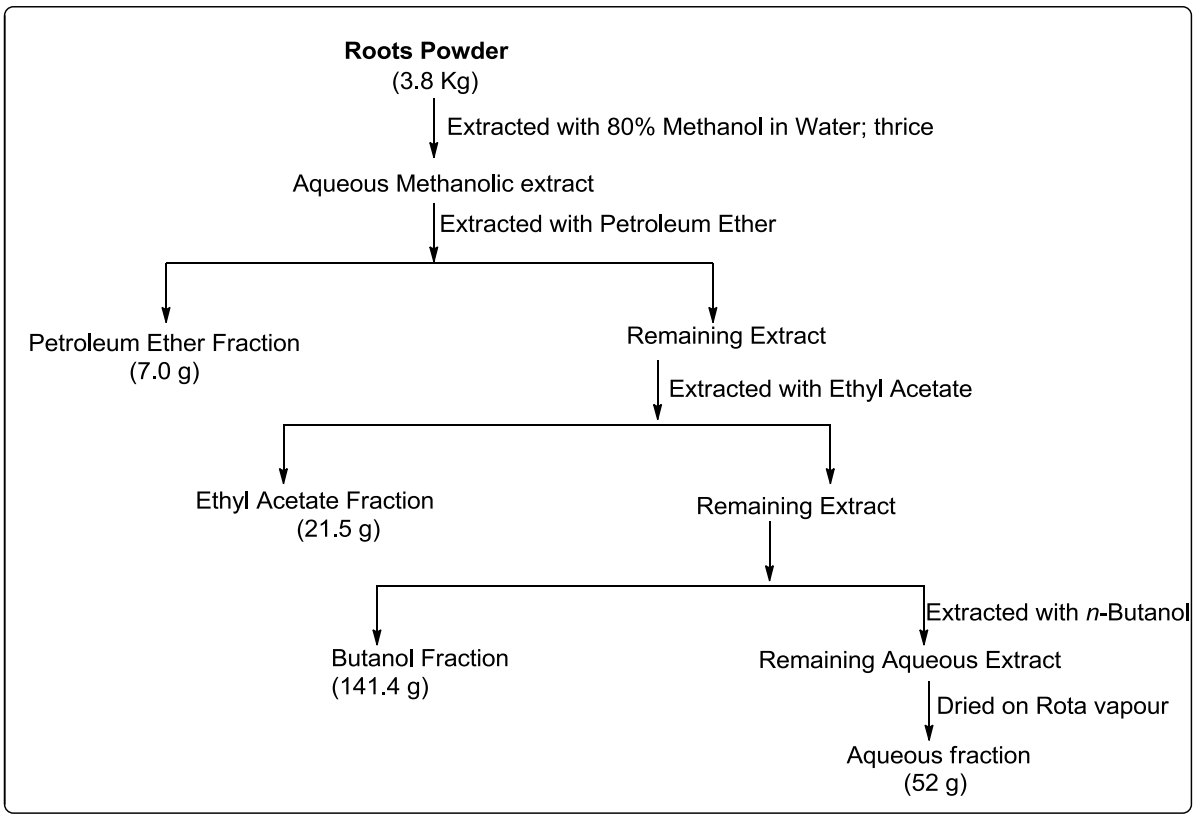



**Figure 4.** Extraction procedure of roots of *P. atrosanguinea*

### 3.5 Photoprotective activity

#### 3.5.1 Determination of Sun protection Factor (SPF)

The efficacy of sunscreen is defined in term of SPF, the higher the SPF, the more effective is the product in preventing sunburn. SPF is defined as the UV energy required to produce a minimal erythema dose (MED) on protecting skin, divided by the UV energy required to produce a MED on unprotected skin.

$$\text{SPF} = \frac{\text{Minimal erthema dose in sunscreen - protected skin}}{\text{Minimal erthema dose in non sunscreen - protected skin}}$$

*In-vitro* SPF determination may play a vital and rational tool in defining photoprotective activity of sunscreen preparation which in turn reduces UV exposure of human subjects and its consequences. Moreover it also reduces the

number of *in-vivo* experiments, when technical test parameters are adjusted and optimized (Dutra *et al.*, 2004; C. D. Kaur and Saraf, 2011).

There are two different methods for the determination of SPF. First method involves the measurement of absorption or the transmission of UV radiation through sunscreen product films in quartz plates or bio-membranes. However, second method is based on spectrophotometric analysis of dilute solutions at different concentration (Dutra *et al.*, 2004). The absorbance values of each dilution of extract were determined between 290-320 nm, at 5 nm intervals, taking methanol: water (80:20) as blank using UV-visible spectrophotometer. SPF was calculated according to the equation developed by Mansur (Mansur *et al.*, 1986).

$$SPF_{spectrophotometric} = CF \times \Sigma EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where EE (λ) indicates an erythemal effect spectrum; I (λ) indicates a solar intensity spectrum; Abs (λ) indicates absorbance of sunscreen product and CF stands for correction factor (= 10). The values of EE x I are constant and represented in Table 3 (Sayre *et al.*, 1979).

**Table 3.** The normalized product function used in the calculation of SPF

| **Wave length (nm)** | **EE X I (normalized)** |
|---|---|
| 290 | 0.015 |
| 295 | 0.0817 |
| 300 | 0.2874 |
| 305 | 0.3278 |
| 310 | 0.1864 |
| 315 | 0.0837 |
| 320 | 0.018 |

EE- Erythemal effect spectrum; I- Solar intensity spectrum

### 3.5.2 Preparation of samples

For sample preparation 100 mg of each extract i.e. aqueous-methanolic (*Pa*-$H_2O$-MeOH), ethyl acetate (*Pa*-AcoEt), *n*-butanol (*Pa*-*n*-BuOH) and aqueous (*Pa*-$H_2O$) extracts were weighed and made up to the volume of 10 ml with methanol: water (80:20) which gave concentration 10,000 µg/ml of each extract. From previously prepared dilution 1ml was taken out and made up the volume up to 10 ml which produced 1,000 µg/ml. Further took 1.2 ml of it and made up the volume up to 10

ml which gave 120 µg/ml of the extract. Similarly for preparation of other concentration e.g. 100, 80, 60 and 40 µg/ml took 1, 0.8, 0.6 and 0.4 ml from above prepared dilution (1,000 µg/ml) and made up to volume 10 ml with methanol: water (80:20) respectively.

### 3.6 Antioxidant assays

#### 3.6.1 Determination of Total Phenol Content

The total phenol content of *P. atrosanguinea* was quantified spectrophotometrically as per the procedure given by Yu and co-workers (Yu *et al*., 2002). The absorbance of blue colored mixture, formed due to the reaction of Folin Ciocalteu reagent (1:1) and 20% sodium carbonate solution with extract and fraction solution, was measured at 765nm (Systronics 2202 UV-VIS Spectrophotometer). The amount of total phenol was calculated as mg Gallic Acid Equivalents (GAE) /g dry weight of extract from calibration curve of gallic acid standard solution.

#### 3.6.2 DPPH radical scavenging activity

The measurement of hydrogen donating capability of extract was assessed using DPPH (2, 2' diphenyl-1- picryl hydrazyl) radical as substrate, following the method described by Blois (Blois, 1958). In this assay, 0.3 ml of extract solution was added to 2.7 ml of 0.1mM methanolic DPPH solution in a cuvette and absorbance was read at 517 nm. The decrease in absorbance at ambient temperature was correlated with the scavenging action of the test compound and compared with rutin (used as standard phenolic compound). The radical scavenging activity was calculated using the equation:

$$\% \text{ DPPH radical scavenging} = (1\text{-}A_S/A_C) \times 100)$$

Where, $A_C$ = Absorbance of Control, $A_S$ = Absorbance of Sample solution.

#### 3.6.3 Superoxide anion radical scavenging assay

For assessing the superoxide anion scavenging ability of different extract/fractions of *P. atrosanguinea*, the method described by Nishikimi (1972) was followed with slight modifications (Nishikimi *et al.*, 1972). The superoxide anions were generated

non-enzymatically in a PMS-NADH system comprising of phenazine methosulphate and reduced nicotinamide adenine dinucleotide, and assayed by development of blue coloured formazan dye upon reduction of nitro blue tetrazolium. Briefly, 1ml of plant extract or fractions of different concentrations (20-200 µg/ml) was mixed with 156 µM NADH (1ml), 60µM NBT (1ml) and 468µM phenazine methosulphate (1ml) in phosphate buffer (pH = 8.3). The reaction was initiated with the addition of PMS. The reaction mixture was incubated at 25°C for 10 minutes. The absorbance of colored complex was measured at 560nm and the inhibition percentage was calculated using the formula-

$$\text{Percentage inhibition} = (1- A_S/A_C) \times 100$$

Where, $A_C$ = Absorbance of Control, $A_S$ = Absorbance of Sample solution

### 3.6.4 Cupric ion reducing antioxidant assay (CUPRAC assay)

The cupric ion reducing potential of different fractions were determined spectrophotometrically (Apak *et al.*, 2004). To the mixture of 1 ml (10 mM) copper (II) chloride, 1 ml (7.5 mM) neocuproine and 1 ml (1.0 M, pH 7) ammonium acetate buffer solution, added 100 µl of extract solution with 1 ml of distilled water as dilution factor to different concentrations of extract/fractions. The reaction mixture was allowed to stand for 30 minutes at room temperature and absorbance was measured at 450 nm. An increase in absorbance indicates the increased reduction ability. Percentage reduction was calculated using the following equation:

$$\text{Percentage reduction} = [1 - (1 - A_S/A_C)] \times 100$$

Where, $A_C$ = absorbance of standard at maximum concentration tested and $A_S$ = absorbance of sample solution

## 3.7 Isolation and characterization of secondary metabolites

Isolation of molecules was performed with the help of column chromatography whereas characterizations were done by NMR, and mass spectroscopy.

### 3.7.1 Isolation

Isolation of molecules was performed with the help of column chromatography whereas characterization was done by NMR and mass spectroscopy. Ethyl acetate extract (15.0 g) was subjected to column chromatography (CC) over silica gel (60-120 mesh, 14.0 g) and initially eluted with pure petroleum ether to give a total of 24 fractions. TLC of each fraction was checked using $60F_{254}$ TLC plates but there was no spot. Again a gradient elution of Pet. Ether: EtOAc, with an increasing proportion of EtOAc (5, 10, and 20%) to give a total of 50 fractions. Fractions 48-50 (10 ml each) were combined together on the basis of a single spot on precoated silica gel 60F254 TLC plates. The combined fractions were dried on a rotavapor yielding **VVR-I** (93 mg) as a yellow semi-solid. Further column elution was done with 25% EtOAc to give a total of 50 fractions. Fractions 17-28 (10 ml each) were combined and dried on a rotavapor yielding 657 mg of a reddish brown solid mixture, which was further chromatographed over silica gel (60-120 mesh, 1 g) and eluted with 25% EtOAc in Pet. ether. Subfractions 13-15 (10 ml each) were combined on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on a rotavapor under reduced pressure yielding **VVR-II** (12 mg) as a yellowish powder. Further, CC was eluted with an increasing proportion of EtOAc (25-50%) to give a total of 72 fractions. Fractions 32-50 were combined and dried on rotavapor under reduced pressure yielding 140 mg of sea green solid mixture, which was further re-chromatographed over silica gel (60-120 mesh, 1g) and eluted with 20% EtOAc in Pet. Ether. Subfractions 2-8 were combined on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on rotavapor under reduced pressure yielding **VVR-III** (25 mg) as a yellow amorphous powder. Further, the column was eluted with 25% EtOAc in Pet. Ether to give a total of 24 fractions. Fractions 2-4 were combined on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on rotavapor under reduced pressure yielding **VVR-IV** (13 mg) as a green powder. Further, the column was eluted with 30% EtOAc in Pet. Ether to give a total of 20 fractions. Subfractions 1-5 were combined as they had the same mixture of compounds. The combined fractions were dried over rotavapor yielding 40 mg of yellowish solid mixture which was again subjected to CC on silica gel (60-120 mesh, 35 mg) using gradient elution of 5%, 10%, 15%, 20%, 25% and 30% EtOAc in Pet. ether to give

a total of 75 fractions. Subfractions 30-32 were combined based on a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on rotavapor under reduced pressure yielding **VVR-V** (3.7 mg) as a white powder. Rest subfractions (1-29 and 33-75) were combined and dried on rotavapor yielding 30 mg yellowish solid mixture which was further re-chromatographed on silica gel (60-120 mesh, 25 mg) using gradient elution of pure Pet. Ether, 10%, 15% and 20% EtOAc in Pet. ether to give a total of 50 fractions. Subfractions, 1-5 (each 10 ml) obtained from elution of pure Pet. ether were combined on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on rotavapor under reduced pressure yielding **VVR-VI (**3.5 mg) as yellowish powder. Whereas, subfractions 25-30 (obtained from 15% EtOAc in Pet. ether) were combined on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. The combined fractions were dried on rotavapor yielding **VVR-VII** (6 mg) as a yellowish amorphous powder. All isolated compounds have been represented in Table 4.

**Table 4.** Isolated compounds from ethyl acetate extract of roots of *P. atrosanguinnea*

| Sr. No. | Compounds | Amount (mg) | Rf value | Fraction (%) | Characterization |
|---|---|---|---|---|---|
| 1 | VVR-I | 93 | 0.54 | 20% E.A. in P.E. | Yes |
| 2 | VVR-II | 12 | 0.71 | 25% E.A. in P.E. | No (Less amount) |
| 3 | VVR-III | 40 | 0.66 | 20% E.A. in P.E. | Yes |
| 4 | VVR-IV | 13 | 0.44 | 25% E.A. in P.E. | No (Less amount) |
| 5 | VVR-V | 3.7 | 0.77 | 25% E.A. in P.E. | No (Less amount) |
| 6 | VVR-VI | 6 | 0.52 | 15% E.A. in P.E. | No (Less amount) |
| 7 | VVR-VII | 3.5 | 0.83 | Pure P. E. | No (Less amount) |

E.A. = Ethyl acetate, P.E. = Petroleum ether

### 3.7.2 Characterization of VVR-I ($C_{46}H_{78}O_2$)

UV: λ max 240 nm, IR: 1744, 1466 and 804 $cm^{-1}$

$^1H$ NMR (400 MHz, CDCl3): 0.89- 0.92 (m, 3H), 1.21 (m, 2H), 1.22-1.29 (m, 2H), 1.29-2.0 (m, 2 x 26H), 2.21 (t, 2H), 3.59 (s, 3H), 5.25- 5.31 (m, 2 x 7H).

$^{13}C$ NMR ($CDCl_3$): 13.11 (C-45), 21.56 (C-24), 21.68 (C-19), 23.91 (C-21, 23, 25, 27), 24.49 (C-8), 24.59 (C-3, 29), 26.17 (C-20, 22, 26), 28.07 (C-4, 44), 28.09 (C-

18), 28.14 (C-17), 28.24 (C-16), 28.31 (C-15), 28.33 (C-14), 28.43 (C-12), 28.51 (C-10), 28.56 (C-11), 28.67 (C-8), 28.74 (C-7, 9, 13), 30.50 (C-6), 30.91 (C-5), 33.07 (C-2), 50.43 (C-1'), 126.07 (C-32), 126.68 (C-34), 126.88 (C-36), 127.02 (C-38), 127.21 (C-40), 127.23 (C-42), 128.72 (C-33), 128.96 (C-35), 129.01 (C-30), 129.18 (C-31, 37, 39), 129.23 (C-41), 130.92 (C-43), 173.36 (C-1).

ESI-MS: *m/z* $[M+H]^+$ calculated for ($C_{46}H_{78}O_2$ + H): 663.11; found: 663.70 corresponding to molecular formula $C_{46}H_{78}O_2$.

### 3.7.3 Characterization of VVR-III ($C_{19}H_{38}O_2$)

$^1$H NMR (400 MHz, CDCl3): 0.85 (m, 2 x 3H), 0.85 (m, 2H), 1.30 (m, 3 x 2H), 1.60 (m, 2H), 1.18 (m, 3 x 2H), 1.22 (m, 2 x 2H), 1.23 (m, 4 x 2H), 2.52-2.71 (m, 2H), 4.0 (t, 2H).

$^{13}$C NMR ($CDCl_3$): 14.11 (C-15, 4'), 22.68 (C-14), 25.87 (C-3, 3'), 26.97 (C-4), 28.54 (C-6), 29.23 (C-7), 29.34 (C-2, 5), 29.50 (C-8, 9), 29.56 (C-10, 11), 29.61 (C-12), 31.89 (C-13), 34.75 (C-2'), 64.94 (C-1), 171.94 (C-1').

ESI-MS: *m/z* $[M-H]^-$ calculated for $(C_{19}H_{38}O_2\text{-}H)^-$: 297.50; found: 297.10 corresponding to molecular formula $C_{19}H_{38}O_2$.

## 3.8 *In-silico* study of polyphenolic compounds

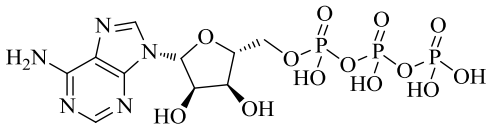
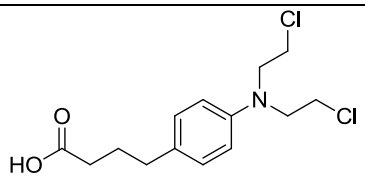
### 3.8.1 Ligand Preparation

All the 28 plant derived polyphenolic compounds (Figure 5) were sketched using ChemBio Draw Ultra 12.0 and saved in .sdf format using ChemBio3D ultra 12.0. Ligands were prepared for docking using LigPrep application of Schrödinger suite 2012 (Kawatkar *et al.*, 2009). LigPrep application of Schrödinger suite involved the addition of hydrogen atoms; removal of unwanted molecules such as water and small ions; neutralization of charge groups, then generate ionization and tautomeric states with Epik; generation of stereoisomers, particularly if the stereo chemical information is missing; generation of low-energy ring conformations; removal of any badly prepared structures and optimization of the geometries.

### 3.8.2 Protein Preparation

The x-rays crystal structure of human MRP-1 (PDB ID 2CBZ) in conjugation with ATP bound to the nucleotide binding domain 1 and human Glutathione-s-transferase p1 (PDB ID 3CSJ) in conjugation with chlorambucil were obtained from the protein data bank (http://www.rcsb.org/pdb) and represented in Table 5. Proteins were prepared for docking using 'protein preparation wizards' application of Schrödinger suite 2012. Protein preparation facility consists of two components, preparation and refinement. After ensuring chemical correctness, the preparation component neutralizes side chains that are not close to the binding cavity and do not participate in salt bridges. The refinement component performs a restrained impact minimization of the target which reorients the side chains and relieves steric clashes to perform docking studies.

**Table 5.** Target macromolecules, PDB codes, ligand names, and ligand structures used in the docking

| Target molecule | PDB ID | Name of Ligand | Structure of ligand |
|---|---|---|---|
| MRP-1 | 2CBZ | Adenosine-5'-Triphosphate |  |
| GSTP1-1 | 3CSJ | Chlorambucil |  |

### 3.8.3 Receptor Grid generation

Receptor grid generation for both proteins (2CBZ and 3CSJ) were done using the output file of the protein preparation wizard. Ligand docking jobs cannot be performed until the receptor grids have been generated. It defines the receptor structure by excluding any co-crystallized ligand that may be present, determine the position and size of the active site. If the co-crystallized ligand is not present in the protein, the site map program is run and highest site score along with site

protein is taken into consideration for a receptor grid generation (Kawatkar, *et al.*, 2009; Schulz-Gasch and Stahl, 2003).

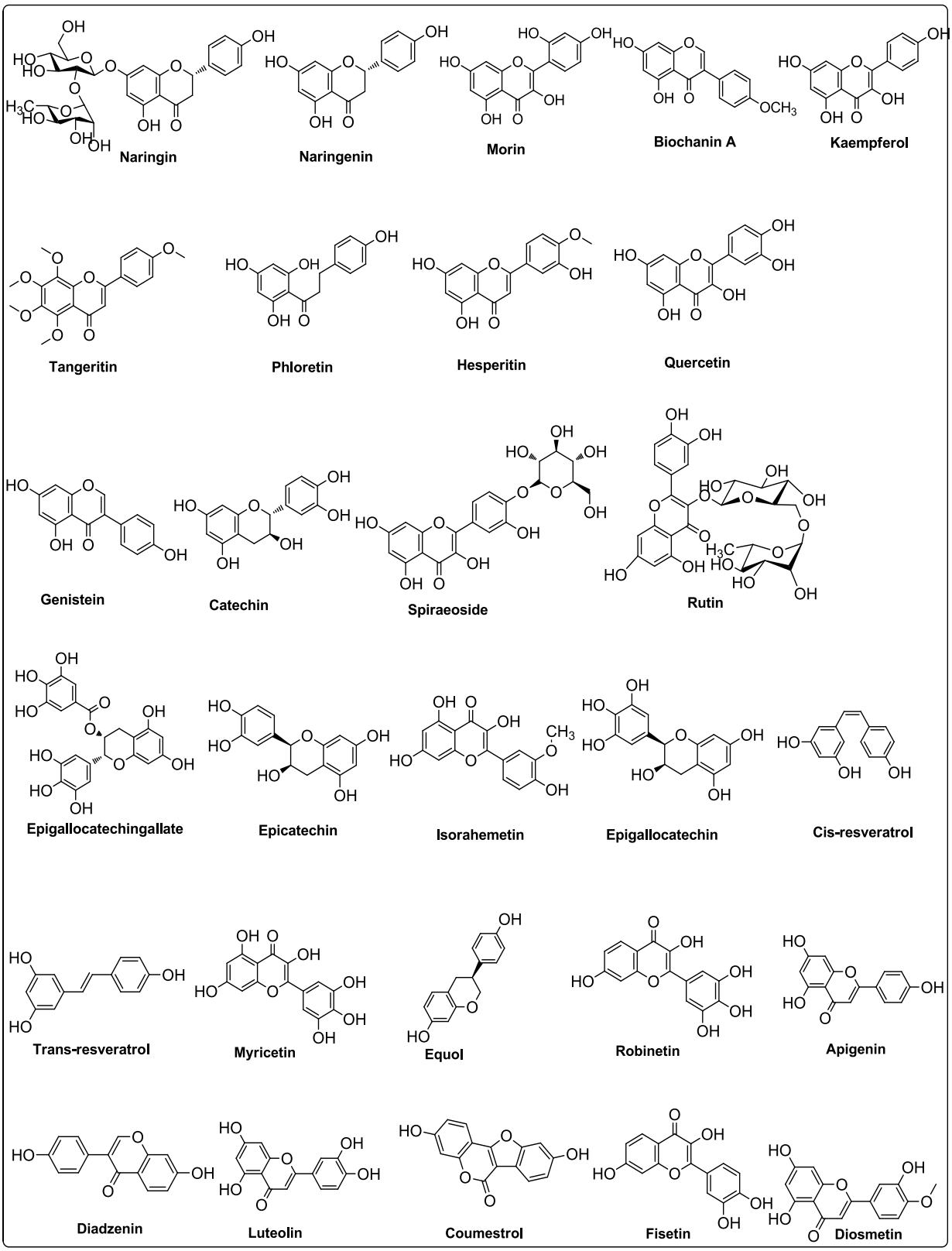



**Figure 5.** Structures of *in-silico* studied natural polyphenolic compounds

### 3.8.4 Glide Docking

Docking was performed using glide docking application (Grid Based Ligand Docking from Energetics, from Schrödinger, L.L.C.) of Schrödinger suite 2012. Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. Glide provides better favourable interactions between one or more ligand molecules and a receptor molecule, usually a protein. High throughput virtual screening (HTVS), standard precision (SP), extra precision (XP) is the three different docking precisions available in the glide HTVS is used for initial screen of millions of compounds (limited conformational search but fast); SP for thousands of compounds (better coverage of conformational space) and XP for tens or hundreds of compounds (high accuracy on docked poses). All the prepared compound and protein were docked using XP docking precision (Friesner *et al*., 2006)

### 3.8.5 QikProp

QikProp designed is an application of Schrödinger suite 2012; it is used for quick, accurate, easy-to-use absorption, distribution, metabolism and excretion (ADME) prediction of chemical compounds. It predicts physically significant descriptions and pharmaceutically relevant properties of organic molecules.

# CHAPTER 4

# RESULTS AND DISCUSSION

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Photoprotective activity

*In-vitro* SPF was determined by the spectrophotometric method developed by Mansur using the UVB region (Mansur *et al.*, 1986). *In-vitro* SPF is useful for screening test during pre-formulation of sunscreen product, as a supplement of *in-vivo* SPF measure. In the present study different extracts of *P. atrosanguinea* (*Pa*) e.g. *Pa*-$H_2O$-MeOH, *Pa*-AcOEt, *Pa*-BuOH and *Pa*-$H_2O$ were evaluated for their photo protective activity by UV spectrophotometry. SPF values were determined for each extract are depicted in Table 6. The SPF ranged from 1.35 to 3.49, 2.67 to 7.31, 1.28 to 3.74 and 1.09 to 2.73 for aqueous methanol, ethyl acetate, *n*-butanol and aqueous extracts respectively. Measurements were taken thrice and values were represented as mean± S.D. It was observed that the extracts *Pa*-AcOEt showed higher SPF (7.319 ± 0.353) at 120 µg/ml which was very appreciative in the terms of concentration of other reported SPF values of extracts. SPF of *Aloe-vera* has been reported in the range of 1.29 to 3.49 and 1.37 to 9.97 at concentration 4000 µg/ml of crude lyophilized and methanolic extracts respectively (Kumar *et al.*, 2009). Photoprotective activity (SPF value) of *P. atrosanguinea* extract is more as compared to *Boerhavia diffusa* (3.539 ± 0.021 to 7.174 ± 0.003) (Ashawat *et al*., 2006) and of *Aloe vera* gel (0.995 ± 0.221) (Ashawat *et al*., 2008). However, SPF value of *Neoglaziovia variegate* extract (27. 68 ± 4.03) (De Oliveira *et al.*, 2013), alcoholic extract of *Camellia sinensis* (18.10 ± 0.05) (C. D. Kaur and Saraf, 2011), leaves extract of *Dracocephalum moldavica* L. (24.79) and flowering tops of *Viola tricolor* L. (25.69) (Khazaeli and Mehrabani, 2008) were higher than *P. atrosanguinea*. The ability of protection against UVB region followed the trend *Pa*-AcOEt > *Pa*-BuOH > *Pa*-$H_2O$-MeOH > *Pa*-$H_2O$. Calculated SPF value for *Pa*-*n*-BuOH, Pa-$H_2O$-MeOH and Pa-$H_2O$ extracts at 120 µg/ml were found to be 3.740 ± 0.223, 3.494 ± 0.362 and 2.725 ± 0.479 respectively. It indicates that *the Pa* - AcOEt extract has high amount of photo protective agents that need to be explored in future. Increments in photoprotective activity of all studied extracts of *P. atrosanguinea* were concentration dependent (Table 6).

**Table 6.** SPF determination of different extracts of *Potentilla atrosanguinea*

| **Aqueous methanolic extract** | | |
|---|---|---|
| **Concentration (µg/ml)** | **Σ EE(λ) x I(λ) x Absorbance** | **SPF * (between 290-320 nm)** |
| 40 | 0.135 ± 0.016 | 1.351 ± 0.168 |
| 60 | 0.186 ± 0.016 | 1.868 ± 0.164 |
| 80 | 0.239± 0.011 | 2.395 ± 0.111 |
| 100 | 0.302± 0.018 | 3.028 ± 0.186 |
| 120 | 0.349 ± 0.036 | 3.494 ± 0.362 |
| **Ethyl acetate extract** | | |
| **Concentration (µg/ml)** | **Σ EE(λ) x I(λ) x Absorbance** | **SPF (between 290-320 nm)** |
| 40 | 0.267± 0.009 | 2.672± 0.092 |
| 60 | 0.407 ± 0.022 | 4.075 ± 0.224 |
| 80 | 0.439 ± 0.034 | 4.393 ± 0.345 |
| 100 | 0.677 ± 0.035 | 6.775 ± 0.350 |
| 120 | 0.731 ± 0.035 | 7.319 ± 0.353 |
| ***n*-Butanol extract** | | |
| **Concentration (µg/ml)** | **Σ EE(λ) x I(λ) x Absorbance** | **SPF (between 290-320 nm)** |
| 40 | 0.127 ± 0.005 | 1.275 ± 0.055 |
| 60 | 0.204 ± 0.011 | 2.049 ± 0.113 |
| 80 | 0.263 ± 0.016 | 2.635 ± 0.161 |
| 100 | 0.299 ± 0.019 | 2.996 ± 0.191 |
| 120 | 0.374 ± 0.022 | 3.740 ± 0.223 |
| **Aqueous extract** | | |
| **Concentration (µg/ml)** | **Σ EE(λ) x I(λ) x Absorbance** | **SPF (between 290-320 nm)** |
| 40 | 0.108 ± 0.014 | 1.086 ± 0.145 |
| 60 | 0.155 ± 0.031 | 1.552 ± 0.319 |
| 80 | 0.198 ± 0.038 | 1.981 ± 0.389 |
| 100 | 0.258 ± 0.048 | 2.585 ± 0.481 |
| 120 | 0.272 ± 0.047 | 2.725 ± 0.479 |

*SPF= CF x Σ EE (λ) x I (λ) x Absorbance, where CF (correction factor) = 10

For commercialization purpose minimum concentration required to produce photoprotective activity play an important role in the maintenance of Pharmacoeconomics especially in developing countries. *Pa*-BuOH extract also showed respectable SPF at all studied concentration and could be utilized as an ingredient of the photoprotective topical formulation. However, *Pa*- $H_2O$ extract have lowest SPF value among all studied extract.

### 4.2 Antioxidant activity

#### 4.2.1 Determination of total phenol content

The study of literature revealed that *Potentilla* species consists of polyphenolics (Flavonoids), triterpenoids, hydrolysable tannins, coumarins, organic acids, phenolic carboxylic acids, sterols, megastigmanes and essential oils (Michał Tomczyk and Latté, 2009a). In literature, there is only one paper which state that aerial part of *P. atrosanguinea* consist of antioxidant activity (Kalia *et al.*, 2008). In this regards, total phenol content and antioxidant activity of aqueous methanolic extracts and fractions of *P. atrosanguinea* were determined. It has been reported in literature that antioxidant activities of polyphenolics mainly depend on the arrangement of functional group around basic skeleton. The main structural feature which influences the antioxidant activities of polyphonic are the number and configuration of H-donating hydroxyl groups (Cao *et al.*, 1997; Sekher Pannala *et al.*, 2001). The total phenolic content of *P. atrosanguinea* was determined by using the protocol given by Yu and co-workers (Yu *et al.*, 2002). In this protocol, Folin–Ciocalteu reagent is used which produces blue colour, due to the formation of phosphomolybdenum tungstate anions, which indicates phenol content in extracts and fractions. It has been reported in literature that the number of hydrogen donating groups in the phenolic compounds was directly proportional to intensity of blue colour that indicated higher total phenol content (Huang *et al.*, 2005; Kaur *et al.*, 2008). The amount of total phenol was calculated as mg GAE/g dry weight of extract from calibration curve of gallic acid (Table 7). The aqueous methanolic extract exhibited higher phenol content of 429.8 mg GAE/g followed by an ethyl acetate fraction (408.33 mg GAE/g) > n-butanol fraction (319.87 mg GAE/g) > aqueous fraction (105.12 mg GAE/g). Total phenol content of methanolic extract of aerial part of *P. atrosanguinea* was reported to be 16.86 ±

0.02 which is very less compared to its root extract (Kalia, *et al.*, 2008). Apart from this, TPC of 80% methanolic root extracts of *P. atrosanguinea* was found to be greater than the TPC of *Potentilla fulgens* (30.22 ± 0.09) (Jaitak*, et al.*, 2010), Iranian *Ocimum* accessions (in between 22.9 to 65.5 mg GAE/g) (Javanmardi *et al.*, 2003) and of methanolic extracts of *Anacardium occidentale* (307.33 ± 0.11 mgGAEg) (Razali *et al.*, 2008). Earlier reports on medicinal plants suggested that total phenol content is directly proportional to the antioxidant activity (Da Silva *et al.*, 2006; Maisuthisakul *et al.*, 2007).

**Table 7.** Total phenol content of different fractions of *P. atrosanguinea*

| **Concentration (µg/ml)** | **mg GAE/g dried weight of extract** |
|---|---|
| Ethyl acetate fraction (120) | 408.333 |
| Aqueous methanolic extract (120) | 429.808 |
| n-Butanol fraction (120) | 319.871 |
| Aqueous fraction(120) | 105.12 |

### 4.2.2 DPPH radical scavenging activity

DPPH radical scavenging activities of different fractions of 80% methanol extract of roots of *P. atrosanguinea* are shown in Figure 6. DPPH radical is stable radical which on reduction by antioxidants produces colour change violet to yellow (Alinezhad *et al*., 2012). The aqueous methanolic crude extract showed highest DPPH radical scavenging activity of 90.04 % at 200 µg/ml concentrations whereas different fractions followed the trend ethyl acetate (88.10%) > *n*-butanol (82.37%) > aqueous (42.94%) respectively at the same concentration. Rutin was taken as reference compound and $IC_{50}$ values for all fractions were calculated. $IC_{50}$ values of aqueous methanolic and ethyl acetate fraction for DPPH assay was comparable as that of rutin (80µg/ml). However, $IC_{50}$ value for *n*-butanol fraction was 120µg/ml. The ethyl acetate of *Anacardium occidentale* were able to inhibit the formation of DPPH radicals with a percentage inhibition of 46.1 ± 0.2 at 400 µg/ml (Razali *et al.*, 2008). But in the present study ethyl acetate extract of *P. atrosanguinea*

exhibited 88.10% DPPH radical inhibition at 200 µg/ml, which clearly indicates that antioxidant activity of *P. atrosanguinea* is better in terms of concentration and percentage inhibition as well. DPPH scavengers have various biological activities as lipid peroxidation inhibitory action, radioprotective activity (Alinezhad *et al*., 2012). The good antioxidant activity of aqueous methanolic extracts may probably have electron donating compounds such as phenols.

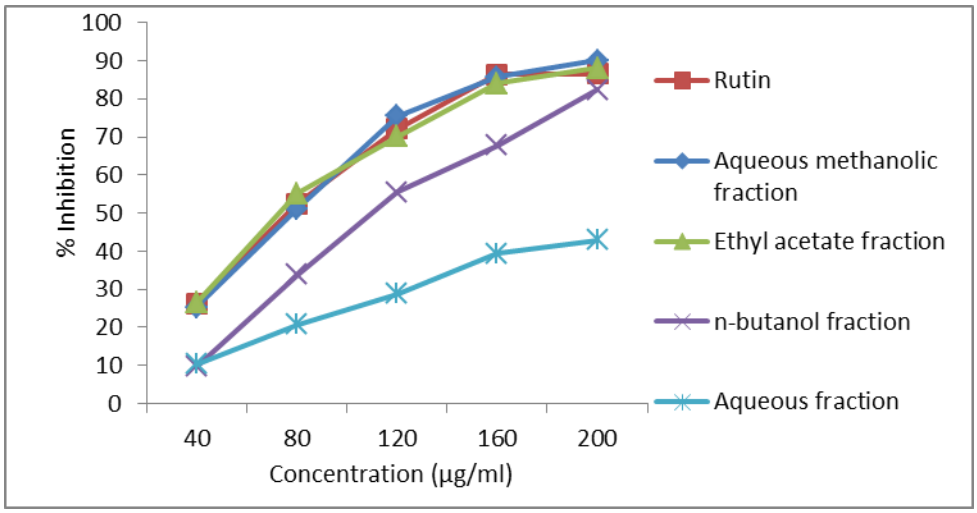



**Figure 6.** Graph depicting % inhibition of DPPH by *P. atrosanguinea*

### 4.2.3 Superoxide anion scavenging activity

Superoxide anion radical is very reactive and dangerous, mainly generated in mitochondria and cardiovascular system (half-life is $10^{-6}$ Sec) and capable of damaging almost each molecule found in living cells (Devasagayam, *et al.*, 2004; H. Li *et al.*, 2009). Superoxide anion radical scavenging activities of different fractions of root extracts of *P. atrosanguinea* were very good as compared to standard compound rutin (63.77%) (Figure 7). The crude aqueous methanolic extract showed highest superoxide anion radical scavenging activity of 78.86% at 200µg/ml whereas ethyl acetate fraction showed higher activity of 77.20% at 160µg/ml followed by *n*-butanol (68.05%) and aqueous fraction (59.24%) at the same concentration. Calculated $IC_{50}$ values of all fractions were found to be more excellent than standard rutin ($IC_{50}$ 150 µg/ml). Aqueous-methanolic, ethyl acetate, *n*-butanol and aqueous fractions were found to have $IC_{50}$ values of 60, 70, 90 and 140 µg/ml respectively. In a previous report on the superoxide anion scavenging

activity of selected red and white wine were found to be in the range of 35- 77% which is less that of *P. atrosanguinea* extracts ( Li *et al.*, 2009).

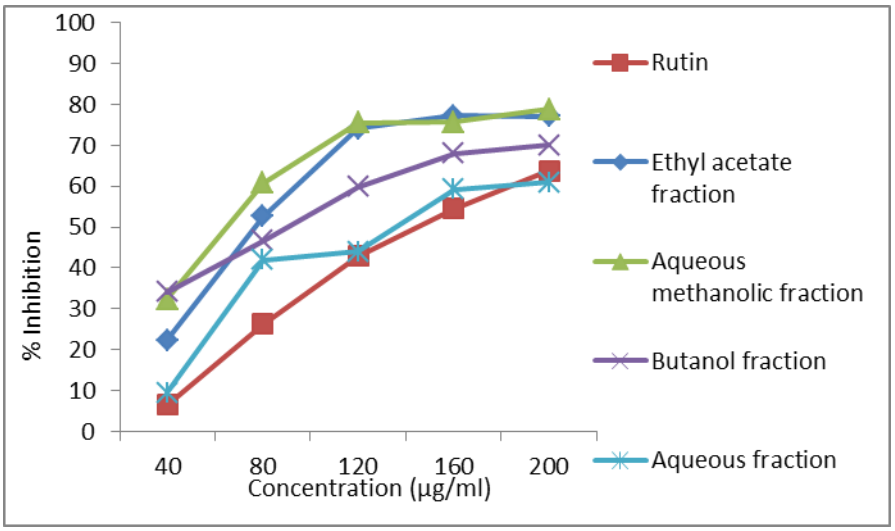



**Figure 7**. Graph depicting % inhibition of superoxide anion scavenging assay by *P. atrosanguinea*

### 4.2.4 CUPRAC assay

Electron donating capacity of any antioxidant was determined by reducing power assay that can reduce oxidized intermediates in lipid peroxidation (Li *et al*., 2009). Previously, Ferric ion reducing antioxidant power (FRAP) assay was used for the determination of reducing the power of antioxidants. But FRAP has two major drawbacks: (1) FRAP assay is conducted at acidic pH (3.6) to maintain iron solubility; (2) FRAP assay does not measure thiol antioxidants, such as glutathione (Li *et al.*, 2009; Prior *et al.*, 2005). Thus it may not meet physiological standards and variation in reducing power might be possible in the living cells. Thus we used CUPRAC assay for the assessment of reducing power of *P. atrosanguinea* extracts. CUPRAC assay is based on reduction of Cu (II) to Cu (I) by antioxidants. The cupric ion reducing potential of different fractions and crude extract was determined and it is represented in Figure 8. The aqueous methanolic extract showed highest reducing potential of 106.9% at 200µg/ml followed by *n*-butanol (79.30%), ethyl acetate (79.03) and aqueous fraction (28.50 %) at the same concentration. As per our knowledge there is not even a single report on CUPRAC assay of any *Potentilla* species in the literature. This is the first report of

CUPRAC assay on any *Potentilla* species. However, previously reported CUPRAC assay of methanolic extracts of *Centaurea polypodiifolia*, *C. pyrrhoblephara* and *C. antalyense* showed absorbance of 0.31 ± 0.03, 0.27 ± 0.05 and 0.32 ± 0.08 at 200 µg/ml (Aktumsek *et al.*, 2013). While aqueous methanolic extracts of *P. atrosanguinea* showed absorbance of 0.398 at 200 µg/ml that is much higher than the previous one.

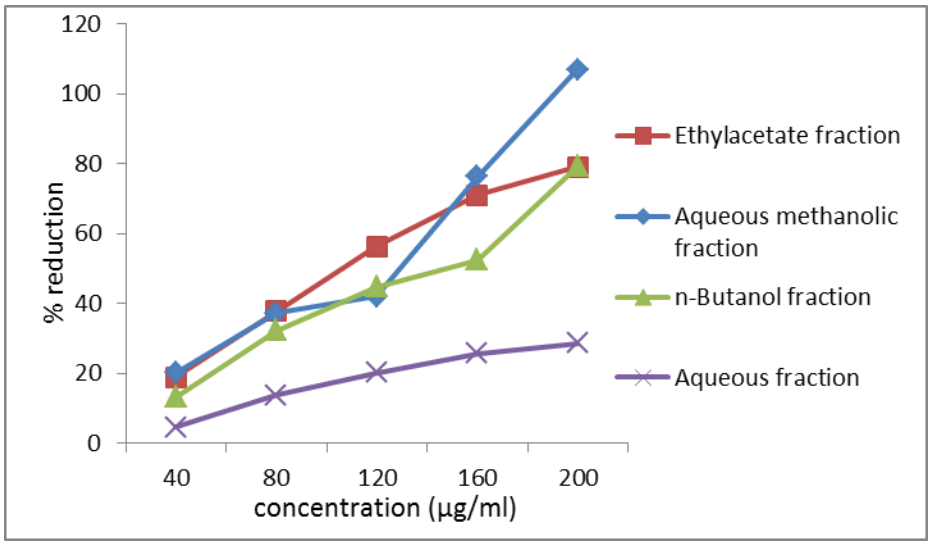



**Figure 8.** Graph depicting the cupric ion reducing potential of *P. atrosanguinea.*

### 4.3 Isolation and characterization of secondary metabolites

#### 4.3.1 Characterization of VVR-I ($C_{46}H_{78}O_2$)

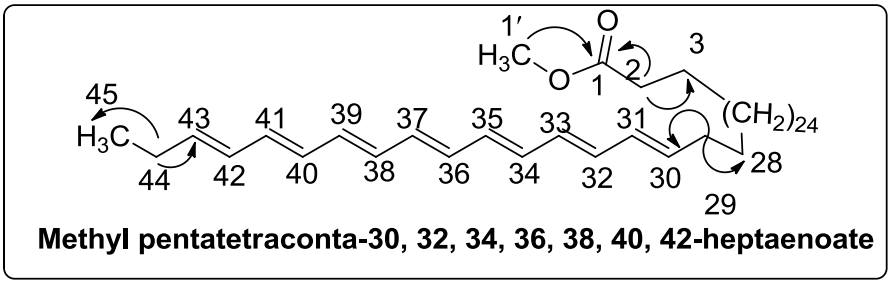



**Figure 9.** VVR-I

Compound **VVR-I**, an amorphous yellowish semi-solid, produced a single black spot on a precoated silica gel $60F_{254}$ TLC plate. Its *m/z* ratio indicated an ion peak at *m/z* 663.70 $[M+H]^+$ (calculated for $[C_{46}H_{78}O_2 + H]$, 663.11) corresponding to molecular formula $C_{46}H_{78}O_2$, with eight degrees of unsaturation. IR band at 1744 $cm^{-1}$ (C=O stretching) indicated presence of carbonyl group in the molecule (See

Appendix A). IR bands 1466 (C=C stretching) and 804 $cm^{-1}$ (C-H bending) indicated the presence of a conjugated double bond in the molecule. Apart from this UV spectrum showed maximum absorption at 240 nm along with 273 and 357 nm (See Appendix A). The $^{13}C$ NMR spectrum, including distortionless enhancement by polarisation transfer (DEPT) spectra clearly indicated the presence of 2-$CH_3$, 29-$CH_2$ and 14-CH. One signal was assigned to quaternary carbon. Furthermore, a proton at $\delta_H$ 3.59 (s, 3H) of carbon $\delta_C$ 50.43 (C-1') showed correlation with $\delta_C$ 173.36 (quaternary carbon, C-1) which clearly indicated the presence of methyl group ($\delta_C$ 50.43 (C-1') at terminal position. Proton $\delta_H$ 2.21 (t, 2H) of carbon δ 33.07 (C-2) showed correlation with $\delta_C$ 24.59 (C-3) and $\delta_C$ 173.36 (C-1') which confirmed the presence of quaternary carbon adjacent to carbon $\delta_C$ 33.07 (C-2). Proton $\delta_H$ 1.21(m, 2H) of carbon $\delta_C$ 28.07 (C-44) showed HMBC correlation with δ 13.11 (C-45) and 130.92 (C-43) which clearly indicated that carbon $\delta_C$ 28.07 (C-44) is attached with terminal methyl group $\delta_C$ 13.11(C-45) and alkene chain, $\delta_C$ 130.92 (C-43) on another side. Similarly, proton $\delta_H$ 1.22 (m, 2H) of carbon $\delta_C$ 24.59 (C-29) showed HMBC correlation with $\delta_C$ 129.01(C-30) and $\delta_C$ 24.49 (C-28) which indicated the presence of unsaturated aliphatic chain on one side and saturated aliphatic chain on another side respectively. IUPAC name of isolated compound **VVR-I** was found to be "methyl pentatetraconta-30, 32, 34, 36, 38, 40, 42-heptaenoate" with molecular formula $C_{46}H_{78}O_2$ and it has been reported for the first time from this plant as novel compound.

### 4.3.2 Characterization of VVR-III ($C_{19}H_{38}O_2$)

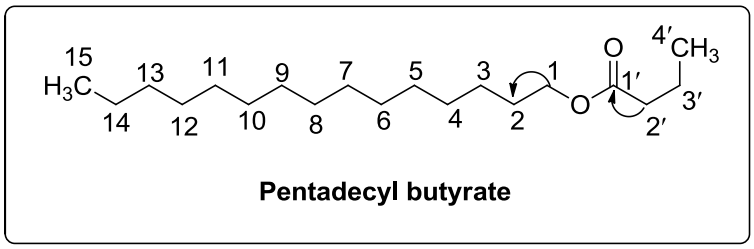



**Figure 10.** VVR-III

Compound **VVR-III**, an amorphous yellow solid, showed a single black spot on a precoated silica gel $60F_{254}$ TLC plate. Its positive *m/z* indicated an ion peak at *m/z* 297.1 $[M-H]^-$ (calculated for $[C_{19}H_{38}O_2 - H]^-$, 297.50) corresponding to molecular formula $C_{19}H_{38}O_2$, with one degree of unsaturation. The $^{13}C$ NMR spectrum,

including distortionless enhancement by polarisation transfer (DEPT) spectra clearly indicated the presence of 2-$CH_3$, 16-$CH_2$ and one quaternary carbon (See Appendix B). Proton $\delta_H$ 0.85 of carbon $\delta_C$ 14.12 (C-15) showed correlation with $\delta_C$ 22.68 (C-14). Proton $\delta_H$ 2.52 (m, 2H) of carbon $\delta_C$ 34.75 (C-2') showed HMBC correlation with $\delta_C$ 171.94 (C-1') clearly indicated the presence of quaternary carbon. Proton $\delta_H$ 4.0 (t, 2H) of carbon $\delta_C$ 64.94 (C-1) showed HMBC correlation with $\delta_C$ 29.34 (C-2) which indicated the presence of saturated aliphatic chain. IUPAC name of isolated compound **VVR-III** was found to be "Pentadecyl butyrate" with molecular formula $C_{19}H_{38}O_2$.

### 4.4 *In-silico* study of polyphenolic compounds

Glide docking of all 28 naturally originated polyphenolic compounds which are considered to be potential candidates for cancer chemotherapy were done to identify the lowest energy conformation and better binding mode of inhibitors against the protein structure. Each compound was docked in each of three different targets and lowest energy docked confirmation was taken into account. Docking energies of all 30 natural compounds with MRP-1, GSTP1-1 are represented in Tables 8 and 9 respectively.

#### 4.4.1 Docking with MRP-1 receptor

Rutin showed very good interaction with MRP-1 receptor and its Glide score (G score), lipophilicity, H-bond and electrostatic energies were -8.47, -1.53, -4.94 and -2.0 kcal/mol respectively. Interaction profile of rutin is represented in Figure 11 in which two hydroxyl groups are attached with ring-A were exposed to solvent in which one hydroxyl group formed hydrogen bond with Trp653 residue. Both hydroxyl groups attached with ring-B was formed hydrogen bonds with Glu694 residue. The sugar molecule attached with C-ring at position 3 showed hydrogen bond interaction with Gly681 residue. Next to rutin, naringin showed good binding pattern with MRP-1 receptor among selected natural compounds and its G score, dock score, lipophilicity, H-bond and electrostatic energies were -7.81, -7.81, -1.68, -4.39 and -1.73 kcal/mol respectively (Table 8). Ring-B of Naringin is exposed to solvent and sugar moiety attached at C-7 showed hydrogen bond interaction with Tyr710, Gln713 and Gln714 residues. *In-vitro* studies of naringin

also support its MRP-1 inhibition property (Leslie *et al.*, 2001). EGCG (epigallocatechingallate), EGC (Epigallocatechin) and spiraeocide also showed good binding pattern with MRP-1 and their G Score were -7.7, -6.8 and -6.41kcal/mol respectively. Tangeritin has very weak interaction with the MRP-1 having G score -2.58 kcal/mol, may be due to presence of methoxy group, as hydroxyl groups were participating in H-bonding.

**Table 8.** Glide score, Lipophilicity, Hydrogen bond and electrostatic energies of naturally derived product docked with MRP-1 (PDB ID 2CBZ).

| Ligand | G Score (Kcal/mol) | Lipophilic EvdW | $E_H$ (Kcal/mol) | $E_{Elect}$ (Kcal/mol) |
|---|---|---|---|---|
| **Rutin** | **-8.47** | **-1.53** | **-4.94** | **-2.0** |
| Naringin | -7.81 | -1.68 | -4.39 | -1.73 |
| EGCG | -7.7 | -1.2 | -4.74 | -1.76 |
| Epigallocatechin | -6.8 | -1.03 | -4.02 | -1.27 |
| Spiraeocide | -6.41 | -1.32 | -3.09 | -2 |
| Robinetin | -5.93 | -1.38 | -2.97 | -1.08 |
| Myricetin | -5.42 | -1.17 | -2.88 | -0.94 |
| Morin | -5.25 | -1.13 | -2.14 | -1.49 |
| Kaemferol | -5.05 | -1.04 | -2 | -1.51 |
| Quercetin | -5.01 | -1.15 | -2.22 | -1.14 |
| Isorhamnetin | -4.78 | -1.6 | -2.12 | -0.61 |
| Catechin | -4.29 | -1.44 | -1.65 | -0.7 |
| Apigenin | -4.13 | -1.09 | -1.4 | -1.14 |
| Diosmetin | -4.09 | -1.16 | -1.15 | -1.28 |
| Hesperitin | -4.09 | -1.16 | -1.15 | -1.28 |
| Luteolin | -4.08 | -1.16 | -1.16 | -1.27 |
| Naringenin | -4.07 | -1.14 | -1.18 | -1.25 |
| Genistein | -4.04 | -1.12 | -1.54 | -0.88 |
| Biochanin_A | -3.99 | -1.1 | -1.52 | -0.87 |
| Phloretin | -3.54 | -1.35 | -1.01 | -0.67 |
| Tangeritin | -2.58 | -1.21 | -0.7 | -0.41 |
| Epicatechin | -5.48 | -0.84 | -3.09 | -1.05 |
| Fisetin | -5.1 | -1.39 | -2.4 | -0.81 |
| Cis-resveratrol | -4.09 | -0.67 | -1.7 | -1.23 |
| Trans-resveratrol | -3.99 | -1.31 | -1.7 | -0.48 |
| Coumestrol | -3.27 | -1.39 | -0.88 | -0.5 |
| Equol | -3.26 | -1.15 | -1.02 | -0.59 |
| Daidzein | -3.17 | -0.95 | -0.92 | -0.8 |

Docking study also revealed that cis - resveratrol (G score - 4.09 kcal/mol) has strong affinity with MRP-1 than trans - resveratrol (G score-3.99 kcal/mol). Similarly epicatechin (G score-5.48 kcal/mol) has strong affinity with MRP-1, than catechin (G score -4.29 kcal/mol).

### 4.4.2 Docking with GSTP1-1 receptor

Docking results of all selected natural compounds with GSTP1-1 (PDB ID 3CSJ) revealed that rutin, spiraeocide, naringin, robinetin and myricetin are good GSTP1-1 inhibitors among all docked molecules as their G scores were -10.78, -9.96, -

9.43, -8.32 and -8.26 kcal/mol respectively (Table 9). Interaction profile of rutin with GSTP1-1 is represented in Figure 12. Ring-A of rutin has hydrophobic interaction with Phe8 residue; ring-B was exposed to the hydrophobic region in which C-3' showed H-bond interactions with water molecules.

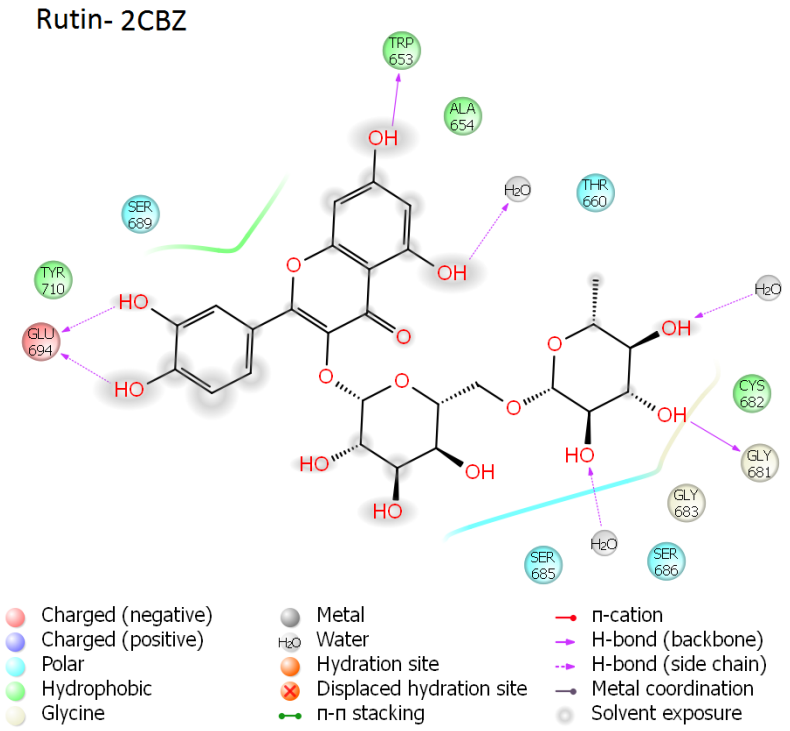



**Figure 11.** Diagram representing the interaction profile of docked rutin with MRP-1

Moreover, sugar residue attached at C-3 position in the ring-C formed hydrogen bonds with water molecules. It clearly showed that rutin has occupied the active site of GSTP1-1, resulting in good binding pattern. In other molecule spiraeocide, Ring-A is exposed to solvent; ring-B hydrophobically interacts with Phe8 residue and substituents at 4' position on ring B is participating in hydrogen bond formation with water molecules. The keto group at position 4 of ring-C interacts with Val35 amino acid that might be responsible of its activity. Interaction profile of naringin showed hydrogen bonding interaction with Tyr7, Thr109, Gly205 residues and ring-B is exposed to solvent. G score of quercetin is -7.36 and its *in-vitro* evaluation of inhibitory effect on human GSTP1-1 have already been reported (Van Zanden *et al*., 2003).

**Table 9.** Glide score, Lipophilicity, Hydrogen bond and electrostatic energies of naturally derived product docked with GSTP1-1 (PDB ID 3 CSJ)

| Ligand | G Score (Kcal/mol) | Lipophilic EvdW | $E_H$ (Kcal/mol) | $E_{Elec}$ (Kcal/mol) |
|---|---|---|---|---|
| **Rutin** | **-10.78** | **-4.54** | **-4.98** | **-1.11** |
| Spiraeocide | -9.96 | -3.62 | -4.78 | -1.13 |
| Naringin | -9.43 | -4.52 | -3.79 | -0.82 |
| Robinetin | -8.32 | -3.42 | -3.84 | -0.4 |
| Myricetin | -8.26 | -3.75 | -3.22 | -0.67 |
| EGCG | -7.99 | -3.59 | -3.74 | -1.15 |
| Morin | -7.45 | -3.78 | -2.42 | -0.45 |
| Quercetin | -7.36 | -4.16 | -2.19 | -0.3 |
| Epigallocatechin | -7.33 | -3.87 | -2.52 | -0.4 |
| Isorhamnetin | -7.17 | -4.39 | -2.14 | -0.27 |
| Phloretin | -7.04 | -3.21 | -2.22 | -1.15 |
| Hesperitin | -6.9 | -3.92 | -2.1 | -0.38 |
| Genistein | -6.84 | -3.84 | -1.84 | -0.46 |
| Epicatechin | -6.83 | -3.79 | -2.06 | -0.42 |
| Luteolin | -6.81 | -3.5 | -2.42 | -0.19 |
| Fisetin | -6.81 | -3.66 | -1.83 | -0.4 |
| Equol | -6.79 | -4.3 | -1.31 | -0.38 |
| Diosmetin | -6.68 | -3.85 | -1.94 | -0.17 |
| Coumestrol | -6.54 | -3.5 | -1.33 | -0.64 |
| Kaemferol | -6.47 | -3.15 | -2.32 | -0.51 |
| Biochanin A | -6.43 | -3.54 | -1.77 | -0.46 |
| Apigenin | -6.4 | -4.19 | -1.28 | -0.29 |
| Catechin | -6.16 | -2.99 | -2.23 | -0.54 |
| Trans-resveratrol | -5.74 | -3.26 | -1.7 | -0.54 |
| Naringenin | -5.7 | -4.01 | -0.7 | -0.12 |
| Daidzein | -5.61 | -4.05 | -0.54 | -0.26 |
| Tangeritin | -5.51 | -4.03 | -0.68 | -0.17 |
| Cis-resveratrol | -5.4 | -2.47 | -1.78 | -0.92 |

All other selected natural compounds showed a good G score with GSTP1-1 rather than MRP-1. Trans-resveratrol had good G score than cis-resveratrol for GSTP1-1 (Table 9) and vice-versa in case of MRP-1 inhibition (Table 8). It might be due to the structure compatibility with these receptors. G score of tangeritin for GSTP1-1 (-5.51 kcal/mol) is better than the MRP-1 (-2.58 kcal/mol) inhibition, as tangeritin had shown poorest interaction with MRP-1 among all docked natural molecules.

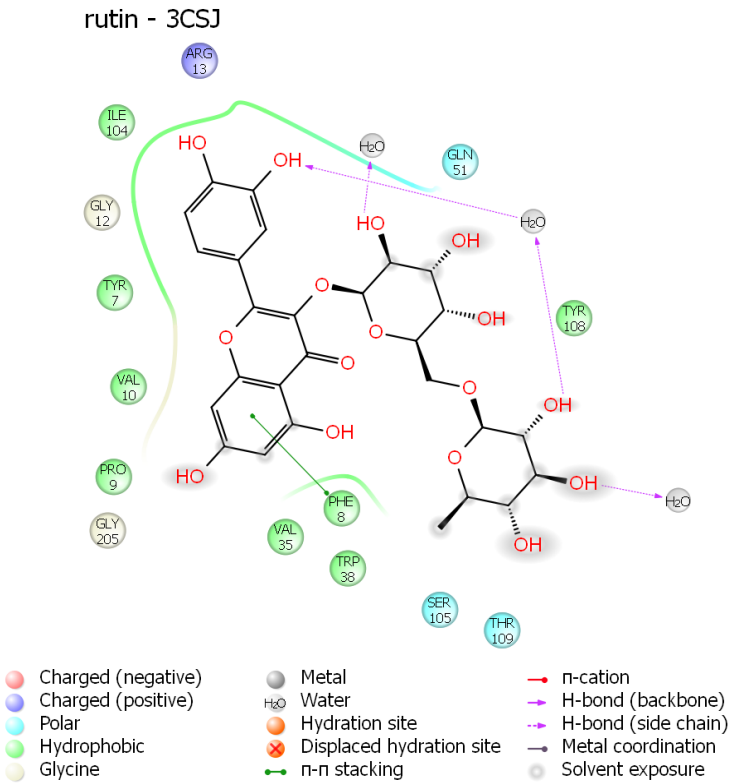



**Figure 12.** Diagram representing the interaction profile of docked rutin with GSTP1-1

### 4.4.3 *In-silico* ADME prediction

ADME properties of all studied natural compounds were predicted using qikprop application and represented in Table 10**.** Percentage oral bioavailability of Equol, Trans-resveratrol, Cis-resveratrol, Diadzein, and Biochanin A were found to be 96.074, 82.443, 82.426, 83.731, and 90.999 respectively. The QPP Caco value of EGCG, epigallocatechin, myricetin, robinetin, naringin, quercetin, rutin, spiraeocide and tangeritin were very poor as a result oral bioavailability of these compounds were also very poor because caco-2 cell is a model for the absorption from the gastrointestinal tract (Press and Di Grandi, 2008). In contrast to QPP Caco, poor Q Plog P o/w value of these compounds is also responsible for their poor bioavailability. The QPP MDCK value of sulforaphane was found to be highest among all studied compounds. MDCK cells are considered to be a good mimic for the blood brain-barrier (Q. Wang *et al.,* 2005). Moreover MDCK permeability assay is a valuable tool for membrane permeability screening (Irvine *et al*., 1999), identification and characterization of P-gp (Doan *et al*., 2002), MRP1 (Brayden and Griffin, 2008), ABCG2 (Xiao *et al*., 2006) substrates and inhibitors. The QPP

MDCK value of EGCG was lowest among all studied compound and it was found to be 0.288 mm/Sec. Besides EGCG; naringin, epigallocatechin, robinetin, quercetin, rutin and spiraeocide also showed the low MDCK permeable rate which can be acceptable as these compounds will not cross the blood-brain barrier and thus will not produce any CNS toxicity.

**Table 10.** Qikprop results of studied natural compounds

| Natural Molecule | Mol. Wt. (130- 725) | H- Bond Donor (0-6) | H-Bond Acceptor (2-20) | Q P log $P_{o/w}$ Range (-2.0- 6.5) | QPP Caco (nm/sec) <25-poor >500-great | Q P log BB range (-3 –1.2) | QPP MDCK (nm/sec) <25-poor >500-great | Q Plog Kp -8.0 - -0.1 | Q Plog $K_{hsa}$ -1.5 -1.5 | Percentage Human Oral Absorption >80 % High < 25% Poor |
|---|---|---|---|---|---|---|---|---|---|---|
| **EGCG** | **458.378** | **8** | **8.75** | **-0.243** | **1.018** | **-4.333** | **0.288** | **-7.514** | **-0.439** | **0** |
| Epicatechin | 290.272 | 5 | 5.45 | 0.494 | 55.271 | -1.845 | 21.634 | -4.688 | -0.405 | 61.023 |
| Epigallocatechin | 306.271 | 6 | 6.2 | -0.17 | 19.959 | -2.348 | 7.195 | -5.578 | -0.546 | 36.26 |
| **Equol** | **242.274** | **2** | **2.25** | **2.761** | **910.085** | **-0.505** | **446.809** | **-2.356** | **0.171** | **96.074** |
| Trans-resveratrol | 228.247 | 3 | 2.25 | 2.012 | 277.056 | -1.293 | 123.547 | -2.892 | -0.159 | 82.443 |
| Cis-resveratrol | 228.247 | 3 | 2.25 | 1.923 | 295.706 | -1.213 | 132.56 | -2.974 | -0.182 | 82.426 |
| Apigenin | 270.241 | 2 | 3.75 | 1.642 | 116.375 | -1.446 | 48.379 | -3.963 | -0.028 | 73.536 |
| Luteolin | 286.24 | 3 | 4.5 | 0.96 | 42.098 | -1.947 | 16.119 | -4.851 | -0.19 | 61.636 |
| Diosmetin | 300.267 | 2 | 4.5 | 1.79 | 118.565 | -1.56 | 49.364 | -4.035 | 0.01 | 74.547 |
| Fisetin | 286.24 | 4 | 5.5 | 0.5 | 49.048 | -1.881 | 19.014 | -4.701 | -0.37 | 60.133 |
| Isorhamnetin | 316.267 | 3 | 5.25 | 1.308 | 86.903 | -1.742 | 35.284 | -4.228 | -0.17 | 69.31 |
| Myricetin | 318.239 | 5 | 6 | -0.282 | 7.564 | -2.822 | 2.521 | -6.319 | -0.492 | 28.063 |
| Robinetin | 302.24 | 5 | 6.25 | -0.156 | 17.716 | -2.39 | 6.325 | -5.591 | -0.505 | 48.377 |
| Coumestrol | 268.225 | 2 | 4.5 | 1.319 | 234.633 | -1.045 | 103.232 | -3.452 | -0.2 | 77.093 |
| Daidzein | 254.242 | 2 | 4 | 1.774 | 391.184 | -0.903 | 179.375 | -2.821 | -0.141 | 83.731 |
| Biochanin A | 286.284 | 1 | 4 | 2.474 | 587.936 | -0.763 | 278.627 | -2.794 | 0.113 | 90.999 |
| Catechin | 290.272 | 5 | 5.45 | 0.475 | 53.278 | -1.91 | 20.792 | -4.695 | -0.419 | 60.627 |
| Genistein | 270.241 | 2 | 3.75 | 1.672 | 166.681 | -1.312 | 71.335 | -3.552 | -0.103 | 76.502 |
| Hesperitin | 300.267 | 2 | 4.5 | 1.789 | 126.052 | -1.518 | 52.741 | -3.986 | 0.003 | 75.015 |
| Kaemferol | 286.24 | 3 | 4.5 | 1.055 | 58.254 | -1.798 | 22.899 | -4.536 | -0.197 | 64.718 |
| Morin | 304.256 | 4 | 6.45 | 0.21 | 30.088 | -2.105 | 11.212 | -5.147 | -0.423 | 54.634 |
| Naringenin | 272.257 | 2 | 4 | 1.201 | 155.677 | -1.083 | 66.258 | -3.902 | -0.194 | 73.214 |
| **Naringin** | **580.541** | **7** | **19.3** | **-1.632** | **5.12** | **-4.136** | **1.653** | **-5.989** | **-1.281** | **0** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Phloretin | 274.273 | 2 | 3 | 2.124 | 95.314 | -1.867 | 38.989 | -3.797 | 0.001 | 74.804 |
| Quercetin | 302.24 | 4 | 5.25 | 0.518 | 21.041 | -2.413 | 7.617 | -5.356 | -0.317 | 53.661 |
| **Rutin** | **608.552** | **9** | **17.9** | **-1.359** | **4.229** | **-3.657** | **1.344** | **-6.439** | **-0.907** | **0** |
| **Spiareocide** | **464.382** | **7** | **13.75** | **-1.486** | **1.834** | **-4.06** | **0.545** | **-7.048** | **-0.897** | **0** |
| **Tangeritin** | **372.374** | **0** | **6.25** | **3.331** | **-5.041** | **-4.642** | **3891.17** | **-0.124** | **0.876** | **-0.075** |

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

# CHAPTER 5

# SUMMARY

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## SUMMARY

*In-vitro* assay guided fractionation of root extracts of *P. atrosanguinea* was successfully performed. Initially dried powdered root was extracted thrice with 80% methanol in water followed by fractionation with different solvents in the increasing order of their polarity. All fractions were dried on rotavapor separately followed by *in-vitro* Photoprotective and antioxidant activities of each fraction. Sunburn, photo aging, and skin cancer are consequences of long term exposure of skin to ultraviolet B (UVB) radiation. Accordingly the *in-vitro* photoprotection ability of different fractions was investigated by spectrophotometric method in the range of 290-320 nm. The ability of protection against UVB (290-320 nm) region followed the trend *Pa*-AcOEt > *Pa*- *n*-BuOH > *Pa*-$H_2O$-MeOH > *Pa*-$H_2O$. Ethyl acetate fraction of *P. atrosanguinea* indicated the highest sun protection factor (SPF) (7.319 ± 0.353) at a concentration of 120 µg/ml. It has been reported in literature that antioxidants can absorb harmful UV radiations and protect skin against photo induced DNA damage by reactive oxygen species, so there is need to determine its antioxidant potential too because that would be additional supportive to this SPF data. Keeping in the view SPF results, total phenol content and antioxidant activities root extracts of *P. atrosanguinea* were determined by three *in-vitro* assays namely DPPH, superoxide anion radical scavenging and CUPRAC assay. The aqueous methanolic extract showed higher total phenol content and antioxidant activity than its fractions. However, the total phenol content and antioxidant power of fractions followed the trend *Pa*-AcOEt > *Pa*- *n*-BuOH > *Pa*-$H_2O$-MeOH > *Pa*-$H_2O$. This clearly indicates that the antioxidant activity is directly proportional to total phenol content. *In-vitro* photoprotective and antioxidant activities for an ethyl acetate fraction were found to be excellent among all other fractions of *P. atrosanguinea*. So the ethyl acetate fraction was further selected for isolation of bioactive molecules using column chromatography. Total 7 molecules were isolated on the basis of a single spot on the precoated silica gel $60F_{254}$ TLC plates. However, only two molecules (**VVR-I** and **VVR-III**) were characterized with their molecular formula $C_{46}H_{78}O_2$ (methyl pentatetraconta-30, 32, 34, 36, 38, 40, 42-heptaenoate) and $C_{19}H_{38}O_2$ (Pentadecyl butyrate) respectively. Out of these molecules, **VVR-I** is novel compound. Apart from these isolated there might be a

lots of other compounds which can be explored in future. In this way, these results indicated the importance of *P. atrosanguinea* extracts as photoprotective agent in sunscreen preparation and may serve as natural source of antioxidants in pharmaceutical industry. Apart from this, *in-silico* study of some reported polyphenolic compounds suggested that rutin may act as dual modulator for MRP-1 and GSTP1-1 receptor, as these two receptors get overexpressed during multidrug resistance in cancer chemotherapy. Although, *in-silico* ADME studies of docked polyphenolic compounds indicated that compound having good affinity for target receptor are lacking bioavailability e.g. rutin has a percent oral absorption of 0% while it had highest binding affinity. So these *in-silico* studies support the need of structural modification which may led to better bioavailability without affecting its therapeutic value.

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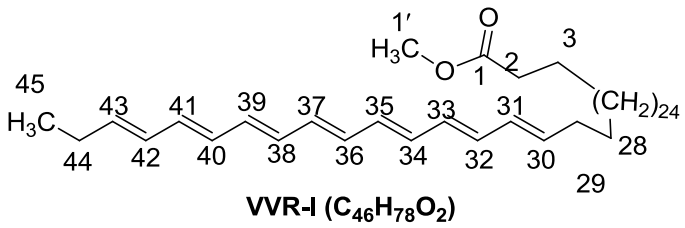
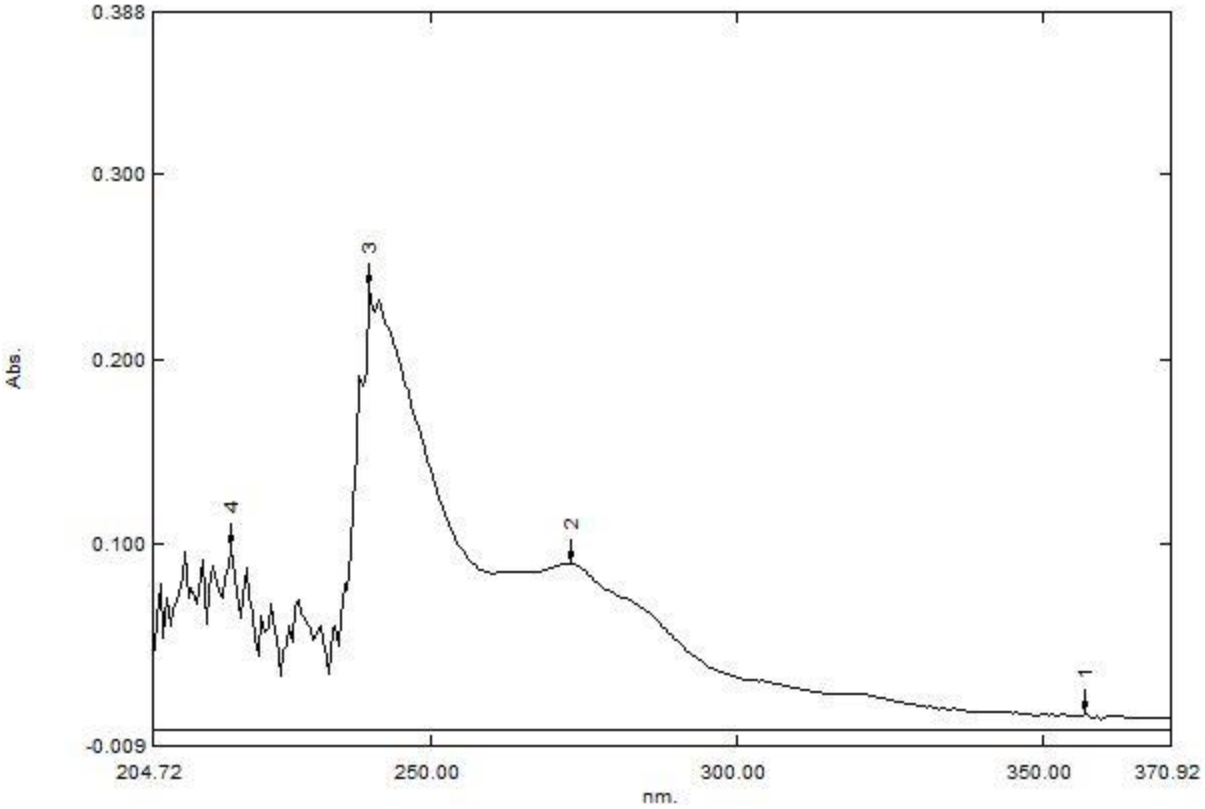
# APPENDIX A

# Spectral Data of VVR-I




**VVR-I ($C_{46}H_{78}O_2$)**

**Methyl pentatetraconta-30, 32, 34, 36, 38, 40, 42-heptaenoate**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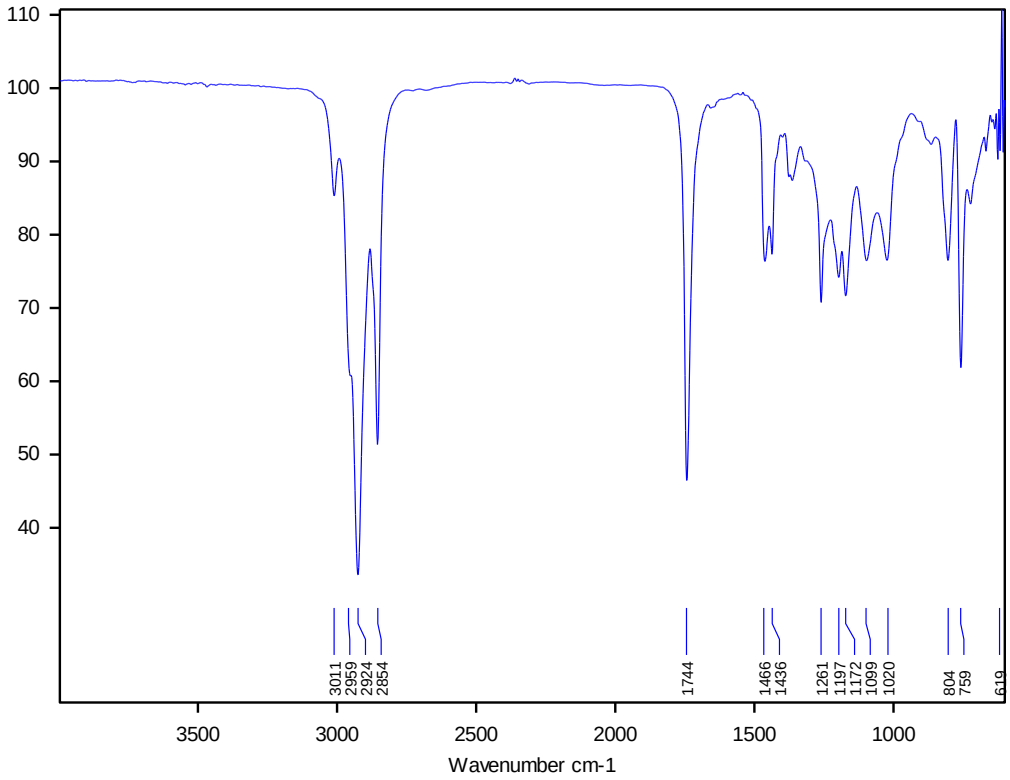
350.00
370.92
nm.
1
2
3
4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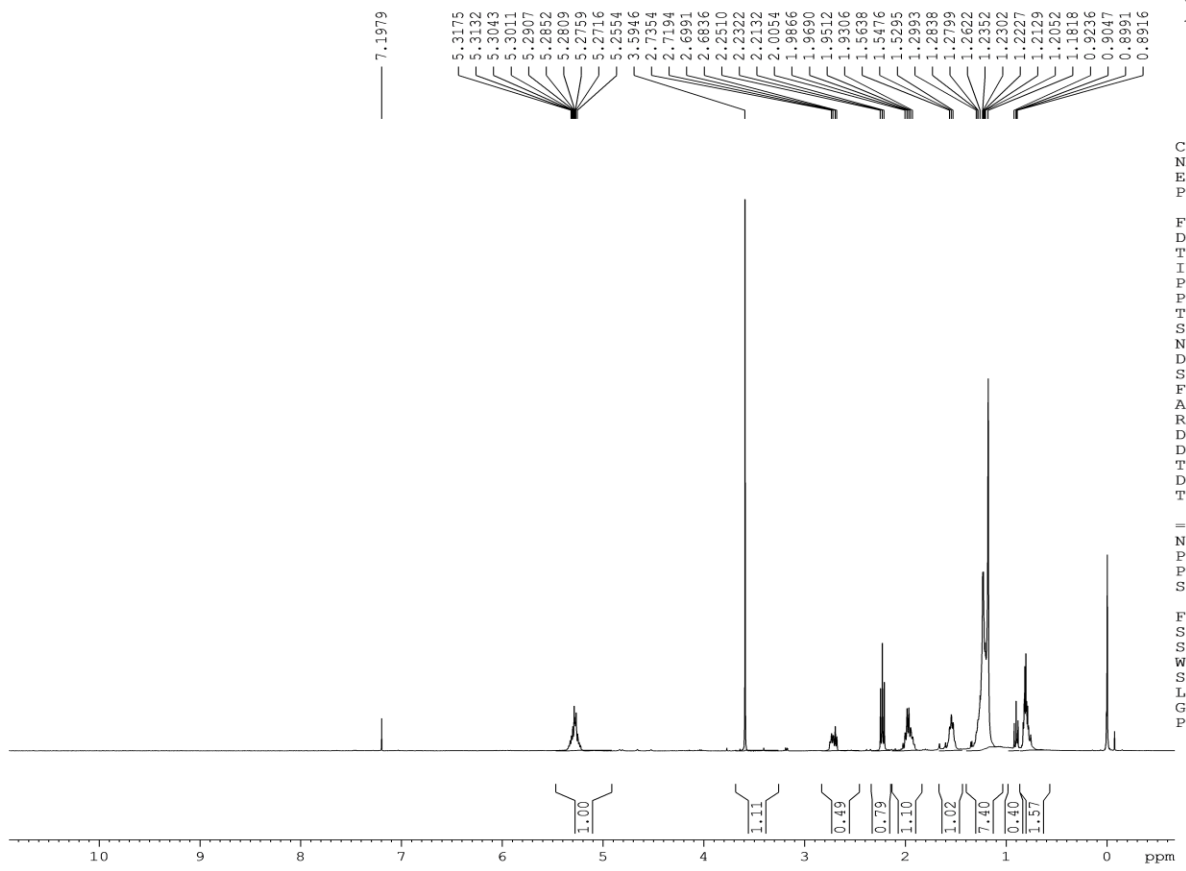
2500
2000
1500
1000
Wavenumber cm-1

VVR-I

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AVANCE II 400 NMR
Spectrometer
SAIF

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PROCNO 1

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DE 6.00 usec
TE 291.5 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 10.90 usec
PL1 -3.00 dB
SFO1 400.1324710 MHz

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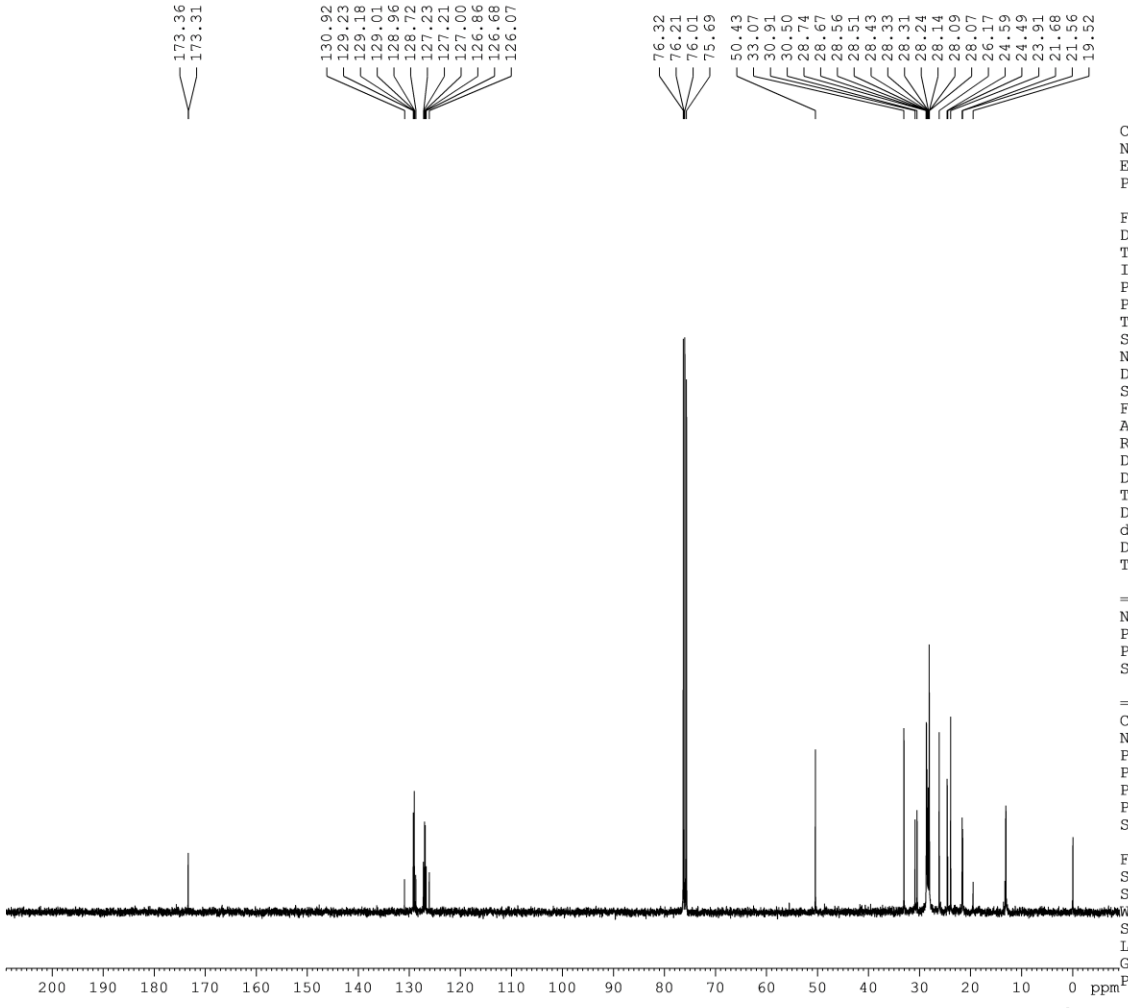
PC 1.00

VVR-I

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SAIF

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DE 6.00 usec
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d11 0.03000000 sec
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TD0 1

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P1 9.60 usec
PL1 -2.00 dB
SFO1 100.6228298 MHz

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PCPD2 80.00 usec
PL2 -3.00 dB
PL12 14.31 dB
PL13 18.00 dB
SFO2 400.1316005 MHz

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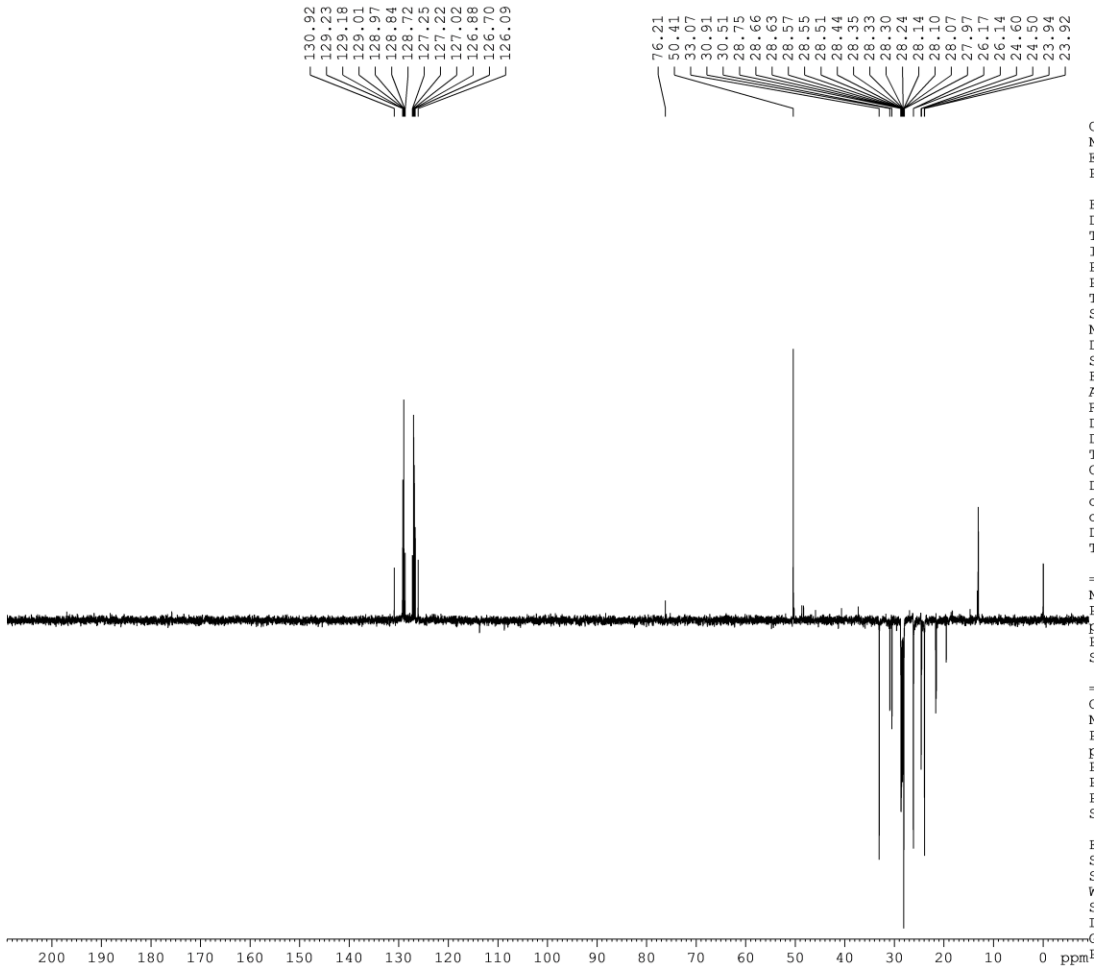
VVR-I




BRUKER
AVANCE II 400 NMR
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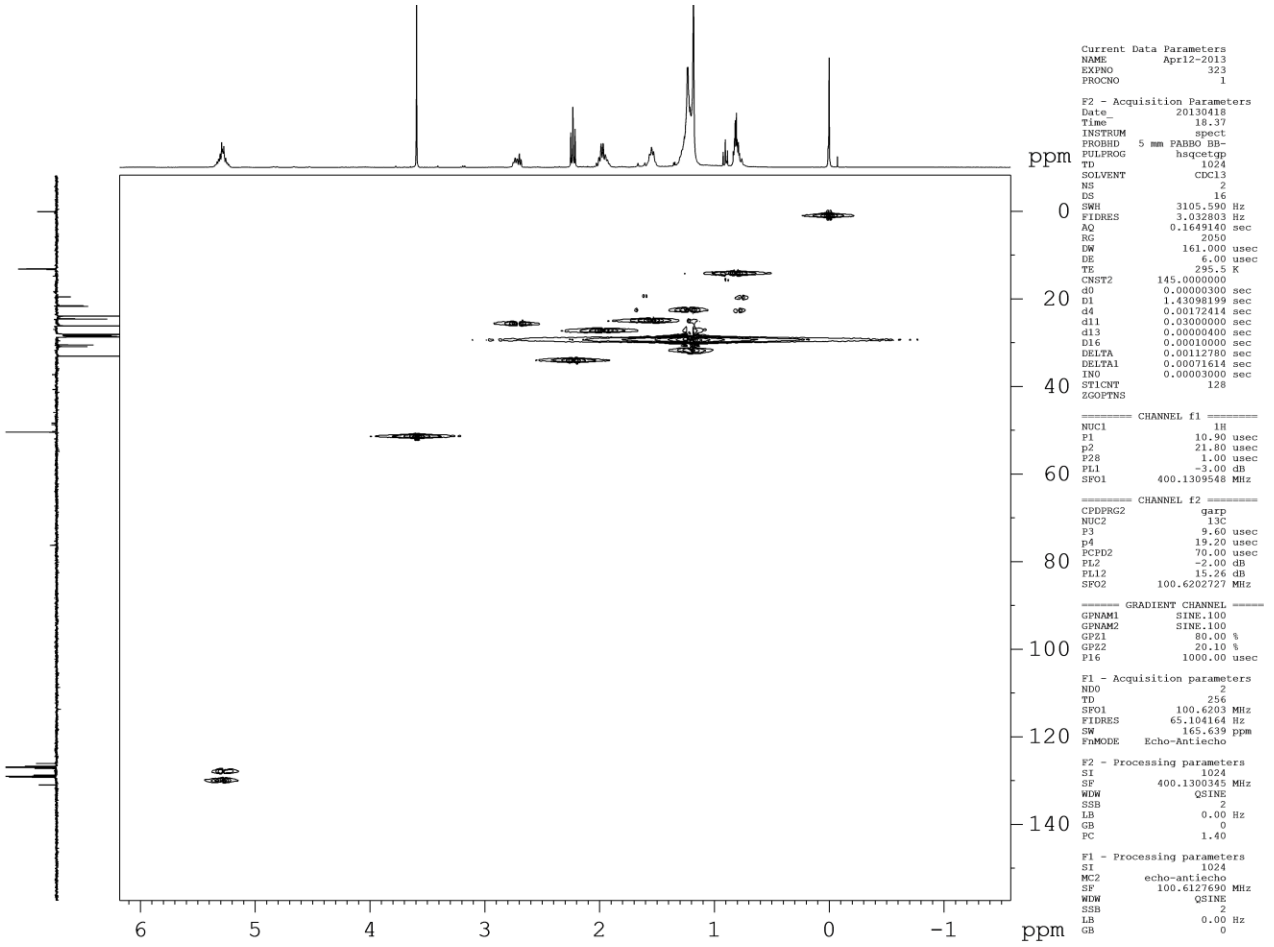
DE 6.00 usec
TE 295.0 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.00001222 sec
TD0 1

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

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

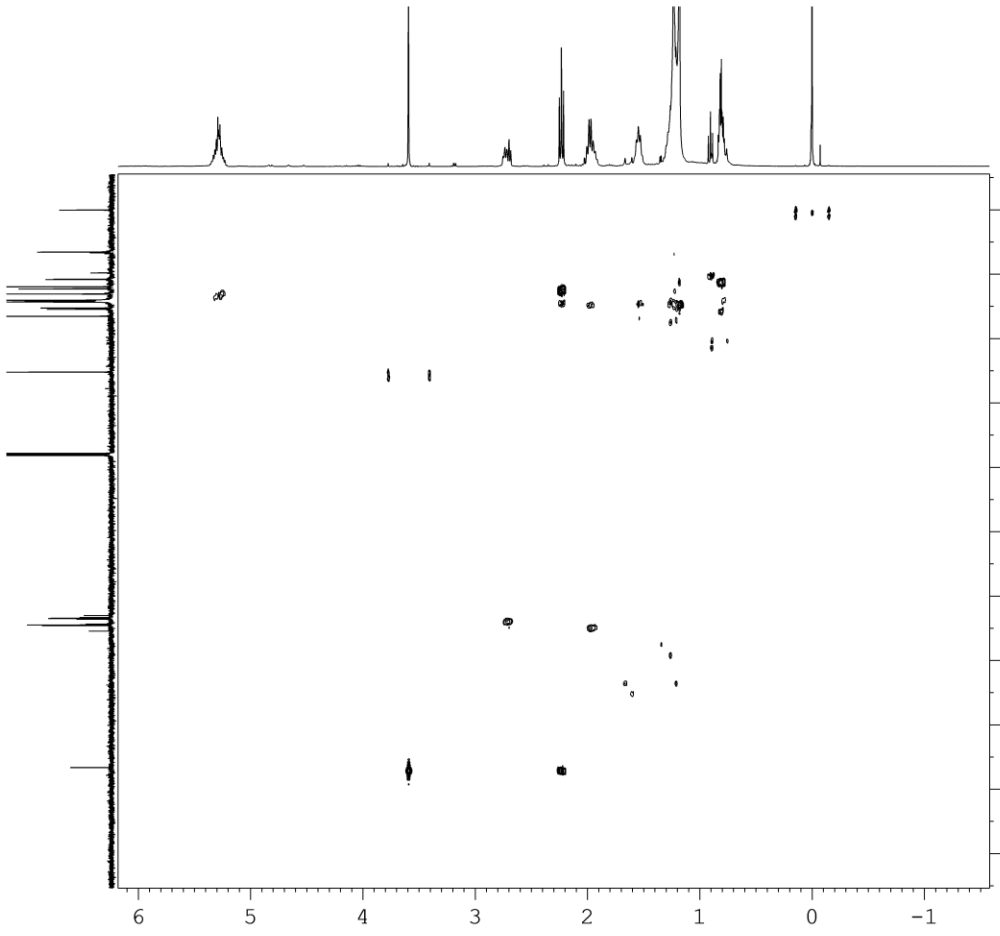
F2 - Processing parameters
SI 32768
SF 100.6128723 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

VVR-I



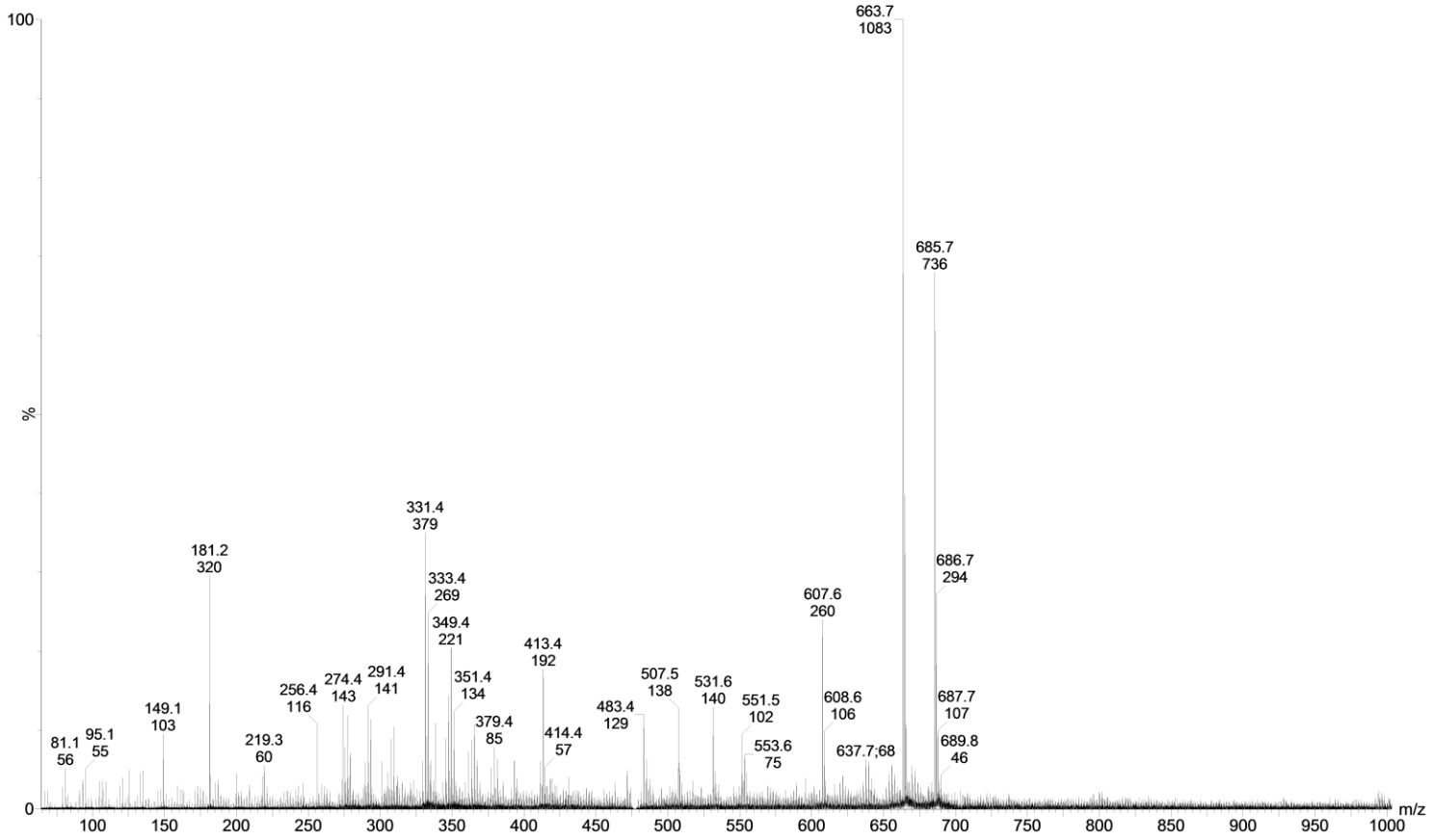

```
Current Data Parameters
NAME          Apr12-2013
EXPNO                323
PROCNO                 1

F2 - Acquisition Parameters
Date_           20130418
Time              18.37
INSTRUM             spect
PROBHD   5 mm PABBO BB-
PULPROG         hsqcetgp
TD                 1024
SOLVENT            CDCl3
NS                    2
DS                   16
SWH           3105.590 Hz
FIDRES        3.032803 Hz
AQ            0.1649140 sec
RG                 2050
DW             161.000 usec
DE               6.00 usec
TE              295.5 K
CNST2        145.0000000
d0           0.00000300 sec
D1           1.43098199 sec
d4           0.00172414 sec
d11          0.03000000 sec
d13          0.00000400 sec
D16          0.00010000 sec
DELTA        0.00112780 sec
DELTA1       0.00071614 sec
IN0          0.00003000 sec
ST1CNT              128
ZGOPTNS

======== CHANNEL f1 ========
NUC1                1H
P1              10.90 usec
p2              21.80 usec
P28              1.00 usec
PL1             -3.00 dB
SFO1        400.1309548 MHz

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

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

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

F2 - Processing parameters
SI                 1024
SF          400.1300345 MHz
WDW              QSINE
SSB                   2
LB               0.00 Hz
GB                    0
PC                 1.40

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

VVR-I



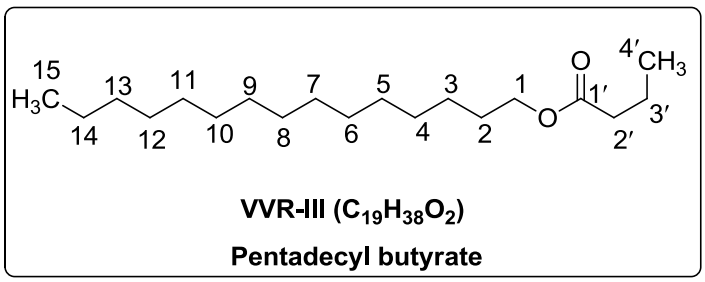

```
Current Data Parameters
NAME          Apr12-2013
EXPNO                324
PROCNO                 1

F2 - Acquisition Parameters
Date_           20130413
Time                3.44
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG     hmbcgpndqf
TD                 4096
SOLVENT            CDC13
NS                    8
DS                   16
SWH           3105.590 Hz
FIDRES        0.758201 Hz
AQ            0.6595060 sec
RG                 2050
DW             161.000 usec
DE               6.00 usec
TE              291.6 K
CNST13         8.0000000
d0            0.00000300 sec
D1            1.22474897 sec
d6            0.06250000 sec
D16           0.00010000 sec
IN0           0.00002240 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                10.90 usec
p2                21.80 usec
PL1               -3.00 dB
SFO1        400.1309548 MHz

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

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

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

F2 - Processing parameters
SI                 1024
SF          400.1300345 MHz
WDW                SINE
SSB                   0
LB                 0.00 Hz
GB                    0
PC                 1.40

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

VVR-I


100
%
0
663.7
1083
685.7
736
686.7
294
687.7
107
689.8
46
331.4
379
181.2
320
333.4
269
349.4
221
413.4
192
607.6
260
608.6
106
507.5
138
531.6
140
551.5
102
553.6
75
637.7;68
483.4
129
379.4
85
414.4
57
351.4
134
256.4
116
274.4
143
291.4
141
149.1
103
219.3
60
81.1
56
95.1
55
m/z
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

# APPENDIX B

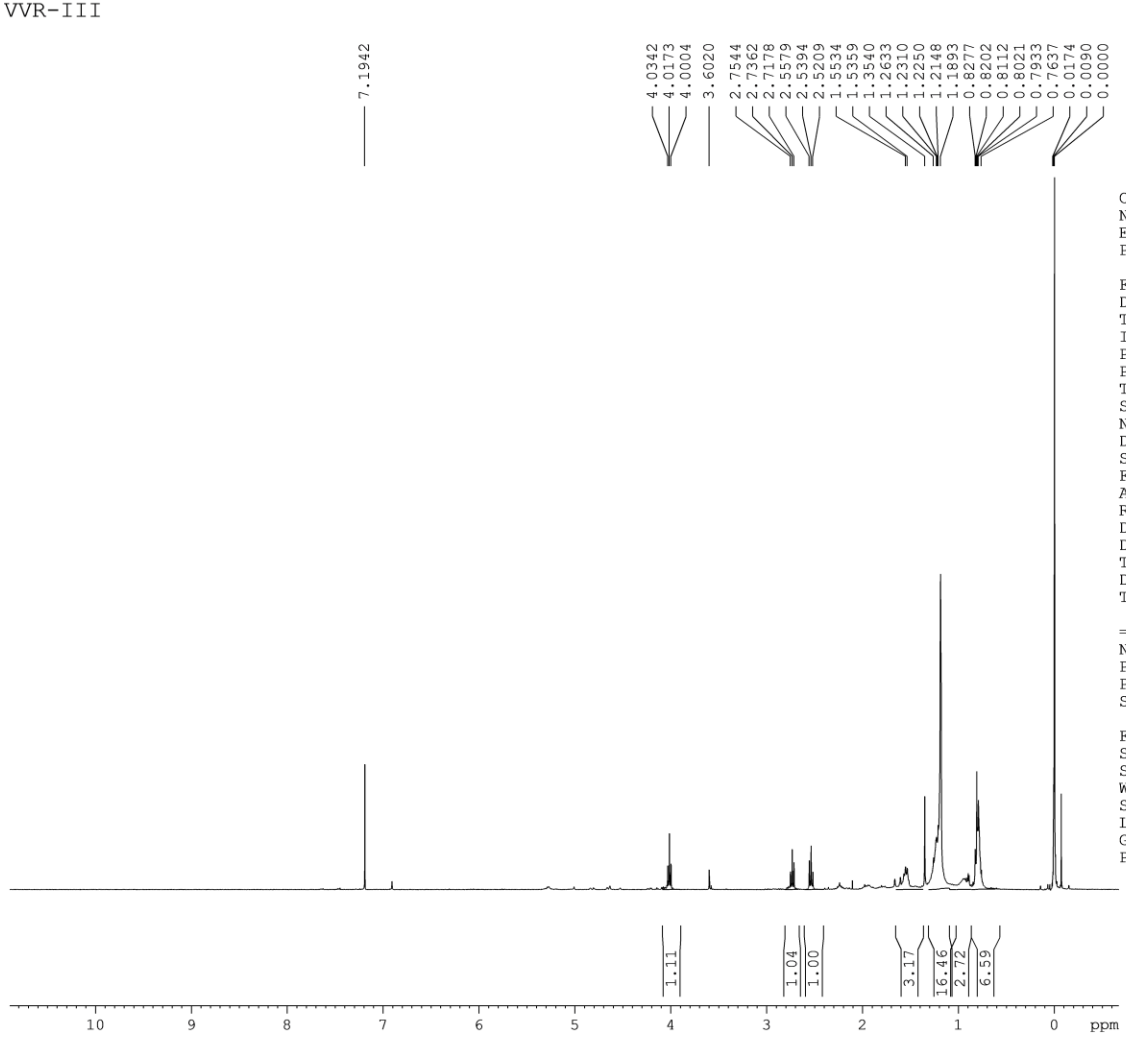
# Spectral Data of VVR-III




**VVR-III ($C_{19}H_{38}O_2$)**

**Pentadecyl butyrate**

VVR-III




BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF

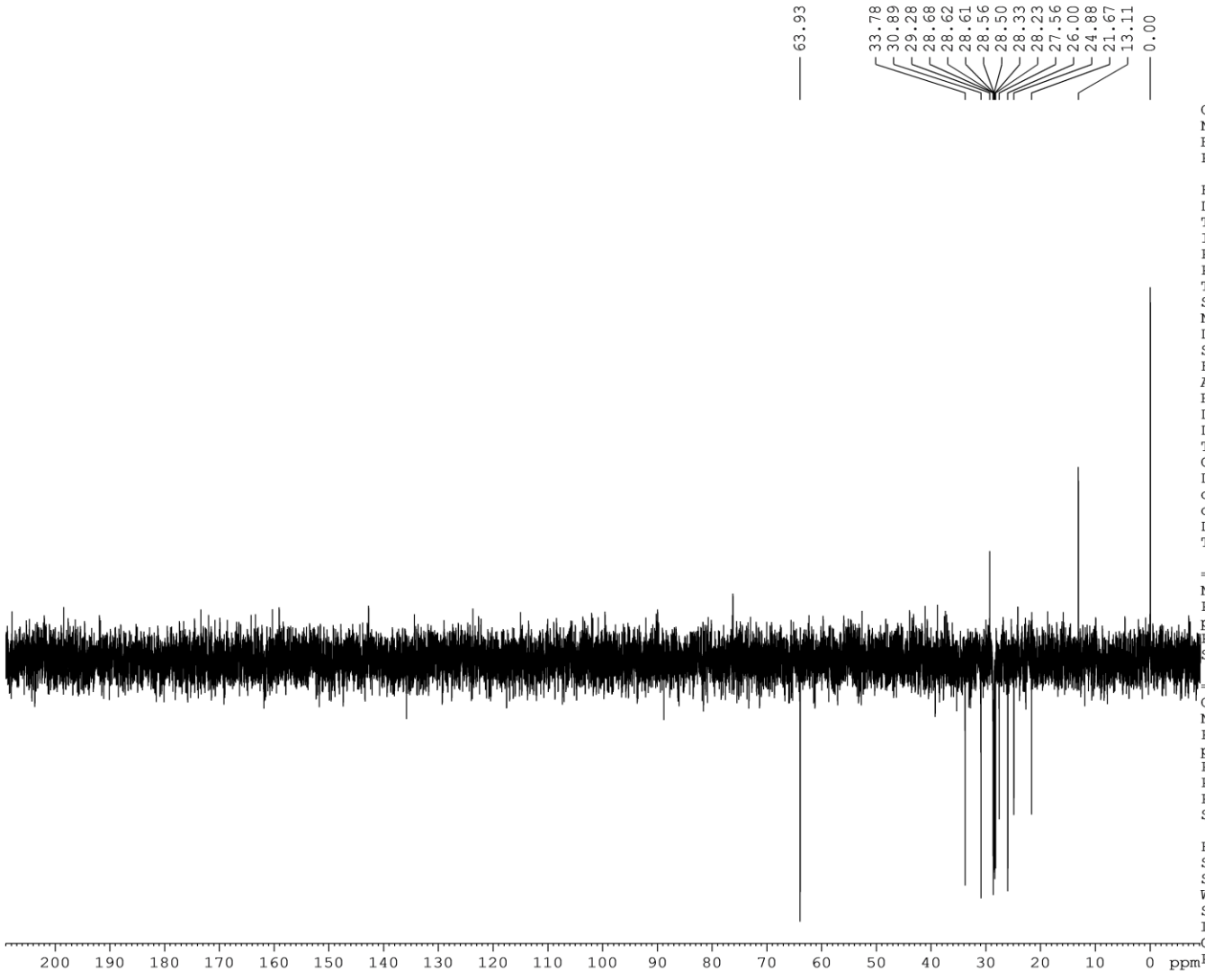
Current Data Parameters
NAME Apr12-2013
EXPNO 330
PROCNO 1

F2 - Acquisition Parameters
Date_ 20130413
Time 4.23
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 12019.230 Hz
FIDRES 0.183399 Hz
AQ 2.7263477 sec
RG 575
DW 41.600 usec
DE 6.00 usec
TE 291.4 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 10.90 usec
PL1 -3.00 dB
SFO1 400.1324710 MHz

F2 - Processing parameters
SI 32768
SF 400.1300360 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

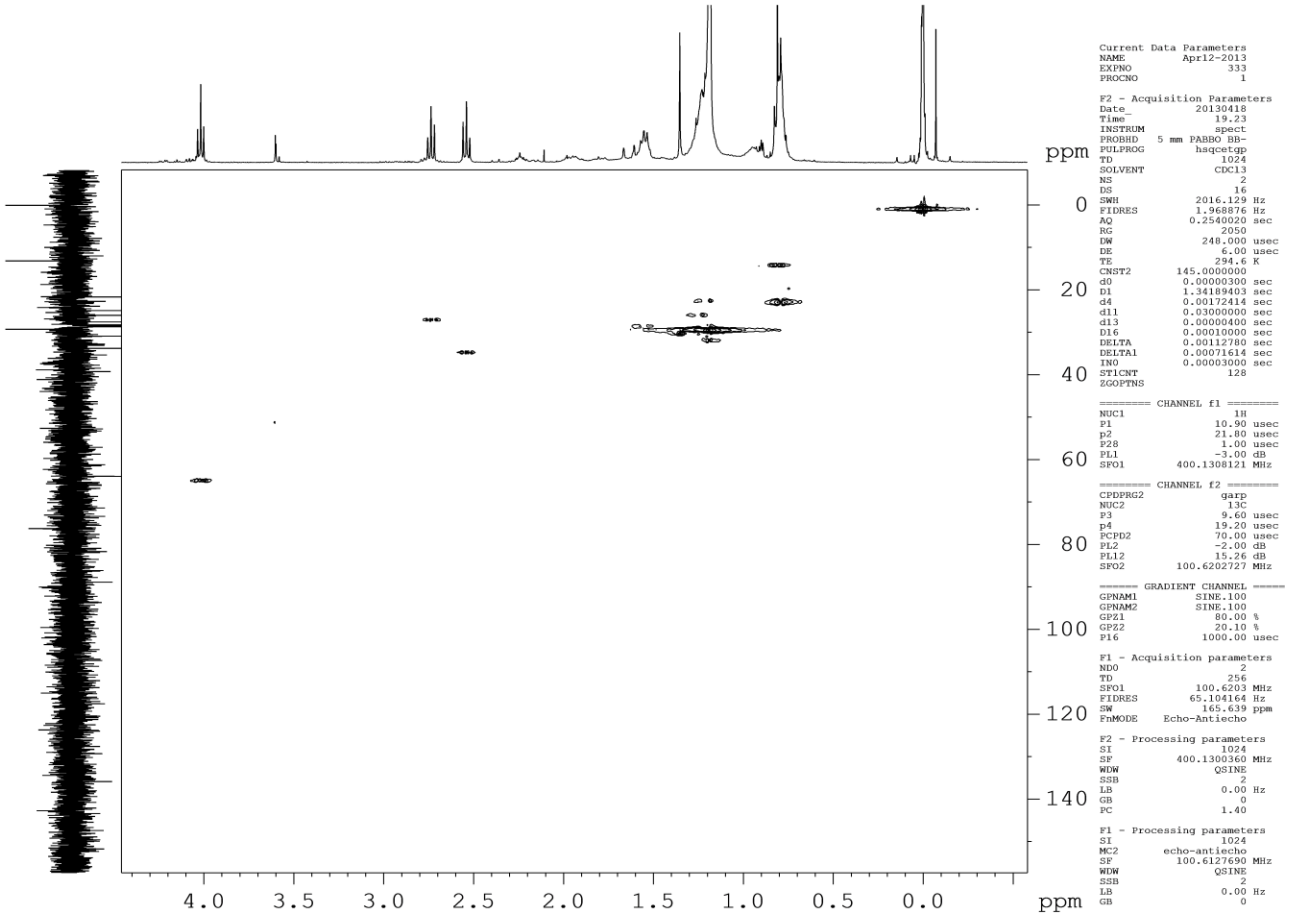
VVR-III




BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF

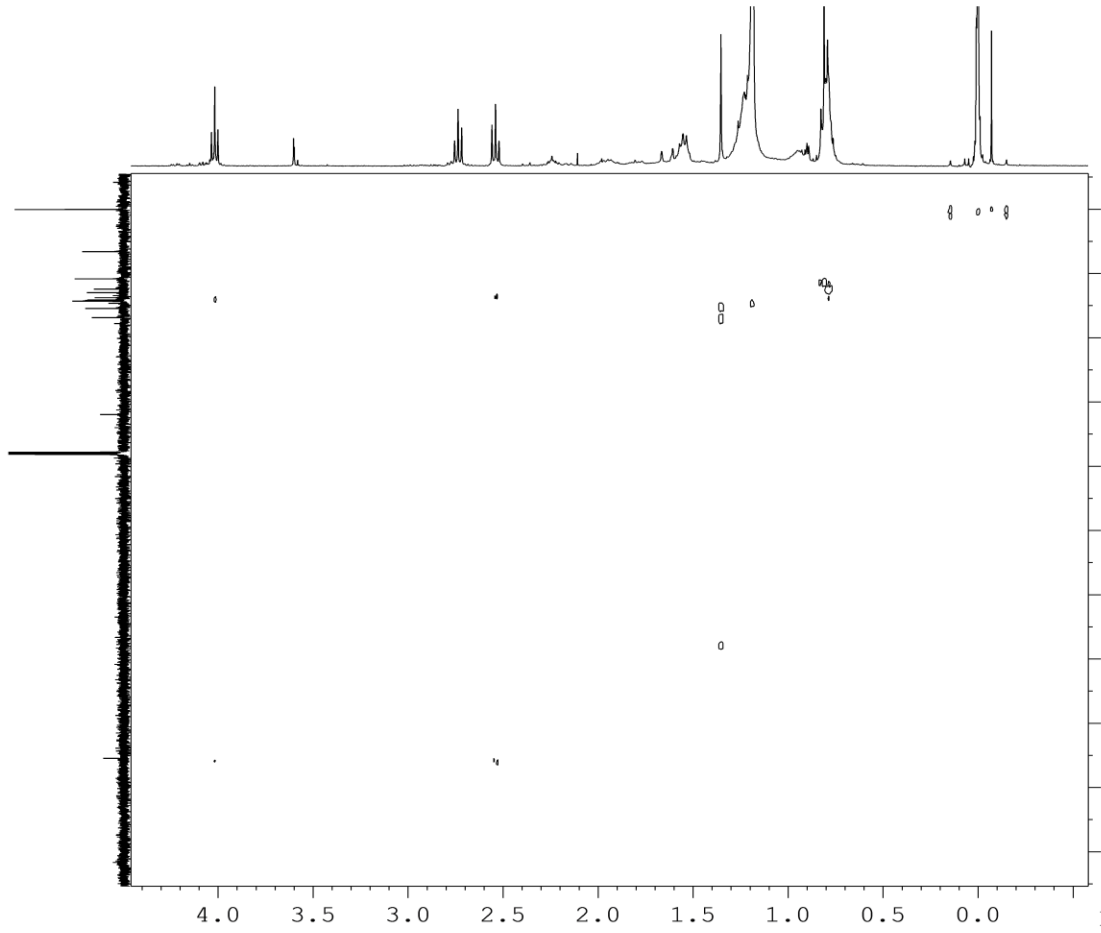
| | |
|---|---|
| Current Data Parameters | |
| NAME | Apr12-2013 |
| EXPNO | 332 |
| PROCNO | 1 |
| F2 - Acquisition Parameters | |
| Date_ | 20130418 |
| Time | 19.22 |
| INSTRUM | spect |
| PROBHD | 5 mm PABBO BB- |
| PULPROG | dept135 |
| TD | 65536 |
| SOLVENT | CDCl3 |
| NS | 512 |
| DS | 4 |
| SWH | 29761.904 Hz |
| FIDRES | 0.454131 Hz |
| AQ | 1.1010548 sec |
| RG | 1030 |
| DW | 16.800 usec |
| DE | 6.00 usec |
| TE | 294.4 K |
| CNST2 | 145.0000000 |
| D1 | 2.00000000 sec |
| d2 | 0.00344828 sec |
| d12 | 0.00002000 sec |
| DELTA | 0.00001222 sec |
| TD0 | 1 |
| ======== CHANNEL f1 ======== | |
| NUC1 | 13C |
| P1 | 9.60 usec |
| p2 | 19.20 usec |
| PL1 | -2.00 dB |
| SFO1 | 100.6228298 MHz |
| ======== CHANNEL f2 ======== | |
| CPDPRG2 | waltz16 |
| NUC2 | 1H |
| P3 | 10.90 usec |
| p4 | 21.80 usec |
| PCPD2 | 80.00 usec |
| PL2 | -3.00 dB |
| PL12 | 14.31 dB |
| SFO2 | 400.1316005 MHz |
| F2 - Processing parameters | |
| SI | 32768 |
| SF | 100.6128735 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 1.00 Hz |
| GB | 0 |
| PC | 1.40 |

VVR-III

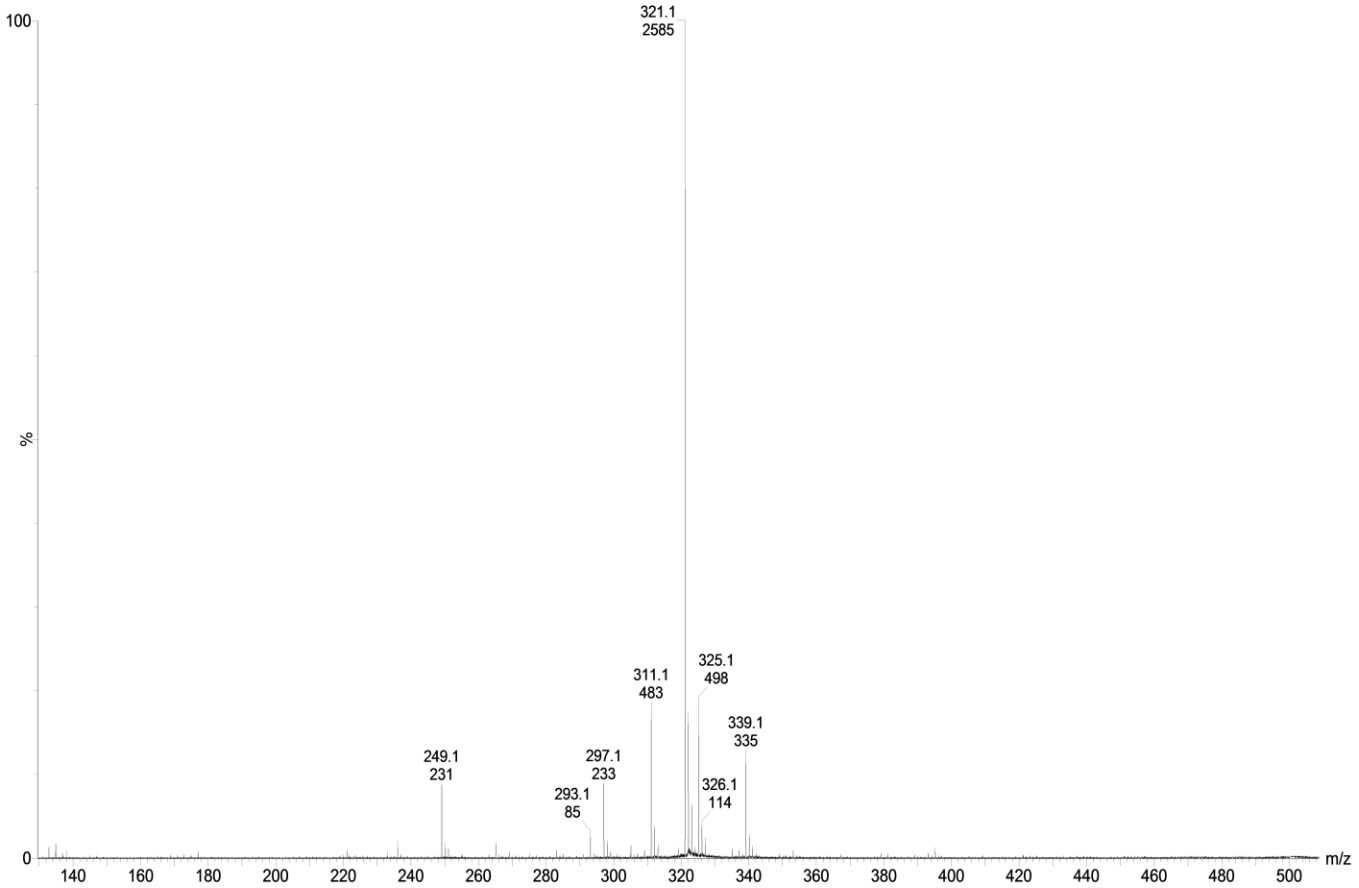



```
Current Data Parameters
NAME          Apr12-2013
EXPNO                333
PROCNO                 1

F2 - Acquisition Parameters
Date_           20130418
Time               19.23
INSTRUM             spect
PROBHD   5 mm PABBO BB-
PULPROG         hsqcetgp
TD                 1024
SOLVENT            CDCl3
NS                    2
DS                   16
SWH            2016.129 Hz
FIDRES         1.968876 Hz
AQ             0.2540020 sec
RG                 2050
DW              248.000 usec
DE                6.00 usec
TE               294.6 K
CNST2        145.0000000
d0             0.00000300 sec
D1             1.34189403 sec
d4             0.00172414 sec
d11            0.03000000 sec
d13            0.00000400 sec
D16            0.00010000 sec
DELTA          0.00112780 sec
DELTA1         0.00071614 sec
IN0            0.00003000 sec
ST1CNT              128
ZGOPTNS

======== CHANNEL f1 ========
NUC1                1H
P1                10.90 usec
p2                21.80 usec
P28                1.00 usec
PL1               -3.00 dB
SFO1         400.1308121 MHz

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

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

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

F2 - Processing parameters
SI                 1024
SF           400.1300360 MHz
WDW                QSINE
SSB                   2
LB                 0.00 Hz
GB                    0
PC                 1.40

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

VVR-III




```
Current Data Parameters
NAME          Apr12-2013
EXPNO                334
PROCNO                 1

F2 - Acquisition Parameters
Date_           20130413
Time                5.21
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG     hmbcgpndqf
TD                 4096
SOLVENT            CDCl3
NS                    8
DS                   16
SWH           2016.129 Hz
FIDRES        0.492219 Hz
AQ           1.0158581 sec
RG                 2050
DW             248.000 usec
DE               6.00 usec
TE              291.6 K
CNST13         8.0000000
d0          0.00000300 sec
D1          0.86839682 sec
d6          0.06250000 sec
D16         0.00010000 sec
IN0         0.00002240 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                10.90 usec
p2                21.80 usec
PL1               -3.00 dB
SFO1        400.1308121 MHz

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

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

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

F2 - Processing parameters
SI                 1024
SF          400.1300360 MHz
WDW                SINE
SSB                   0
LB                0.00 Hz
GB                    0
PC                 1.40

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


100
%
0
321.1
2585
325.1
498
311.1
483
339.1
335
249.1
231
297.1
233
326.1
114
293.1
85
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
m/z

# APPENDIX C

# PUBLICATIONS

## PUBLICATIONS

1. **Gupta, V. K.**, Bhalla, Y., & Jaitak, V. (2013). Impact of ABC transporters, glutathione conjugates in MDR and their modulation by flavonoids: an overview. *Medicinal Chemistry Research* DOI: 10.1007/s00044-013-0612-6.
2. Bhalla, Y., **Gupta, V. K.**, & Jaitak, V. (2013). Anticancer activity of essential oils: A review. *Journal of the Science of Food and Agriculture* DOI: 10.1002/jsfa.6267.
3. **Gupta, V. K.** & Jaitak, V. (2013). Photoprotective activity of dried roots of *Potentilla atrosanguinea* Lodd. – An alpine plant of western Himalaya. *Natural Product Research* (Communicated).
4. **Gupta, V.K.,** Kaur, R. & Jaitak, V. (2013). Total phenol content and antioxidant activity of *Potentilla atrosanguinea* Lodd. - A Himalaya Cinquefoil. *Pharmaceutical Biology* (Communicated).
5. **Gupta, V.K.** & Jaitak, V. (2013). Molecular docking Study of Natural Derived Products on MRP-1, GSTP1-1 and BCRP mediated Multidrug Resistance in Cancer Chemotherapy (under preparation)