Kaunas University of Technology

Faculty of Chemical Technology

Isolation of higher value-added components from *Potentilla fruticosa* and *Hierochloe odorata* by means of high-pressure and enzyme-assisted extraction techniques

Master's Final Degree Project

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Kiran Subbarayadu. Isolation of Higher Value-Added Components From *Potentilla Fruticosa* and *Hierochloe Odorata* by Means of High-Pressure and Enzyme-Assisted Extraction Techniques. Master's Final Degree Project /Doc., dr. Michail Syrpas; Faculty of Chemical Technology, Kaunas University of Technology.

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Summary

Potentilla fruticosa is a shrubby perennial plant, native to Northern areas of America and Europe. It is commonly consumed as an herbal tea and exhibits great antioxidant potential as well as antifungal, antibacterial and anti-inflammatory properties. *Hierochloe odorata* is an aromatic perennial grass, widely distributed in West Asia and Europe, whose roots and aerial parts have a sweet smell. *H. odorata* extracts have shown to have high antioxidant activity and insect-repellent properties. The aim of this work was to isolate and analyze functional characterization of high-added value fractions of *P. fruticosa* and *H. odorata* through the development of biorefining schemes utilizing conventional, high-pressure, and enzymatic treatment techniques.

The chemical composition of *P. fruticosa* and *H. odorata* was studied using various basic methods. Conventional and high-pressure extraction techniques like Solid liquid extraction and supercritical carbon dioxide extraction, pressurized liquid extraction and enzyme assisted extraction were applied on *P. fruticosa* and *H. odorata*. Defatted *P. fruticosa* and *H. odorata* residues after supercritical carbon dioxide extraction were used for further extraction techniques. PLE conditions were optimized using response surface methodology to maximize the extraction yield and phenolic content. Enzyme assisted extractions of defatted plant material and water residue from PLE of *P. fruticosa* and *H. odorata* were performed.

Total phenolic content was determined using Folin-Ciocalteu's technique and antioxidant activity was measured by DPPH• and ABTS•+ assays. Initial solid plants materials, SLE and SFE-CO_2 residue antioxidant activity were analyzed using QUENCHER procedure. SLE, SFE-CO_2, PLE, enzyme treated extracts total phenolic content was determined using Folin-Ciocalteu's technique. Antioxidant activity was measured by DPPH• and ABTS•+ assays.

The phytochemical characterization of volatile compounds of *P. fruticosa* and *H. odorata* SFE-CO_2 extracts were analyzed by GCxGC/TOF MS, chemical compounds of *P. fruticosa* and *H. odorata* PLE extracts were analyzed using UPLC/ESI-QTOF-MS/MS.

Subbarayadu, Kiran. Aukštesnės pridėtinės vertės komponentų išskyrimas iš sidabražolės (*Potentilla fruticosa*) ir kvapiosios stumbražolės (*Hierochloe odorata*) taikant aukšto slėgio ir fermentinės ekstrakcijos metodus. Magistro baigiamasis projektas / Doc., dr. Michail Syrpas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

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Santrauka

Aukštesnės pridėtinės vertės komponentų išskyrimas iš sidabražolės (*Potentilla fruticosa*) ir kvapiosios stumbražolės (*Hierochloe odorata*) taikant aukšto slėgio ir fermentinės ekstrakcijos metodus

Potentilla fruticosa yra daugiametis krūminis augalas, kilęs iš Šiaurės Amerikos ir Europos, vartojamas kaip žolelių arbata bei pasižymintis dideliu antioksidaciniu potencialu, taip pat priešgrybelinėmis, antibakterinėmis ir priešuždegiminėmis savybėmis. *Hierochloe odorata* yra aromatinė daugiametė žolė, plačiai paplitusi Vakarų Azijoje ir Europoje, kurios šaknys ir plaukeliai turi saldų kvapą. *H. odorata* ekstraktai turi didelį antioksidacinį potencialą ir pasižymi vabzdžius atbaidančiomis savybėmis. Šio darbo tikslas yra išskirti ir išanalizuoti aukštesnės pridėtinės vertės *P. fruticosa* ir *H. odorata* frakcijų funkcinę charakteristiką, sukuriant jų biorafinavimo schemas taikant tradicinius, aukšto slėgio ir fermentinės ekstrakcijos metodus.

Cheminė *P. fruticosa* ir *H. odorata* sudėtis buvo tiriama naudojant įvairius pagrindinius metodus. *P. fruticosa* ir *H. odorata* buvo tiriamios tradiciniais ir aukšto slėgio ekstrakcijos metodais, naudojant maceraciją (SLE), superkrizinę skysčių ekstrakciją anglies dvideginiu (SFE-CO_2), ekstrakciją suspaustais skysčiais (PLE) bei fermentinę hidrolizę. Po superkrizinės skysčių ekstrakcijos nuriebalintos *P. fruticosa* ir *H. odorata* liekanos buvo naudojamos tolesnėms ekstrakcijoms. PLE sąlygos buvo optimizuotos naudojant paviršiaus atsako metodiką, siekiant maksimaliai padidinti ekstrakcijos išeigą ir fenolinių junginių kiekį. Atlikta *P. fruticosa* ir *H. odorata* pradinės žaliavos ir nuriebalintų vandeninių liekanų po PLE fermentinė hidrolizė.

Bendras fenolinių junginių kiekis buvo nustatytas naudojant *Folin-Ciocalteu* reagentą, o antioksidaciniam potencialui įvertinti naudoti $DPPH^{\bullet}$ ir $ABTS^{\bullet+}$ metodai. Pradinės žaliavos, SLE ir SFE-CO_2 liekanų antioksidacinis potencialas buvo nustatytas naudojant QUENCHER procedūrą. SLE, SFE-CO_2, PLE ir fermentais apdorotų ekstraktų bendras fenolinių junginių kiekis taip pat buvo nustatytas naudojant *Folin-Ciocalteu* reagentą. Antioksidacinis aktyvumas buvo nustatytas $DPPH^{\bullet}$ ir $ABTS^{\bullet+}$ metodais. *P. fruticosa* ir *H. odorata* SFE-CO_2 ekstraktų lakiųjų komponentų fitocheminė sudėtis buvo analizuojama GCxGC/TOF MS, o PLE ekstraktų cheminė sudėtis buvo analizuojama naudojant UPLC/ESI-QTOF-MS/MS .

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List of abbreviations

ABTS●+	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation
ANOVA	Analysis of variance
AUC	Area under the curve
CCD	Central composite experimental design
DPPH●	2,2-diphenyl-1-picrylhydrazyl radical
5,8-DHC	5,8-dihydroxycoumarin
DW	Dry weight
EAE	Enzyme-Assisted Extraction
GAE	Gallic acid equivalents
GC	Gas chromatography
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
MeOH	Methanol
MS	Mass spectrometry
PBS	Phosphate buffered saline
PLE	Pressurised liquid extraction
PS	Pilot scale
R^2	Determination coefficient
ROS	Reactive Oxygen Species
RSM	Response surface methodology
SCF	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SFE-CO_2	Supercritical carbon dioxide extraction
SFE-CO_2-SLE	Solid liquid extraction on SFE-CO2 residue
SLE	Solid liquid extraction
SLE-Ace	Solid liquid extraction using acetone
SLE-EtOH	Solid liquid extraction using ethanol
SLE-Hex	Solid liquid extraction using hexane
SLE-H20	Solid liquid extraction using water
TEAC	Trolox equivalent antioxidant capacity
TE	Trolox equivalent
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC	Ultra-high performance liquid chromatography
Vs	Versus

Introduction

In recent years there is an enormous demand for food ingredients and food additives of natural origin. This is also due to the fact that the widely applied chemical counterparts are in several cases linked with adverse health effects. Whereas, the functional properties of natural additives could be potentially beneficial for the improvement of human health.

Many scientists are searching for natural, bioactive and potent compounds which have the antioxidant capacity and are safe for human consumption. It is a significant concern to produce these bioactive compounds in a sustainable and environment-friendly manner since most of the conventional methods are harmful to the environment[1].

Oxidation of food is related with loss of nutritional value, food quality, and safety, as well as organoleptic characteristics such as flavor, color, and texture, and finally. Oxidation processes are caused by Reactive Oxygen Species (ROS), they are the free radicals with unpaired electrons, in most of the cases, ROS are harmful to cells and leads to the development of several diseases like cancer, heart diseases, and age-related diseases. The use of synthetic antioxidants in the food industry is under strict regulation because of the unknown knowledge of their safety. So, there is an interest in natural food additives among the consumers, although the economic expense in the production cost of such products is relatively high. The beneficial properties of antioxidants are currently an important topic of research among scientists[2].

Potentilla fruticosa is a species of hardy deciduous flowering shrub in the *Potentilla* genus of the family Rosaceae which has long been applied in traditional medicine and been confirmed to possess relatively high concentrations of phenolic acids and flavonoids with powerful radical scavenging capacity[3][4]. The contents of hyperoside, catechin and ellagic acid are incredibly high in *P. fruticosa* leaves[5]. The radical scavenging capacities of *Potentilla fruticosa* are also proven to be higher than that of synthetic antioxidants [2]. *Potentilla fruticosa* is widely used in medicine, cosmetic and tea industries.

Hierochloe odorata, commonly known as sweetgrass, is an aromatic member of the Gramineae family native to arctic and temperate regions throughout the northern hemisphere. *Hierochloe odorata* extracts have been reported that could retardation lipid peroxidation[6]. This herb is known to contain coumarin and its derivatives 5,8-dihydroxycoumarin and 5-hydroxy-8-O-β-d glucopyranosycoumarin. However, it is chemical composition, and biological properties have not been investigated extensively. It was also reported that *in vitro* analysis showed that 5,8-dihydroxycoumarin antioxidant activity is higher than that of a well-known natural antioxidant rosmarinic acid[6].

Three different high-pressure and fractionation procedures such as Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE) and Enzyme-Assisted Extraction (EAE) techniques were evaluated, and the antioxidant capacity of the obtained fractions was determined with *in vitro* antioxidant and radical scavenging activity. These experiments show the distribution of the antioxidants in different fractions of extracts. These results lead to some suggestions for specific extraction procedures, which could be used for the isolation of the antioxidants[6].

Aim

To isolate and perform fuctional characterization of high-added value fractions of *Potentilla fruticosa* and *Hierochloe odorata* through the development of biorefining schemes utilizing conventional, high-pressure, and enzymatic treatment techniques.

Objective

1. To determine chemical composition of *Potentilla fruticosa* and *Hierochloe odorata*
2. To obtain non-polar extracts of *Potentilla fruticosa* and *Hierochloe odorata using* supercritical carbon dioxide extraction (SFE-CO_2)
3. To perform solid-liquid extraction (SLE) with different polarity solvents to isolate non-polar and polar fractions from *Potentilla fruticosa* and *Hierochloe odorata* plant material and SFE-CO_2 residues.
4. To optimize pressurised liquid extractions of SFE-CO_2 residues of *Potentilla fruticosa* in order to obtain the maximum yield and total phenolic content of acetone, ethanol and water fractions and to perform pressurised liquid extractions on SFE-CO_2 residues of *Hierochloe odorata*.
5. To evaluate enzyme-assisted extraction (EAE) for recovery of polar fractions from *Potentilla fruticosa* and *Hierochloe odorata* residues after SFE-CO_2 and PLE water extraction.
6. To measure Total phenolic content and *in vitro* radical scavenging capacity ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays) of starting plant materials, SLE (residue and extracts), SFE-CO_2 (residues and extracts), PLE extracts and EAE extracts.
7. To characterize the non-polar profile of *Potentilla fruticosa* and *Hierochloe odorata* from SFE-CO_2 extracts by GCxGC/ TOF MS.
8. To characterize compounds of *Potentilla fruticosa* and *Hierochloe odorata* from SFE-CO_2 and PLE extracts using UPLC/ESI-QTOF-MS.

1 Literature review

1.1 Potentilla fruticosa

1.1.1 General characteristics

The genus Potentilla is composed of about 500 species in which fifty of them are found in North America, and seventy-five species belongs to the European region and consists of mostly boreal herbs and shrubs of the family Rosaceae[7]**.** *Potentilla* is the prominent genus and mainly occurs in European, arctic regions, but few species are south temperate. However, some species are also found in alpine and high mountain regions of the tropics and South America[7].

Potentilla fruticosa or *Dasiphora fruticosa* is a shrubby perennial plant, native to Northern areas of America and Europe. *P. fruticosa* is commonly known as Shrubby cinquefoil, and other familiar names include yellow hardhack, black bush and yellow rose[8]. These plants are densely leafy, the leaves divided into five or pinnate leaflets, flowers are pale to bright yellow[9]. *P. fruticosa* is illustrated in **Figure 1.1**

Figure 1.1. Picture of *P. fruticosa.* Imported from the flora of China[10].

Potentilla species are used in traditional medicine, consumed as a herbal tea and exhibits great antioxidant potential as well as, antibacterial, antiviral, hepatoprotective nature, and anti-inflammatory properties are due to the presence of higher amounts of tannins and phenolic compounds[4].

1.1.2 Chemical profile and bioactivity of *P. fruticosa*

Ganenko *et al.* studied the epigeal organs of *P. fruticosa* for the isolation of polar and non-polar compounds. Two substances that belonged in the flavonoids, were extracted using 70% ethanol as a solvent, the compounds were quercetin (3,3',4',5,7-pentahydroxyflavone), and quercitrin (3,3',4',5,7-pentahydroxyflavone 3-o-α-L-rhamnopyranoside)[11]. The same authors did later investigations on the polar extracts, and few more flavonoids were identified and were similar to the mentioned compounds namely, quercetin 3-o-α-L-arabinopyranoside, quercetin-3-o-β-d-galactopyranoside, Terniflorin, tribuloside and catechin[12]. The same authors also identified in the non-polar extracts the triterpenoids, epiursolic acid, 2α-hydroxyursolic acid, and tormentic acid[13].

In 2003, G. Miliauskas *et al.* investigated twelve medicinal and aromatic plants for the radical scavenging activity using DPPH• and ABTS•+ assays and found that methanol extracts of *P. fruticosa* had the highest antioxidant capacity and thoroughly scavenged ABTS•+ and DPPH• radicals among the studied plants. Relatively low amounts of flavonoids and flavonols were determined in *P. fruticosa*, but it contained the highest amount of phenolic compounds among the studied plants, confirming that phenolic compounds play a role in the radical scavenging activity of *P.fruticosa* extract[14].

G. Miliauskas *et al.* identified several compounds in the various fractions of *P. fruticosa* using various spectroscopic techniques, and also structures were determined. Quercetin-3-*α*-arabinofuranoside was isolated from ethanol- butanol fraction. Kaempferol-3-*O*-*β*-(6´´-*O*-(*E*)-*p* coumaroyl) glucopyranoside from ethanol-butanol and water-butanol extract. kaempferol-3-*β*-rutinoside, quercetin-3- *β*-rutinoside (rutin), quercetin-3-*β*-glucopyranoside, quercetin-3-*β* -galactopyranoside, quercetin- 3-*β*-glucuronopyranoside and catechin from ethanol-butanol extract. Rhamnetin-3-*β*-glucopyranoside, rhamnetin-3-*β*-galactopyranoside, and rhamnetin- 3-*α*-arabinofuranoside, ellagic acid isolated from ethanol-butanol extract[15]. Catechin and ellagic acid were found to be the most active radical scavengers in DPPH• and ABTS•+ assays in polar media, showing greater activity than Rosmarinic acid[15]. All the identified compounds are shown in **Table 1.1**. The structures of some important radical scavengers are presented in **Figure 1.2**.

Table 1.1. Radical scavengers of *P. fruticosa*[15]

Sl No	Compounds
1	Quercetin-3-β-glucopyranoside
2	Quercetin-3-β-galactopyranoside(hyperoside)
3	Quercetin-3-β-rutinoside(rutin)
4	Quercetin-3-β-glucuronopyranoside
5	Quercetin-3-α-arabinofuranoside
6	Rhamnetin-3-β-glucopyranoside
7	Rhamnetin-3-β-galactopyranoside
8	Rhamnetin-3-α-arabinofuranoside
9	Kaempferol-3-β-rutinoside
10	Catechin
11	Ellagic acid
12	Kaempferol-3-O-β-(6"-O-(E)-pcoumaroyl) glucopyranoside

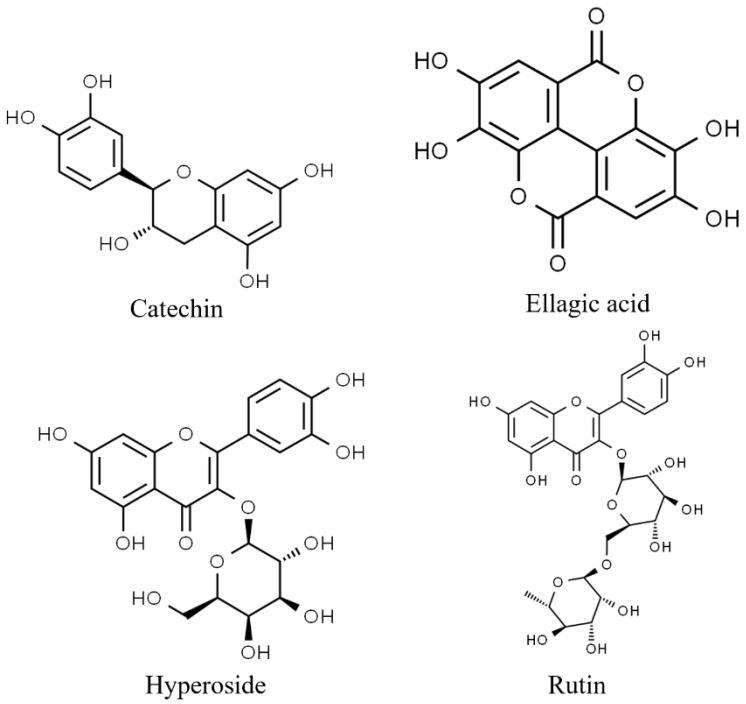


Figure 1.2 Structures of radical scavengers in *P.fruticosa*[16][17][18][19]

Michal Tomczyk *et al.* reported that the TPC in aqueous extracts of *P. fruticosa* was found to be 116.3 ± 3.9 mg of gallic acid equivalents (GAE)/g DW. The aerial parts of *P. fruticosa* also showed high concentrations of tannins, proanthocyanidins, phenolic acids and flavonoid compounds (167.3 ± 2.0, 4.6 ± 0.2, 16.4 ± 0.8, 7.0 ± 1.1 mg/g DW), respectively calculated in quercetin units.[20]. S.Wang *et al.* reported that the acetone extracts of *P. fruticosa* leaves also possessed high antioxidant activity in the DPPH•, ABTS•+ and FRAP assays (16.87 μg/mL, 2763.48 and 1398.70 μmol Trolox equivalent/g of extract, respectively)[5]. Another author also reported that ABTS•+ scavenging capacity was in the range of 303.048 ± 15.67 to 1309.74 ± 75.25 μmol Trolox equivalent /g in *P. fruticosa* ethanol extracts[21].

Z. Luo *et al.* investigated nine sub-fractions of *P. fruticosa* crude extracts of leaves using ethanol and butanol as solvents and determined higher amounts of hyperoside (17.67mg/g), ellagic acid (4.77mg/g) and (+)-catechin (4.52mg/g) contents. Crude extracts of leaves contained the total phenolic content of 349.03 ± 6.82 mM GAE 100/g and also showed more significant radical scavenging activity, these results suggest that (+)-catechin, ellagic acid and hyperoside play a crucial role in the antioxidant capacity of *P. fruticosa*[22]. D. Yu *et al.* also studied the ethanol extracts of leaves, flowers, and stems of *P. fruticosa* from two different production areas of China, similar to Z. Luo *et al.* study, in which the results indicated that the higher contents of hyperoside, catechin, ellagic acid, and rutin in leaves, flowers and stems of *P. fruticosa* correlated well with the radical scavenging activity of these extracts[23]. *Zehua Liu et al.* studied the synergistic effect of *P. fruticosa* acetone extracts (PFE) combined with green tea polyphenols (GTP) in combination to enhance the antioxidant activity in real food applications. The combination of PFE and GTP in a ratio of 3:1 demonstrated the best synergistic effect as indicated by the radical scavenging assay. This was probably due to the presence of high amounts of catechin,

hyperoside, caffeic acid, rutin, ellagic acid from in the PFE, and epigallocatechin and epigallocatechin gallate in GTP[24].

1.1.3 Pharmacological activity and applications of *P. fruticosa*:

The antibacterial and antifungal activities of aqueous extracts from aerial parts of *P. fruticosa* were investigated, and resulting tests showed antimicrobial activity against *H. pylori* with minimal inhibitory concentration(MIC) of 0.1 mg/ml[25]. Extracts inhibited the growth of Gram+ bacteria (*M. luteus, S. aureus, B. subtilis*), but was unsuccessful to inhibit the growth of Gram- bacteria[25].In another study, *P. fruticosa* showed the best activity against the gram-negative bacteria *Pseudomonas aeruginosa* and fungus *Candida albicans* of 6.25 mg/mL and 0.78 mg/mL MIC values, respectively. However, failed to prevent the growth of *Escherichia coli* and *Klebsiella pneumoniae* and also showed the most potent antifungal activity on Alternaria brassicae, the activities are due to the presence of hyperoside in acetone extract[5].

P. fruticosa aqueous extracts from ultrasonication method effectively inhibited the complete growth of oral bacteria with the MIC of 3.2 mg/mL against the test *Streptococcus mutans* and also inhibited the dental plaque formation *in vitro*, this suggests that aqueous extracts from *P. fruticosa* extract can prevent dental caries. Also suggests that *P. fruticosa* aqueous extracts can be used in the development of pharmaceutical products[20].The antimicrobial activities of ethanolic extracts from different parts of *P. fruticosa* were studied and found ethanolic extracts exhibited significant antifungal and antibacterial activities, with the EC_{50} value ranging from 0.61 to 6.00 mg/mL MIC and also showed higher antifungal activity with the leaves extracts. These results suggest that *P. fruticosa* extracts can serve as a source to produce the microbicide for use in post-harvest storage of fruits[1].

In a study conducted by G. Miliauskas *et al.* in which *P. fruticosa* extracts are added to fermented sausages prepared in Dutch style to study the effect on lipid oxidation. The ethanol–butanol fraction are applied to the sausages, as these fractions contained a higher amount of phenolic compounds, which possess strong radical scavenging properties by quercetin, kaempferol, and rhamnetin, and catechin[15]. The activity of *Potentilla* extracts was low in the sausage due to the presence of lipophilic media, since the found antioxidants are hydrophilic. Thus these kinds of extracts can only be successfully used in more hydrophilic products like salad dressings, emulsions[26].

1.2 Hierochloe odorata

1.2.1 General Characteristics

Hierochloe odorata is a plant of the genus Hierochloe, family Gramineae, the root, and the aerial parts have a sweet smell[27]. *H. odorata* is a perennial plant that grows on the slopes of mountains and is widely distributed in West Asia, Europe and from Alaska to Newfoundland, and also native to northern Europe[28].

H. odorata is commonly known as sweet grass, or vanilla grass, and as bison grass by Polish vodka producers[29][27]. *H. odorata* grows to a height of about 20cm, since they lack in rigid stem and the leaves grow horizontally more than 100 cm in length[30]. A picture of *H. odorata* is shown in **Figure 1.3.**

Figure 1.3 Picture of *H. odorata* [31]

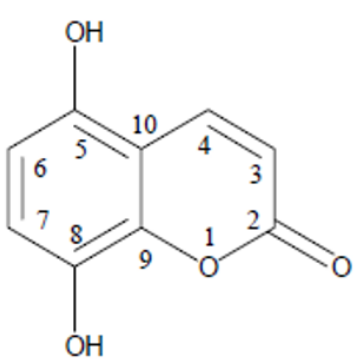
H. odorata is also used in the production of vodka from Poland known as Zubrowka[28] *H. odorata* was used as tea by Native Americans to treat cough and sore throats[32]. The sweet smell is due to the presence of Coumarin, which is a natural anticoagulant and also toxic, causing liver injury and hemorrhages[33].

1.2.2 Chemical profile and bioactivity

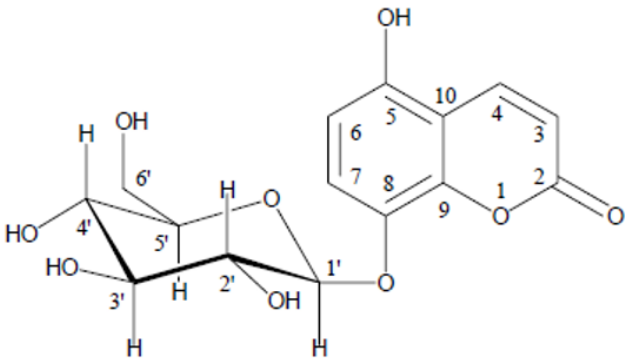
Swewart *et al.* conducted several studies on ethanol extracts and steam distillation extract of *H. odorata* to analyze the biosynthesis pathway of coumarin formation with radioactive isotopes. They demonstrated that cinnamic, o-coumaric, shikimic acids act as possible precursors. Further investigation showed that there is a higher chance of conversion of cinnamic acid into o-coumaryl glucoside as an intermediate compound.[34] Having the uncertainties about the intermediate compound formations, further studies by the same author showed that o-coumaryl glucoside and coumarin are both metabolic intermediates rather than the end products which supports the ortho-hydroxylation theory of coumarin biosynthesis[35].

A study conducted by Yoshitaka *et al.* on the root and aerial parts of *H. odorata* ethanol extracts examined by Gas Chromatography, and High-performance liquid chromatography (HPLC) resulted in the identification of several volatile compounds[28]. The essential oil was rich in coumarin, 10.3 % in the roots and 24.9 % in the aerial parts.[28]. Other main compounds identified were 3-methyl butanal, 3-methyl butanol, furfural, and aliphatic acid ethyl esters and minor constituents were cishex-3-enoic acid, trans-hex-2-enoic acid and Massoia lactone.[28]

Pukalskas *et al.* performed multistep fractionation and identified two compounds from aerial parts of *H. odorata* which have greater DPPH• and ABTS•+ free radical scavenging activity than natural antioxidant rosmarinic acid. The structures of these compounds was elucidated by NMR as 5,8-dihydroxycoumarin, and 5-hydroxy-8-O-α-D-glucopyranosyl-benzopyranone (**Figure 1.4**)[36].



5,8-dihydroxycoumarin



5-hydroxy-8-O-α-D-glucopyranosyl-benzopyranone

Figure 1.4 Radical scavengers isolated from Hierochloe odorata[36]

1.2.3 Pharmacological activity and toxicity of *H.odorata*

W. Luczaj *et al.* studied the effect of *H. odorata* extracts on rat liver intoxicated with ethanol. The metabolism of ethanol causes the generation of free radicals which damage the liver cell components and also decreases the activity of enzymes such as superoxide dismutase, catalase, glutathione peroxidase in cells via lipid peroxidation[37]. When *H. odorata* extracts were administered to the intoxicated rats, they successfully prevented the damage of liver cell membranes[37]. Dobrzynska *et al.* further confirmed these results showing that *H. odorata* extracts can prevent lipid peroxidation in rat liver cells when intoxicated with ethanol[38].

Traditionally, the Flathead Indians from western Montana were using *H. odorata* as an insect repellent by burning the plant from one end to produce enough smoke[39]. In a study conducted by Charles *et al.*, the insect repellent properties of *Hierochloe odorata* extracts were analysed[40]. The crude extracts produced from *H. odorata* via hydrodistillation showed higher levels of mosquito biting deterrence when compared to diethyltoluamide(insect repellent), due to the presence of Phytol and coumarin which are responsible for biting deterrency[40].

The antioxidant activity of acetone extracts of *H. odorata* was tested in refined rapeseed oil, and its oxidative deterioration was measured at different storage periods. It was shown that 0.05 to 0.2% concentrations of *H. odorata's* acetone extracts were efficient in preventing oxidation of rapeseed oil.[41] Zainuddin *et al.* studied the antioxidant activities of *H. odorata,* and *Salvia officinalis* acetone extracts in emulsions of lard and rapeseed oil and found that stability against autoxidation was significantly increased by both extracts and also in combination. Furthermore, oil stability increased with the addition of citric acid or ascorbyl palmitate in combination with plant extracts, which resulted in higher antioxidant activities in emulsions. They also observed that the removal of essential oils by steam deodorization produces bland, tasteless products and also the antioxidative activity became lower than that of the original extracts[42].

Damasius *et al.,* studied the effect of several spice extracts on cooked meat samples in order to analyze the effect on heterocyclic amines formation in meat samples since these Maillard reaction products are

known to be carcinogenic[43]. When 5,8-dihydroxycoumarin(5,8-DHC) from *H. odorata* extract was used in a meat sample, even though it possessed strong antioxidant properties, it could not reduce the formation of heterocyclic amines in cooked meat[43].

A study conducted by Nemeikaite *et al.* analysing the cytotoxic nature of 5,8-dihydroxycoumarin isolated from *H. odorata,* showed that this product possesses the oxidative stress-type cytotoxicity in Lamb kidney fibroblast cell lines by forming extracellular H_2O_2 through autoxidation of 5,8-DHC[44]. Slapsyte *et al.* studied the *in vivo* genotoxic effect of 5,8-DHC isolated from *H. odorata* in rat bone marrow, using different genetic end-points, i.e. chromosome aberrations (CAs) and micronuclei (MN)[45]. *In vitro* study of CAs and sister chromatid exchanges (SCEs) were performed on human lymphocytes and *in vivo* somatic mutations and recombination in *Drosophila melanogaster* wing cells. 5,8-DHC did not affect the increase of CAs frequency in rat bone marrow cells but influenced a significant increase of MN, and slight mutagenicity was observed after 120 hr of treatment in *Drosophila melanogaster.* 5,8-DHC influenced both Chromosome aberrations and Sister chromatid exchanges *in vitro* in human lymphocytes, indicating a clear dose dependency. Thus, 5,8-DHC is classified as weakly genotoxic both *in vivo* and *in vitro*[45].

1.3 The extraction of bioactive compounds from plants

Most of the bioactive compounds such as antioxidants are distributed in a wide range of polarity in various parts of plants, and different polarity solvents are being used in order to isolate active compounds[46]. These antioxidants can be divided into two groups by their polarity: low-polar antioxidants like α-Carotene, β-Carotene, lycopene, lutein and the polar antioxidants like flavonoids, phenolics, anthocyanins and lignins [47].

In order to isolate these bioactive compounds from plants, several sample preparation procedures are required.Sample preparation techniques like Initial drying, freeze drying of the plant materials are carried out, and later techniques like homogenization are applied to allow greater diffusion of solvents inside the plant matrix[46]. Thermal pre-treatment like drying at high temperatures can lead to a loss of bioactivity, while freeze-drying prevents the degradation of active compounds[48].

Extraction of bioactive compounds can be achieved by different methodologies depending on the target molecules and their physicochemical properties. Extraction through various solvents in the increasing order of their polarity is preferably used[46]. The variability of extracted components depends both on the solvent and the extraction conditions. Phenolic compounds are readily soluble in aqueous and alcoholic mixtures, and ethanol is mostly preferred for alcoholic extraction[49]. The non-polar solvents like acetone, hexane and others are used to obtain non-polar compounds[48][49]. Frequently applied techniques include: Soxhlet extraction, solid-liquid extraction (SLE), hydro-distillation, Ultrasound-assisted extraction, microwave-assisted extraction (MAE), pressurised liquid extraction (PLE), supercritical fluid extraction (SFE), enzyme-assisted extraction (EAE)[50].

Active compound enrichment is often performed by techniques such as ion chromatography, size-exclusion chromatography, column chromatography, electrodialysis, membrane filtration, nanofiltration[46]. Finally, phytochemical, qualitative and quantitative analysis is performed to identify and purify active compounds through various high throughput techniques like Gas Chromatography, High-performance liquid chromatography and mass spectrometry[49].

1.4 High Pressure and sustainable extraction methods

1.4.1 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a modern technique that involves the use of supercritical fluids to extract essential oils, active compounds from various plant and food sources as a replacement to the traditional extraction methods[51]. A supercritical fluid is formed when the solvent is heated and pressurized above its critical temperature (T_c), and critical pressure (P_c) **Figure 1.5.** This acquired property of the solvent has better transport properties than liquids and diffuse quickly throughout the solid materials and remarkably remove analyte[52].When the supercritical fluid reaches its critical point, the viscosity becomes similar to gas and density is similar to a liquid, but the diffusivity is in-between between fluid and liquid and is shown in **Table 1.2**. Thus, supercritical fluid has better solvating power than liquids and gases[53].

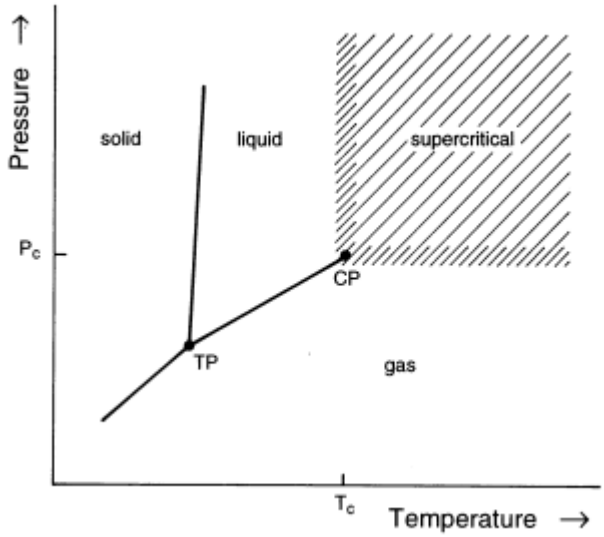


Figure 1.5 Pressure-temperature phase diagram of the supercritical fluid[54].

SFE is one of the clean, environmentally friendly and "green" techniques among sustainable methods of extraction[55].

Table 1.2 Order of magnitude of physical properties for gas, supercritical fluid and liquid[56]

Type of Fluid	Volumetric Mass Density ($g \cdot cm^{-3}$)	Viscosity (cP)	Diffusivity ($cm^{-2} \cdot s^{-1}$)
Gas	10^{-3}	10^{-2}	0.2
Supercritical state	0.5	5×10^{-2}	5×10^{-4}
Liquid	1	1	10^{-5}

Supercritical carbon dioxide extraction (SFE-CO_2) is one of the widely used method for extraction, in which the solvent CO_2 being Generally Recognised as Safe (GRAS) to use and also does not interfere with the active compounds during extraction[52]. CO_2 in nature has a relatively low critical temperature of 31.2ºC (T_c) and critical pressure of 7.38 MPa (P_c) and at its critical conditions acts as a non-polar solvent[57]. SFE-CO_2 extraction system consists of a CO_2 pump, CO_2 supply system, modifier pump**,** extraction vessel, pressure release valves, extract separator, CO_2 condenser and heat exchangers. The

schematic representation of SFE-CO_2 extraction is illustrated in **Figure 1.6**. The extraction vessels are fitted with pressure and temperature control system for stepwise depressurization of the system[58][52].

Since CO_2 behaves as a non-polar solvent at critical conditions, so there is a requirement of modifiers in order to extract a broader range of compounds such as phenolic and flavonoid compounds which are polar. Usually, ethanol or methanol is added along with the CO_2 through a different pump to the extraction cell[59]. Ethanol increases the recovery of polar components to a greater extent[60].

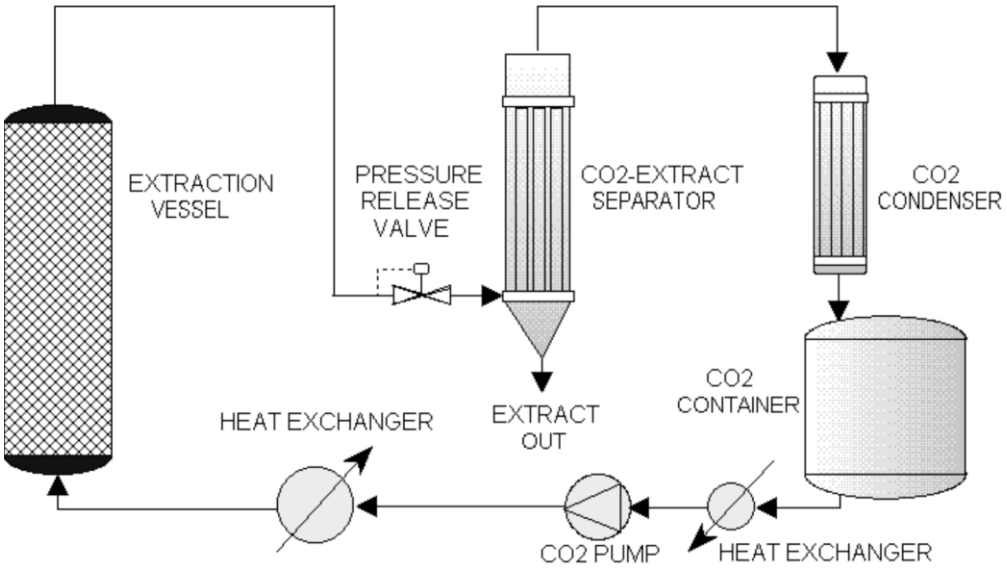


Figure 1.6. Schematic representation of the supercritical CO_2 extraction system[61]

Temperature and pressure play a prominent role in solvent properties like density and solubility[51]. When the pressure reaches its critical pressure, the solubility of compounds increases further decreasing the temperature. Moreover, at high pressures, the solubility of compounds increases with increase in temperature[62]. In general, the operating conditions to isolate compounds like antioxidant, flavour and aroma compounds is in the range of temperature from 40 to 70 ºC and pressure from 10 to 50 Mpa[59][63].

The application of supercritical fluids on food materials is ongoing from the late 1960s, and it been very successful in its approach. There has been developments and trends in supercritical extraction starting from most famous decaffeination of coffee beans, hop extraction for the beer industry to the isolation of nutraceuticals and some biorefining methods in recent years[62]. The advantages include high extraction efficiency, selectivity, absence of solvent residues. However, SFE results in extracts containing both active and inert compounds; thus, in certain cases, there is a requirement for fractionation. Scientists are coupling SFE with Supercritical fluid fractionation (SFF) or Supercritical fluid chromatography (SCF) to obtain highly active pharmaceutical products for commercial uses[62]. Pilar *et al.* isolated functional ingredients from rosemary by selective supercritical fluid fractionation preparative-SCF, which resulted in a 40% enrichment of antioxidants[64]. Fernando *et al.* isolated eicosapentaenoic acid and ocosahexaenoic acid from fish oil and algae oil, with 95% and 80% purity each by employing SCF[65]. Monica *et al*. showed a two-fold increase in thymol content when SCF technique was used to fractionate thyme extracts[66].

Some of the drawbacks of supercritical CO_2 extraction includes its high capital and operational costs since working at high pressure requires high energy requirements[59]. Moreover, the supercritical extraction results in more non-polar compounds due to the CO_2 properties, so there is a requirement of modifiers like ethanol to isolate some essential polar antioxidants and other compounds which further complicates the process in terms of purifying compounds[52].

1.4.2 Pressurized liquid extraction (PLE)

Due to reduced solvent usage and high extraction rates PLE is generally considered as a green extraction technique[67]. Extraction is performed at high temperature and pressures in order to achieve better efficiency when compared to traditional techniques like Soxhlet extraction and solid-liquid extraction, in which these techniques utilize more organic solvents and also require higher extraction time hurting the environment[68]. This technique is also referred to as accelerated solvent extraction (ASE), pressurized solvent extraction, high-pressure solvent extraction and subcritical solvent extraction. If water is used for extraction, then this technique is called Pressurized Hot Water Extraction and sub-critical water extraction[69].

The principle of PLE is that the desorption of analyte from the solid matrix and then it is diffused into matrix-solvent interference, later the analyte reaches the flowing solvent and is collected[70]. Extraction efficiency depends on the nature of the analyte and location within the matrix[70]. A schematic diagram of a pressurized liquid extractor is shown in **Figure 1.7.** The PLE extraction system consists of solvent storage units, extraction cell (where the sample is placed), programmable temperature and pressure controllers, heating oven and pumps, gas cylinder, collection vials and valves[71].

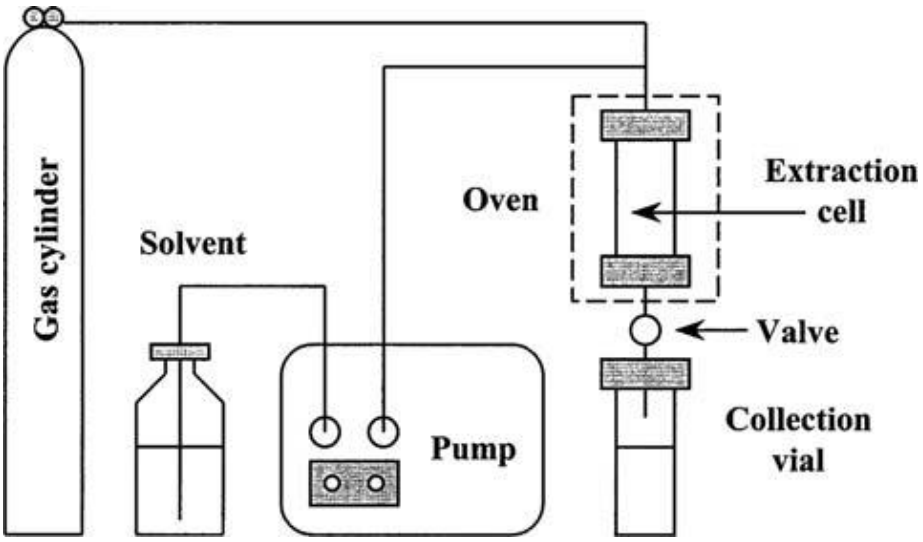


Figure 1.7. Schematic representation of a PLE system Adapted from Sjaak de *et al.* [71]

The selection of solvent is of high importance in order to recover a wide range of compounds having different polarity, most of the time ethanol and water is used in PLE system to extract polar compounds like phenolics, flavonoids, sugars, protein molecules. However, hexane, acetone and few other non-polar solvents can be used in a PLE system to isolate lipophilic compounds, and mixtures of these solvents can also be used for extraction[72]. The working procedure of the PLE system is by pumping solvent into extraction cell filled with biomass(sample) where the cell is controlled by the stipulated amount of pressure and time under certain heating conditions. Extraction can be either static or dynamic, and at the

end of extraction cycle nitrogen is purged for few minutes to remove remaining solvent from the extraction cell, and the analyte is collected in vials[73].Extractions can be performed by both static and dynamic mode of solvent system or in combination. In static mode, the prolonged exposure of solvent allows high recovery of analytes operating at high temperatures, but in dynamic mode, the solvent is pumped continuously increasing mass transfer but consuming more solvent than static mode[67].

Pressurized liquid systems operate in the temperature range from 40 to 200 ºC and pressure from 5 to 21MPa[67]. At these working conditions, the surface tension, viscosity of the solvents gradually reduce increasing the solvating power of the solvents, thus improving the rate of extraction. High temperatures help the penetration of solvent into the matrix and increase the solvent wetting ability, thus improves the diffusion of analyte into the solvent [68]. Pressure plays a crucial role in maintaining solvent in the liquid state when subjected to high temperatures above their boiling points[72]. The accumulation/clogging of the sample affects the extraction rate, so in order to facilitate the extraction efficiency several times filters, dispersing agents or drying agents are used, these agents are mostly inert material such as diatomaceous earth [68]. These dispersing agents reduce solvent consumption and also enhance solubility[70].

Subcritical water extraction uses hot water as solvent for extraction at a sufficient temperature and pressure (20 to 200 ºC and 22MPa), under these conditions the water remains in liquid form and the polarity of the water is considerably reduced. At these conditions, the surface tension and viscosity of water is reduced, which gives the capacity to extract medium polarity compounds[71][74]. The problem of steam degradation of valuable compounds in PLE systems can be eliminated using subcritical extraction system[73].

The advantages of pressurized liquid extraction are reduced solvent consumption, short extraction time, a wide range of working conditions, user-friendly, fully automated systems which give better reproducibility of results and multiple extraction cycles[70]. Some of the limitations are the high cost of equipment and operation costs. Moreover, working at higher temperatures can cause the degradation of active compounds in the analyte[75][70].

Kamali *et al.* and Golmakani *et al*. compared PLE with Soxhlet extractions to isolate the maximum amount of antioxidants and flavonoids from aerial parts of *Dracocephalum kotschyi* and *Scutellaria pinnatifida,* found that PLE extracts had higher radical scavenging ability than those from Soxhlet extraction and also had higher extraction yield[76][77]. Subcritical water extraction was performed in order to study the functional characterization of *Origanum vulgare* leaves at the highest extraction temperature of 200 ºC. However, the phenolic and antioxidant compounds were not degraded and showed similar activity like extracts at a lower temperature when analysed under chromatographic techniques[78]. These results indicate that PLE can be a highly efficient technique as compared to traditional extractions techniques like solid-liquid extraction, Soxhlet extraction.

1.4.3 Enzyme-assisted extraction (EAE)

Enzyme-assisted extraction (EAE) is a potential alternative method for extracting bioactive compounds from plant materials. Some phytochemicals or bioactive compounds are bound within the polysaccharide-lignin complex of the cell wall in plants with higher affinity and cannot be easily extracted by traditional extraction techniques[79]. Enzymes are highly specific in their action and act as catalysts which aid in biological reactions, and they also can disrupt or disintegrate cell walls which result to the recovery of those inbound bioactive compounds[80]. This utilization of enzymes can be

used as a pre-treatment step to breakdown cell walls of plant matrix[80]. Certain enzymes are being used for EAE like cellulase, α-amylase, pectinase, hemicellulase, and xylase based on the type of plant or food material being analyzed. Enzyme extractions, have shown higher recovery and reduced solvent usage than non-enzymatic extraction methods. Hence, pre-treating raw material with enzymes is an efficient way of extraction[81].

EAE is being applied successfully in various areas like juice processing, oil processing, flavor production, beer clarification, isolation of bioactive compounds from medicinal plants, antioxidant extraction, colorant productions, protein isolation [82]. Various researchers had conducted comparative studies between conventional extraction and EAE and found promising results. For example Meyer *et al*. compared the phenolic content of grape pomace after wine production found that enzyme-treated pomace gave a better recovery of phenolics (6.05mg/ml GAE) than Soxhlet treatment with 70% acetone (4.615mg/ml GAE)[83]. A similar study was performed on black currant pomace in which pectinolytic enzymes were treated for recovery of phenolic compounds. However, only when the particle size was reduced from 1000 µm to 125 µm, there was a notable increase in phenolic compounds[84]. Sheetal *et al.* applied EAE to isolate lycopene from tomato peels using cellulase and pectinases and saw a drastic increase of yield when compared with untreated peels with a high percentage of 224% with pectinase and 198% with cellulase[85]. Several parameters like extraction rate, recovery rate, solvent consumption were tested with non-enzymatic methods on the extraction of proteins from olive leaves treated with cellulase enzyme which gave significant results of increased recovery rate and reduced solvent consumption[86]. A higher amount of tocopherols were detected in oil when sesame seeds were extracted using aqueous enzyme assisted extraction when compared to conventional Soxhlet extraction[87].

The most important factors which affect the ability of enzymatic extractions are the surface area of materials, particle size, pH, working temperatures. It is also expensive to use a higher quantity of enzymes to process large amounts of raw materials[82]. Furthermore, at high temperatures, enzymes can undergo degradation and lose their activity[79]. EAE is a promising technique and adds benefit to the existing technologies.

1.4.4 Processing and technology of *P. fruticosa*

P. fruticosa contains several bioactive compounds like hyperoside, ellagic acid, catechins, rutin, quercetins etc., which have potential health effects like antioxidant property, anti-inflammatory effects, anticarcinogenic, antimicrobial, antifungal property. It would be beneficial if correct extraction techniques are employed to extract these compounds[2][21][5][20]. Kalpana *et al.* compared microwave assisted extraction (MAE), ultrasound-assisted extraction and Soxhlet extraction techniques on aerial parts of *Potentilla atrosanguinea* and found that microwave assisted extraction gave higher bioactive phenolic constituents when compare to Soxhlet extraction and also had higher radical scavenging activity in the ABTS•+ and DPPH• assays. Moreover, the solvent consumption and extraction time for MAE was found to be lower than Soxhlet extraction[88]. Most of the studies from several authors give hard evidence that usage of high pressure and temperature techniques give better recovery of potentially bioactive compounds from selected plant materials[89]. Hence, it would be a more sensible approach if these techniques are applied to *P. fruticosa* to isolate bioactive compounds. Currently, there is a lack of data in the literature of pressurized liquid extraction and Enzyme-assisted extraction on *P. fruticosa*. PLE may be directed to compounds that have high and medium polarity extraction, depending on the solvent used in the process. Moreover, there are no reports on the bio-refinery study of *P. fruticosa.*

1.4.5 Processing and technology of *H. odorata*

H. odorata shown to contain high amounts of coumarins, like 5,8-dihydroxycoumarin and 5-hydroxy-8-O-β-d-glucopyranosyl-benzopyranone, which are of antioxidant in nature[34][36]. Coumarins are naturally occurring flavor (like vanilla) substances, some of the medical applications are using coumarins as blood thinners which regulate in blood clotting[90]. Coumarins are also proven to be genotoxic, carcinogenic in mice and also results in liver toxicity[91]. Currently, coumarin is banned by the FDA from using it as a food additive[92]. However, coumarin in low doses can be used in pharmaceutical applications[90].

Grigonis *et al*. compared different extraction techniques like MAE, Soxhlet extraction, SFE-CO_2 techniques to isolate antioxidant compounds from *H. odorata* through multi-step enrichment process. Ethanol extractions gave high antioxidant recoveries when compared to less polar solvents acetone and ethyl acetate. SFE, when coupled with modifier solvent (30%), gave compounds of medium polarity. When two-step SFE extraction was performed, the extracts yielded with almost 22.5% of 5,8-dihydroxycoumarin and 5-hydroxy-8-O-β-d-glucopyranosyl-benzopyranone[93].

Currently, there is a lack of data in the literature for pressurized liquid extraction and enzyme-assisted extraction on *H. odorata*. PLE may be directed to compounds that have high and medium polarity extraction, depending on the solvent used in the process. Moreover, there are no reports on the bio-refinery study of *H. odorata.*

2 Materials and Methods

2.1 Plant material

The dried *P. fruticosa* (blossoms) and *H. odorata* plant materials were ground in a centrifugal high-speed roto mill at 8000 rpm (Restch ZM 200, Resch GmbH, Haan, Germany) into the fraction of 0.5 mm. The ground material was stored in hermetically dried glass jars, in a dark well-ventilated storage place until extraction and fractionation were performed.

2.2 Chemicals and reagents

Acetone, Analytical/HPLC grade hexane, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$, 99 %), catalytic tablet (K_2SO_4, $CuSO_4$), Conc. H_2SO_4, NaOH, H_3BO_4, Na_2CO_3, 2,2-Diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$, 99%), 3,4,5-trihydroxybenzoic acid (gallic acid, 99 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97 %), analytical/HPLC grade methanol (*Sigma-Aldrich, Poole, UK*), hexane (*PENTA Chemikalien, Mainaschaff, Germany*), Folin-Ciocalteu's phenol reagent 2M, nitrogen liquid(*AGA SIA, Riga, Latvia*), carbon dioxide, nitrogen gases (99.9%, *Gaschema, Jonava region,* Lithuania), cotton-wool (Bella-cotton, Poland), microcrystalline cellulose 20 µm (Sigma-Aldrich, St. Louis, MO, USA), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), sodium carbonate (99.5 %, AppliChem, Darmstadt, Germany), ultrapure water obtained by Millipore purification system (Billerica, MA, USA), ethanol (99.5%) (VWR Chemicals, Fontenay-sous-Bois, France).

2.3 Determination of chemical composition

2.3.1 Determination of Ash Content

Ground *P. fruticosa* and *H. odorata* in a fraction of 0.5 mm and weight of 1±0.1 g were placed in a dry constant weight crucible. Experiments were performed in triplicates. Crucibles with ground samples were placed on the electric hotplate and heated until the smoke stopped to form. After this, crucibles with samples were transferred and kept in the muffle under 600-650 °C temperature. Ash (mineral) content (%) was calculated using **Equation 1** below and expressed in a g/100 g of dry weight (DW):

$$\boldsymbol{x = \frac{(k_1 - k_2) \times 100}{(k_1 - k)}}\text{; g/100 g of (DW); } \textbf{(1)}$$

where: k – the weight of the crucible, g; k_1 – the weight of the crucible with the sample before drying, g; k_2 – the weight of the crucible with the sample after drying, g.

2.3.2 Determination of oil content by Soxhlet-Hex extraction

Ground *P. fruticosa* and *H. odorata* in a fraction of 0.5 mm and weight of 2±0.01 g were placed in cellulose extraction thimbles. Soxhlet extraction was performed in automated Soxhlet extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany). Cellulose extraction thimbles were inserted into an inner tube of Soxhlet extraction apparatus for the extraction. All extractions were performed in triplicates, and the non-polar fraction was isolated using hexane solvent. Total extraction time was 360 min, extraction rate one cycle per 5 min, and the temperature was 80º C. Hexane was evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland). After the organic solvent evaporation, extracts were kept under nitrogen flow for 10 to 15 min to evaporate residues of hexane. Extract yields were determined gravimetrically (±0.001 g) and expressed as a % of dry weight.

2.3.3 Determination of Protein content by Kjeldahl method

The samples of each plant materials *P. fruticosa* (1.0± 0.05 g) and *H. odorata* (1.0 ± 0.05g) of 0.5 mm fraction were weighted to the Kjeldahl flask. The plant materials were heated in the flasks with 20 ml concentrated H_2SO_4 and the tablet of catalyst (K_2SO_3 3.4 g, $CuSO_4$ 0.4 g) for 2 hours until it becomes transparent. Then the solution was distilled using automatic steam distillation system under the following conditions –3 sec NaOH, 3 sec H_3BO_4, the time of distillation was 300 min, the intensity of the steam was 80 %. After distillation, the solution was collected into the flask, followed with the addition of Toshiro indicator and titration with 0.1 N HCl solution until the color changes from light green to violet. 20 ml Conc. H_2SO_4 was used as a control sample and analyzed in the same manner, which was described above. The protein content was calculated using the following **Equation 2** (with expression in a percentage):

$$\%P = \frac{(V_1 - V_2) \times N_{HCL} \times 1.4007 \times 6.25}{W}\text{; } \textbf{(2)}$$

where: V_1 – the volume of standard HCl required for the sample, ml; V_2 - the volume of standard HCl required for the blank, ml; N_{HCl} – normality of acid standard; 1,4007 – milliequivalent weight of Nitrogen*100; W – the weight of the sample, g, 6.25- conversion factor.

2.3.4 Determination of water content

Ground *P. fruticosa* and *H. odorata* in a fraction of 0.5 mm and weight of 0.6±0.05 g and 0.5±0.05g were placed in a dry, constant weight glasses with cap closed. Experiments were performed in triplicates, periodically stirred and dried in the oven at the 100-105 °C temperature. After 24 hr samples were cooled in the desiccator for 30 minutes and then weighted gravimetrically (±0.001 g) until the constant weight after 42 hrs of drying the water content (%) was calculated using the **Equation 3** below and expressed in a g/100 g of dry weight (DW)

$$x = \frac{(w_1 - w_2) \times 100}{(w_1 - w)}; \text{ g/100 g of DW; } (\mathbf{3})$$

where: w – the weight of the glass with a cap and rod, g; w_1 – the weight of the glass with a cap, rod, and the sample before drying, g; w_2 – the weight of the glass with a cap, rod and the sample after drying

2.4 Conventional extraction techniques

2.4.1 Solid-liquid extraction (SLE)

Solid-liquid extractions with different solvents (hexane, acetone, ethanol, and water) were performed in a thermostatically controlled shaker. *P. fruticosa* and *H. odorata* plant materials with a size of 0.5 mm and weight of 10 g were loaded into dry glass bottles. 100 ml of different solvents (hexane, acetone, ethanol, and water) was poured on the different ground plants. Glass bottles with solvents and ground samples were shaken at 800 rpm. All extractions were performed in Triplicates. Every extraction took 360 min and was performed hexane (at 60 °C), acetone (at 40 °C), ethanol (at 60 °C) and water (at 100 °C). After extraction, the bottles are rapidly cooled down, centrifugated (9000 rpm, 10 min) and then filtered (Whatman filter paper 1). All the organic solvents were evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland) and water extracts were freeze-dried. After organic solvent evaporation, extracts were kept under the nitrogen flow for 15 min to evaporate residues of organic solvents. All extracts were kept at -20 ºC protected from light until the next analysis. SLE-Hex, SLE-Ace, SLE-EtOH, SLE-H_2O extracts yields were determined gravimetrically (±0.001 g) and expressed in a % of dry weight DW.

2.4.2 Solid-liquid extraction (SFE-CO_2-SLE)

Solid-liquid extractions with different solvents (acetone, ethanol, and water) were performed in a thermostatically controlled shaker. *P. fruticosa* and *H. odorata* SFE-CO_2 residue material with a size of 0.5 mm and weight of 10 g were loaded into dry glass bottles. 100 ml of different solvents (acetone, ethanol, and water) was poured on the different ground plants. Glass bottles with solvents and ground samples were shaken at 800 rpm. All extractions were performed in Triplicates. Every extraction took 360 min and was performed acetone (at 40 °C), ethanol (at 60 °C) and water (at 100 °C). After extraction, the bottles are rapidly cooled down, centrifugated (9000 rpm, 10 min) and then filtered (Whatman filter paper 1). All the organic solvents were evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland) and water extracts were freeze-dried. After organic solvent evaporation, extracts were kept under the nitrogen flow for 15 min to evaporate residues of organic solvents. All extracts were kept at -20 ºC protected from light until the next analysis. SFE-CO_2-SLE-Ace, SFE-CO_2-SLE-EtOH, SFE-CO_2-SLE-H_2O extracts yields were determined gravimetrically (±0.001 g) and expressed in a % of dry weight DW.

2.5 Isolation of bioactive compounds from *P. fruticosa* and *H. odorata* by high-pressure extraction techniques

2.5.1 Pilot-scale supercritical CO_2 extraction (SFE-CO_2)

Supercritical CO_2 extraction was performed in a supercritical fluid medium and a big Pilot Scale extractor Helix extraction system (Applied Separation, Allentown, PA, USA) using 10L stainless steel extraction vessel. A surrounding heating jacket controlled the temperature of the extraction vessel. The volume of CO2 was measured by a digital mass flow meter in standard liters per minute (SL/min) at a standard state (PCO_2=100 kPa, TCO_2=20°C, ρCO_2=0.0018 g/mL) and the CO_2 flow was kept constant for all experiments were at 2 SL/min. The process consisted of static (30 min) and dynamic extraction steps. The extracts were collected into glass bottles and kept at -20 °C temperature before analysis.

The following conditions were applied to *P. fruticosa* (0.5 mm particle size) extraction: the pressure was set at 45 MPa, the temperature was set at 60°C and dynamic extraction time was for 6hr. The extraction vessel was filled with 2400 g of *P. fruticosa* ground material. For exhaustive extraction in this system, static and dynamic extraction times were prolonged to 30 min and 360 min, respectively.

The following conditions were applied to *H. odorata* (0.5 mm particle size) extraction: the pressure was set at 40 MPa, the temperature was set at 60°C and dynamic extraction time was for 4hr. The extraction vessel was filled with 2642.7g of *H. odorata* ground material. For exhaustive extraction in this system, static and dynamic extraction times were prolonged to 30 min and 240 min, respectively.

2.5.2 Pressurized liquid extraction (PLE)

Pressurized liquid extraction was applied to defatted Plant residues after supercritical carbon dioxide extraction. PLE was performed in a pressurised liquid extraction apparatus Dionex ASE 350 (Sunnyvale, CA, USA). The sample was placed in a Dionex stainless-steel extraction cell (2.9 mm diameter) which was equipped with a stainless-steel frit and a cellulose filter at the ends of the cell to avoid solid particles in the collection vial. During all performed extractions, the cells were preheated for 5−7 min to ensure that the sample reached thermal equilibrium at 10.3 MPa pressure and desired temperature before static extraction in 3 cycles. A flush volume of 100 % of the cell was used; finally, the cell was purged with nitrogen for 60 s to collect the extract in the collection vial. Organic solvents were removed from the extracts in the rotary vacuum evaporator at 40 °C, and the residue was finally dried in an incubator at 50°C. If the solvent contained water, it was removed by freeze drying.

For each extraction, dry stainless-steel extraction vessel (cm^3) was filled with diatomaceous earth and cellulose filters in each end. 10±0.01 g of SFE-CO_2 residue plant materials in a fraction of 0.5 mm were mixed with 10±0.01 g of diatomaceous earth and placed in the middle of the vessel, and diatomaceous earth was used as a dispersive agent and was extracted consecutively applying different polarity solvents, namely acetone, ethanol and water.

2.5.3 Experimental design

P. fruticosa (< 0.5 mm particle size), residue after SFE-CO_2 extraction was used to extract with the PLE system. The 10±0.0001 g of *P. fruticosa* residue was placed in a cell for extractions with acetone at different temperatures (60, 90, 120 °C) and static extraction durations (15, 30, 45 min). The central composite design was applied to achieve the highest yield and maximal TPC value. After acetone extraction at optimal conditions (62 °C, 45 min), *P. fruticosa* residue was used to extract with ethanol at

different temperatures (40, 60, 80 °C) and static extraction durations (15, 30, 45 min). The central composite design was applied to achieve the highest yield and maximal TPC value. After Ethanol extraction at optimal conditions (75 °C, 45 min), *P. fruticosa* residue was used to extract with water at different temperatures (100, 115, 130 °C) and static extraction durations (45 min) to obtain maximal yield and TPC contents. Diatomaceous earth was added to the samples (1:1 ratio).

In order to determine the optimum conditions of pressurized liquid extraction, two independent factors – time and temperature were selected using a rotatable central composite experimental design (CCD) with three levels for each variable. Thus, pressurized liquid extraction was optimized using a 3-level factorial design 3^2 studying the effect on extraction yield, total phenolic content (mg GAE/g extract). The effect of the independent variables on the response values was analyzed using simple error, considering a level of confidence of 95% for all the runs. Response optimization was carried out by the combination of experimental factors (pressure, temperature, time), looking for maximizing the response (yield, %).

All models were evaluated considering the per cent variation explained by the residual standard deviation (RSD), determination coefficient (R^2) and lack-of-fit test for the model from the analysis of variance table, as the significance criteria. Standardized Pareto chart was used to analyse the effect of each factor and its statistical significance for each response values at a 99% confidence level. Response surfaces were obtained by accepting significances at $p \leq 0.05$. By the combination of two experimental factors, looking to maximize yield, total phenolic content.

H. odorata residue after SFE-CO_2 extraction (0.5 mm particle size) was used to extract with the PLE system. The optimal conditions which were obtained for *P. fruticosa* was used to perform PLE extractions, i.e. for acetone extraction at optimal conditions (62 °C, 45 min), for ethanol extraction at optimal conditions (75 °C, 45 min) and water extraction at optimal conditions (130 °C, 45 min) were used respectively.

The PLE system was washed out between each extraction to avoid any carry-over from the previous run. After pressurized liquid extraction, extracts were evaporated using nitrogen flow and freeze dryer (Labconco Corporation, Missouri, USA), depending on the used solvent. Extracts were stored at -20 °C temperature and protected from the light until further analysis.

2.6 Enzyme-assisted extraction (EAE)

Enzyme-assisted extractions of PLE-water residues and SFE-CO_2 residues of *P. fruticosa* and *H. odorata* was performed by suspending 10±0.1g sample were loaded into dry glass bottles. To the bottle, 100 mL of 50 mmol/L sodium acetate buffer (pH 3.5) and 0.6 mL of cellulolytic (Viscozyme L) enzyme was added. These bottles were incubated in a thermostatically controlled shaker (800 rpm, 40ºC, 7 hr). After this process, these tubes were immersed into the boiling water bath for 10 min in order to stop the enzyme activity, rapidly cooled and centrifuged at 9000 rpm for 10 min. The Resulting water-soluble supernatants and water-non-soluble solid residues were collected, the supernatants were freeze-dried and kept at -20ºC until further use.

The *P. fruticosa* and *H. odorata* SFE-CO_2 residues after the treatment with EAE were subjected to 3-step Pressurized liquid extraction for further extraction of bioactive compounds. The same optimal PLE conditions which were used for the PLE initial plant material optimization was also used here.

2.7 *In vitro* antioxidant activity

2.7.1 Measurement of total phenols content (Folin-Ciocalteu method)

Total phenols content (TPC) of the SLE extracts of ***P. fruticosa*** and ***H. odorata*** were determined by using Folin-Ciocalteu assay with some modifications[94]. The working solution was prepared by diluting commercial Folin-Ciocalteu's reagent in distilled water (1:9, v/v). 750 µL diluted Folin-Ciocalteu's solution was mixed with 150 µL of the sample. MeOH was used for the blank. After 3 minutes, 600µL of Na_2CO_3 (75g/L) was added into the solution, left in the dark for 120 min at 25 °C.

QUENCHER: Total phenolic content (TPC) of ***P. fruticosa*** **and** ***H. odorata*** initial plant material and SFE-CO_2 residues were measured by directly applying it to the solid particles[95]. Since most of the samples had high antioxidant activity, they were diluted with inert material – microcrystalline cellulose. 750 µL diluted Folin-Ciocalteu's solution was mixed with 10 mg of sample and 150 µL distilled water in a test tube. After 3 minutes, 600 µL of Na_2CO_3 (75g/L) was added to neutralize the mixture, vortexed in the dark for 120 min at 25 °C, centrifuged at 4500 rpm 5 min.

Absorbance was measured at 760 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY, USA).

Gallic acid solutions were used to obtain the calibration curve in the 10µg/ml to 80µg/mL concentration range. Extracts (**Equation 4**) and QUENCHER (**Equation 5**) calibration curve equations:

$$f(x) = 0.0109x - 0.115; R^2 = 0.9858; (\mathbf{4})$$

$$f(x) = 0.0107x - 0.1017; R^2 = 0.9912; (\mathbf{5})$$

Total phenols content (TPC) was expressed in an mg of gallic acid equivalents (GAE) per g of extract or DW of plant material. All analysis was performed in six replicates.

2.7.2 $ABTS^{\bullet+}$ cation radical assay

The Trolox equivalents antioxidant capacity (TEAC) method was adapted from Re *et al*. with some modifications[96]. The phosphate buffered saline (PBS; 75 mmol/L, pH 7.4) was prepared by dissolving 8.18 g NaCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, 0.15 g KCL in 1 L distilled water. The $ABTS^{\bullet+}$ radical solution was prepared by reacting 50 mL of $ABTS^{\bullet+}$ (2 mmol/L PBS) and 200 L $K_2S_2O_8$ (70 mmol/L) and left for 15-16 hours in the dark at 25 °C before use. Then, the working solution was prepared by diluting $ABTS^{\bullet+}$ radical solution with PBS to obtain 0.700 (±0.01) AU at 734 nm. 1500 L of $ABTS^{\bullet+}$ solution was mixed with 25µ L of *P. fruticosa* and *H. odorata* and SLE extracts, SFE-CO_2 extracts, PLE extracts, EAE extracts or MeOH (blank) in an Eppendorf vial and left for 2 hours in the dark at 25 °C.

QUENCHER. For SLE, SFE-CO_2 residues quencher analysis, 10 mg of sample or cellulose (blank) was mixed with 1500µL of $ABTS^{\bullet+}$ solution and 25 µL MeOH, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. Absorbance was measured at 734 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY). Trolox solutions (25µL) at various concentrations (0-1500 mol/l) were used to obtain the calibration curve. Extracts (**Equation 6**) and QUENCHER (**Equation 7**) calibration curve equations:

$$f(x) = 0.0608x - 2.0335; R^2 = 0.9927; (\mathbf{6})$$

$$f(x) = 0.0594x - 0.5245; R^2 = 0.9936; (\mathbf{7})$$

TEAC of samples was calculated through dose-response curves for Trolox. Results were expressed as TEAC values are expressed in mg of Trolox per g of extract or DW of plant material. All analysis was performed in six replicates.

2.7.3 $DPPH^{\bullet}$ radical scavenging assay

$DPPH^{\bullet}$ radical scavenging assay method was adapted from Brand-Williams *et al*. with some modifications[97]. The working solution was prepared by mixing 1000 L $DPPH^{\bullet}$ methanolic solution (~ 89.7 mol/L, final absorption 0.800 ± 0.1 AU at 517 nm) and 500µ L of SLE extracts or MeOH (blank). The mixtures were kept for 2 hours in the dark at 25 °C.

QUENCHER. For initial plant material, SLE residues and SFE-CO_2 quencher analysis 10 mg of sample or cellulose (blank) was mixed with 500 L MeOH and 1000 L 89.7 mol/L $DPPH^{\bullet}$ methanolic solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. The absorbance of all the samples was measured at 517 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY, USA). Trolox solutions (500 L) in various concentrations (0-50 mol/L MeOH) were used to obtain the calibration curve. Extracts (**Equation 8**) and QUENCHER (**Equation 9**) calibration curves equitation:

$$f(x) = 1.3919x + 3.1271; R^2 = 0.996; (\mathbf{8})$$

$$f(x) = 1.4797x - 2.2; R^2 = 0.9893; (\mathbf{9})$$

TEAC of samples was calculated using dose-response curves for Trolox. Results were expressed as TEAC values in mg of Trolox per g of extract or DW plant material. All analysis was performed in six replicates.

2.8 Chemical characterization of *P. fruticosa* and *H. odorata*

2.8.1 Analysis by UPLC/ESI-QTOF-MS

The Phytochemical profile of SFE-CO_2 and PLE extracts under optimal conditions of *P. fruticosa*, and *H. odorata* extracts were determined using an ultrahigh-performance liquid chromatography (UPLC) system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA), coupled to a quadrupole-time-of-flight mass spectrometer (q-TOF MS) Agilent 6540 that was equipped with an orthogonal ESI source (Agilent Jet Stream, AJS, Santa Clara, CA, USA), and controlled by a PC running the Mass Hunter Workstation software 4.0 (MH) from Agilent. A chromatographic method was carried out using a Zorbax Eclipse Plus C18 column (2.1 × 100mm, 1.8 μm particle diameter, Agilent Technologies, Santa Clara, CA) at 30 °C. The mobile phase composition was water (+0.1% formic acid, A) and acetonitrile (+0.1% formic acid, A). This method was developed for establishing phytochemical profiling. The gradient program was as follows: 0 min, 0% B; 12 min, 80% B; 14 min, 100% B; 16 min, 100% B; 17 min, 0% B. A flow rate of 0.5 mL/min and an injection volume of 5 μL were employed. The analysis was performed in negative ion and positive ion mode. The mass spectrometer was used in MS and MS/MS modes for the structural analysis of all compounds. MS parameters were the following: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 350 ºC; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto MS/MS modes were set to acquire m/z ranging between 50-1100 and 50-800 amu, respectively, at a scan rate of 5 spectra per second. Extracts were dissolved at a concentration of 1 mg/mL in methanol for liquid chromatography analysis. The compounds were identified using the METLIN database.

2.8.2 Analysis by GCxGC/TOF MS

SFE-CO_2 extracts of *P. fruticosa* and *H. odorata* were analysed by Two-Dimensional Gas Chromatography/Time-of-Flight Mass Spectrometry (GCxGC/TOF MS). Analyses were performed using LECO Pegasus 4D system, consisting of an Agilent 7890 GC hardware control system, a GERSTEL Multipurpose Sampler MPS (*Gerstel GmbH, Mulheim a der Ruhr, Germany*), TOF MS detector (LECO, St. Joseph, MI, USA). Volatile compounds were separated using primary BPX-5 column (30m, 250µm, 0,25µm film thickness) (*SGE Analytical Science, Australia*) linked with a secondary column, BPX-50 (1.580 m, 100 m i.d., 0.1 µm film thickness). Flow path was: GC oven - length 29.9 m, internal diameter 250 m, maximum temperature 360 °C, film thickness 0.25 µm; Modulator length – 0.1 m, internal diameter 250 m, maximum temperature 360 °C, film thickness 0.25 µm; Secondary column – 1.58 m, internal diameter 100 m, maximum temperature 330 °C, film thickness 0.1 µm; Detector - Modulator length – 0.21 m, internal diameter 100 m, maximum temperature 330 °C, film thickness 0.10 µm. The carrier gas was helium. Target flow was 1 mL/min. Front inlet septum purge flows 3 mL/min. Column front inlet purge time – 30 sec, flow 20 mL/min, the actual flow to the inlet during pre-run and during a run before purge time 11 mL/min.

The oven temperature program was as follows: 40 °C (1 min) then ramped to 300 °C at 7 °C/min (for 5 min); the secondary oven program was the following: 65 °C (0.2 min) then ramped to 295 °C at 15 °C/min (for 5 min). The transfer line temperature was 250 °C. The total GC method time – 1312 s. The mass range used for identification was from 35 to 550 m/z units, the TOF MS acquisition rate was ten spectra/sec. Detector voltage was set at 1550 V, and ion source temperature of 250 °C. Data from the GC×GC-TOFMS system was collected by Chroma TOF software v.4.22 (LECO).

Identification of compounds: The minimum similarity accepted was 800. Minimum molecular weight allowed was 33, the maximum was 550. For tentative identification of compounds, MS and RI methods have been used. By comparing their mass spectra with those know components stored in the Adams, mass spectral libraries. RI: by comparing obtained retention indexes with those reported from PubChem, chemspider Adams and NIST databases. Unique mass was used for the area calculation. The relative percentage of the chemical compounds of SFE-CO_2 extracts of *P. fruticosa* and *H. odorata* was expressed as a percentage by peak area.

2.9 Statistical analysis

Microsoft Excel 2016 was used for calculating mean values and standard deviations. GraphPad Prism 7.04 software was used for one-way analysis of variance (ANOVA), followed by the Tukey's test in order to compare mean values and their significance (p-value < 0.05).

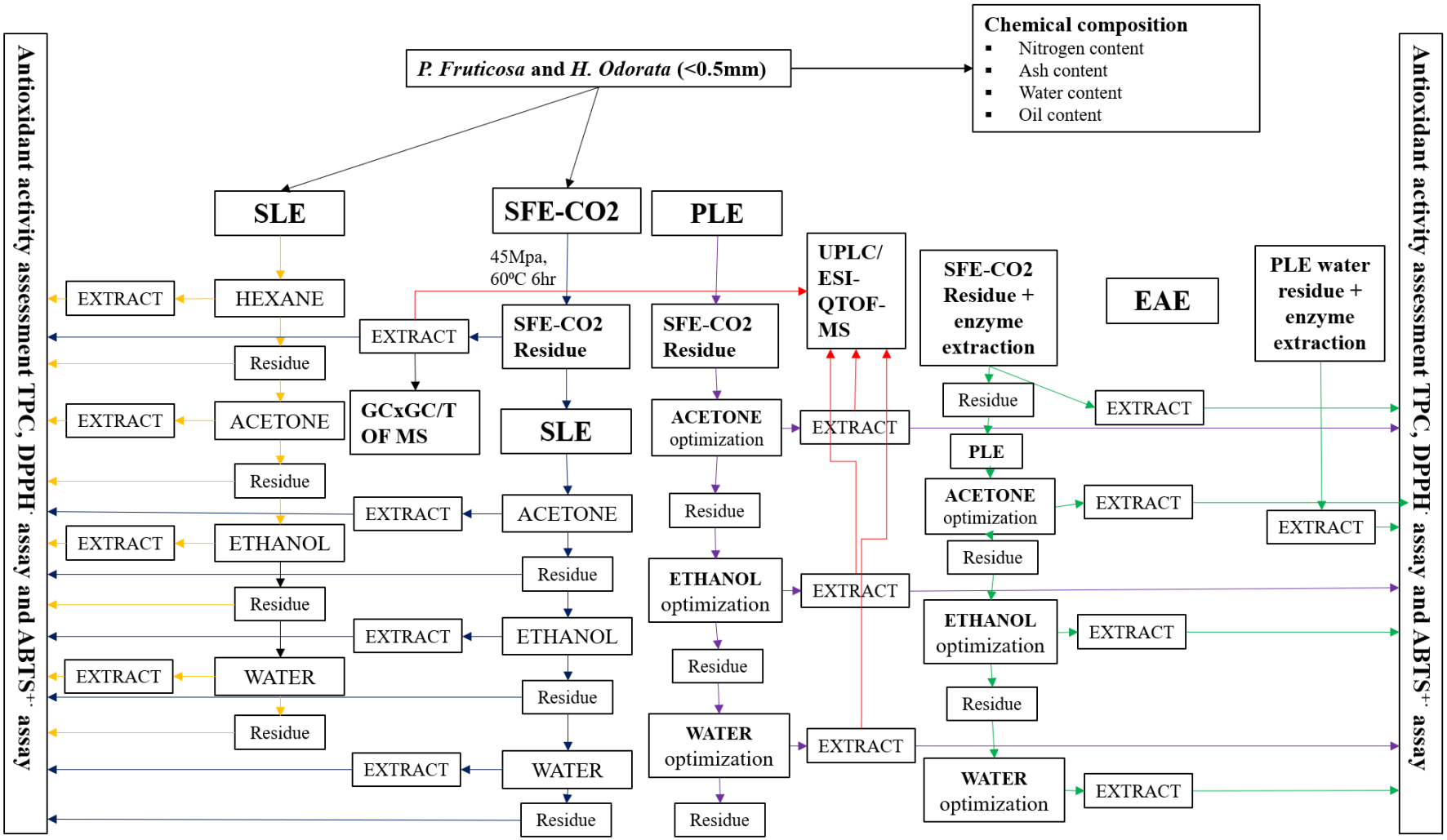


Figure 2.1 Biorefining scheme for *P.fruticosa* and *H.odorata*

3 Results and discussion

3.1 Chemical composition

In a first step of this study, the chemical composition of *P. fruticosa* and *H. odorata* was characterised and are presented in **Table 3.1.** The protein content of the samples ranged between 10.54% and 14.31%. *H. odorata* had the highest protein content of 14.31%. The oil content of the plant material was determined by Soxhlet-Hex extraction technique and ranged between 2.68% and 3.79 %. D. Grigonis *et al.* have also reported that *H. odorata* has 3.0 % oil content which is in range with the obtained value of this study[93]. According to Sengul *et al.*, an another species called *Potentilla anatolica*, has 3.22 % content of oil, which can be comparable to *P. fruticosa* [98].

Table 3.1 Chemical composition of *P. fruticosa* and *H. odorata*

	Potentilla fruticosa	***Hierochloe odorata***
Protein content, %	10.54±0.47	14.31±1.24
Oil content %	2.68±0.16	3.79±0.16
Ash content, %	4.10±0.04	5.45±0.10
Water content, %	9.59±0.16	7.21±0.07

Values represented as mean ± standard deviation.

The ash content of *P. fruticosa* and *H. odorata* was 4.10% and 5.45% respectively. According to Petr Macek *et al.* another species *Potentilla palustris* has 4.8% of ash content in their leaves[99]. The water content of *P. fruticosa* and *H. odorata* was 9.59 % and 7.21% respectively.

3.2 Conventional extraction techniques.

3.2.1 Solid-liquid extraction (SLE)

The effect of the solvent type on SLE yield was examined and is shown in **Table 3.2**. The results showed that different solvents significantly affected the extraction yield when subjected to 4-step SLEs. The highest extraction yield (14.37 %) for *P. fruticosa* was obtained using ethanol, and the lowest was on hexane (3.65%). In a study conducted by Miliauskas *et al.*, *tert*-Butyl methyl ether (non-polar solvent) extraction yield was lower compared to polar solvents ethanol and water extraction, which were used to extract the *P. fruticosa* residues after one 2-step extraction with *tert*-Butyl methyl ether. It indicates that *P. fruticosa* contains high proportion of polar compounds, and it is also evident in this research[15]. The highest extraction yield was obtained on *H. odorata* (7.67 %) with ethanol as a solvent, and the lowest was on hexane (4.85%) for initial plant materials.

Table 3.2. SLE extraction yields of *P. fruticosa* and *H. odorata*

Yield %	**SLE-Hex**	**SLE-Ace**	**SLE-EtOH**	**SLE-H_2O**
P. fruticosa	3.65 ± 0.23^{a}	6.06 ± 0.14^{b}	14.37 ± 0.29^{c}	8.31 ± 0.00^{d}
H. odorata	4.85 ± 0.07^{a}	6.84 ± 0.10^{b}	7.67 ± 0.36^{c}	6.87 ± 0.01^{b}

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$).

The extraction yields with acetone on *H. odorata* was higher than *P. fruticosa,* indicating that *H. odorata* contains more non-polar compounds. However, when ethanol and water were used the extraction yield was almost double in *P. fruticosa* (14.37 and 8.31 %, respectively) when compared to *H. odorata* indicating that *P. fruticosa* contains more polar compounds. In a study conducted by Pukalskas *et al.,* fractionations of *H. odorata* was carried out using methanol, acetone, hexane and water under different time factors indicated that methanol fractions had the highest yield followed by water and acetone and when extracted with n-hexane it gave 3% of yield which is also similar in this research[36].

3.3 Isolation of bioactive compounds from *P. fruticosa* and *H. odorata* by using high-pressure extraction techniques

3.3.1 Pilot-scale supercritical CO_2 extraction (SFE-CO_2)

Supercritical CO_2 extraction was performed in a big Pilot Scale extractor (Applied Separations, PA, USA). The following conditions were set to *P. fruticosa* (0.5 mm particle size) extraction condition: the pressure was set at 45 MPa, temperature at 60°C, for exhaustive extraction in this system, static and dynamic extraction times were prolonged to 30 min and 360 min, respectively. The extraction vessel was filled with 2400 g of *P. fruticosa* ground material. The yield (%) obtained for *P. fruticosa* is 2.46±0.12. The following conditions were set to *H. odorata* (0.5 mm particle size) extraction: the pressure was set at 40 MPa, the temperature was set at 60°C, static and dynamic extraction times were prolonged to 30 min and 240 min, respectively. The extraction vessel was filled with 2612.7g of *H. odorata* ground material and yield (%) obtained for *H. odorata* is 2.10±0.23. D. Grigonis *et al.* reported that supercritical fluid extraction of *H. odorata* at 35MPa and 40ºC resulted in a yield of 2.5% in a small-scale extractor[100]. Moreover, the authors studied the effect of extraction conditions (pressure, temperature) with ethanol (varying concentrations) as a modifier on the extract yield%. The authors compared SFE-CO_2, Soxhlet extraction (acetone) and Microwave-assisted extraction yields via one step and two step extractions. SFE-CO_2 with 20% ethanol as modifier showed higher extraction yield (6.3%) than Soxhlet yield (6%)[93].

3.3.2 Solid-liquid extraction for SFE-CO_2 residues (SFE-CO_2-SLE)

SFE-CO_2 residues of *P. fruticosa* and *H. odorata* were subjected to 3-step Solid-liquid extractions (SLE) with increasing polarity solvents, starting from acetone, ethanol and finally water. The results also showed that different solvents significantly affected the extraction yield, just like the SLE with initial plant materials and is presented in **Table 3.3**. It was also proved that the extraction yield of *P. fruticosa* and *H. odorata* was the highest using ethanol. The highest extraction yield was obtained on *P. fruticosa* (14.85 %) using ethanol, and the lowest was from SFE-SLE-Acetone (6.78%). The highest extraction yield (7.73 %) obtained from *H. odorata* was also using ethanol. On the other hand, extraction yields with Acetone on *H. odorata* was higher than *P. fruticosa,* indicating that *H. odorata* contains more non-polar compounds. However, when ethanol was used, the extraction yield increased to more than double in *P. fruticosa* (14.85%) when compared to *H. odorata,* indicating that *P. fruticosa* contains more polar compounds. A bar graph comparing yield% between SLE and SFE-

CO_2-SLE for *P. fruticosa* and *H. odorata* are presented in **Appendix 1**. in **Figure 3.10** and **Figure 3.11**

Table 3.3. SLE extraction yields of SFE-CO_2 residues of *P. fruticosa* and *H. odorata*

Yield%	SFE-SLE-Ace	SFE-SLE-EtOH	SFE-SLE-H_2O
P. fruticosa	6.78±0.02[a]	14.85±0.04[b]	8.32±0.02[c]
H. odorata	7.16±0.13[a]	7.73±0.29[b]	7.02±0.06[a]

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$)

3.3.3 Optimisation of Pressurized liquid extraction (PLE)

The PLE optimisation of *P. fruticosa* and *H. odorata* was performed on SFE-CO_2 residues of *P. fruticosa* and *H. odorata*. After removing most of the non-polar (lipophilic) constituents via SFE-CO_2, a 3-step fractionation procedure with solvents namely acetone, ethanol and water were applied to isolate the remaining polar constituents. Central Composite design CCD experimental design using response surface methodology (RSM) was applied to study the effect of two parameters, i.e. extraction time and temperature. By using a 2-level factorial design, the effect on extraction yield, total phenolic content (mg GAE/g extract) was studied in order to maximise it. The scheme of Pressurized liquid extraction under optimal conditions are represented in **Figure 3.1**. Similar 4-step fractionation of *Bergenia crassifolia* roots and leaves were carried out by Pressurised liquid extractions on defatted materials after SFE-CO_2 with solvents starting from hexane, acetone, ethanol and water and the obtained results are comparable with the present study[101].

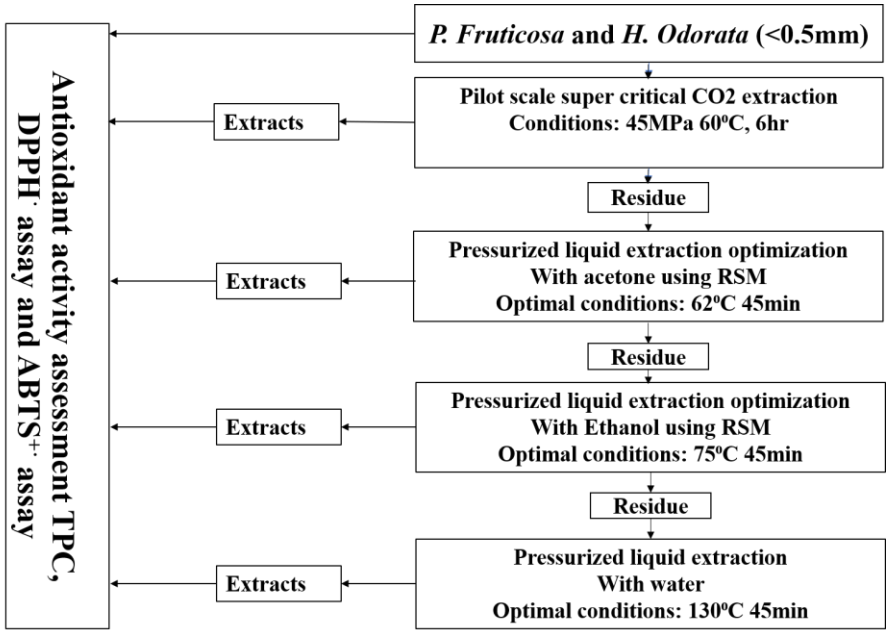


Figure 3.1 Schematic representation of PLE optimization of *P.fruticosa* and *H.odorata* SFE-CO_2 residues

3.3.3.1 PLE Acetone optimisation of *P. fruticosa* SFE-CO_2 residue:

Effect of time(t) and temperature(T) on the extraction yield of *P. fruticosa* SFE-CO_2 residue:

The PLE with acetone gave extract yield varied from 7.05±0.06 to 16.47±0.19 g/100 g DW. The highest yield with acetone (16.47±0.19 g/100 g DW) is obtained using 120 °C and extraction time of 45 min. The application of CCD with extraction time (15,30,45 min) and temperature (60, 90, 120 ºC) for the experiment produces the response surface design, which is presented in **Table 3.4.**The plot in **Figure 3.2** shows the influence of the two parameters on Acetone extraction of *P. fruticosa* SFE-CO_2 residues. A more abundant yield of the extract is produced by increasing the extraction temperature and extraction time. When the temperature is kept at 60 °C, increasing the extraction time from 15 min to 45 min generates a significant amount of yield (from 7.05 to 11.0 g/100 g DW). A better significant growth can be achieved at high temperatures (above 100 °C), when the extraction is prolonged (45 min), raising the temperature from 60 to 120 °C increases the extract yield from 11.0 to 16.47 g/100 g DW. When the time is kept constant at 15 min, increasing the extraction temperature from 60 to 120 °C generates almost twice as large yield of extract (from 7.05 to 14.17 g/100 g DW). The central point of experimental design, which was considered at 90 °C for 30min gave a yield ranged from 12.01 to 12.69 g/100 g DW.

Table 3.4. Experimental design conditions and the response of each extract studied for PLE Acetone optimisation of *P. fruticosa*

P.fruticosa			**Variables**		**Response factors**	
	Run	**Block**	**Time (min)**	**Temperature ºC**	**Yield (g/ 100g DW)**	**TPC (mg GAE/ g) DW [b]**
12	1	Block 1	30	60	9.98	60.69
13[a]	2	Block 1	30	90	12.45	46.41
4	3	Block 1	30	120	15.37	39.69
6	4	Block 1	15	120	14.17	48.28
1	5	Block 1	15	60	7.05	56.48
3[a]	6	Block 1	30	90	12.61	47.78
11	7	Block 1	45	60	11.0	75.02
9	8	Block 1	15	90	10.3	49.21
5[a]	9	Block 1	30	90	12.69	48.49
2	10	Block 1	45	90	14.32	58.14
7[a]	11	Block 1	30	90	12.54	47.75
8[a]	12	Block 1	30	90	12.01	48.70
10	13	Block 1	45	120	16.47	36.04
Optimal conditions			45	62	11.67	77.43

[a] Central point of experimental design; [b] mg gallic acid equivalents/g DW;

Similar PLE optimization technique was studied by Nora et al. on raspberry pomace in which the residues after SFE-CO2 were subjected to hexane optimization from 30 to 110 ºC, 10.3Mpa and 10,15 and 20 min extraction times and the yield varied from 13.6% to 14.8% which can be related to the present study[102]. Similar results can be replicated with lower yield and shorter extraction time. These response surface plots are of great benefit in making the extraction more efficient, it can be done by increasing the extraction time at lower temperatures or applying high temperature for a short extraction time.

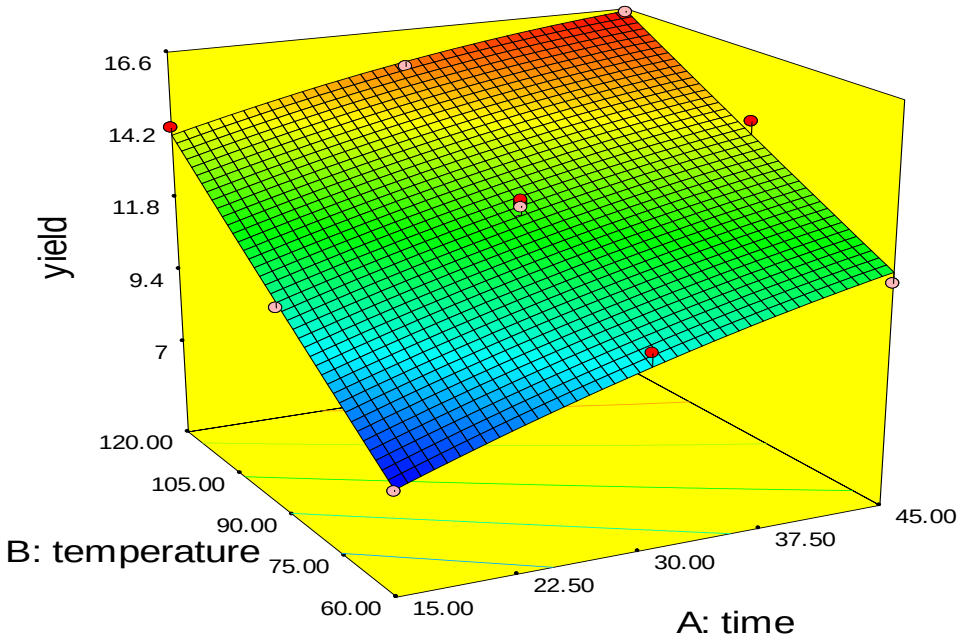


Figure 3.2. The response surface plots for the effects of PLE with acetone on the yield % of *P. fruticosa* SFE-CO_2 residue

Both independent variables (T and t) are significant in the evaluated central composite design (**Table 3.5**). Their importance can arrange them for the extract yield in the following order: extraction temperature > extraction time. ANOVA of the model shows that the model is significant according to the Student test (p<0.05); the lack of fit, however, is not significant. The determination coefficient is R^2=0.9876. An adequate precision of 38.44 indicates an adequate signal of the model. Thus, it can be used to navigate through the design space. Predicted and actual PLE Acetone extraction yields are also presented in **Appendix 2** in **Figure 3.12**. The dots of the predicted and actual values are close to 45° straight line; thus, it shows a normal distribution and confirms that the model is a perfect fit. The results from CCD can be used to compose the second-order polynomial model equation (**10**).

$$Yield\% = 12.51 + 1.71 \times t + 3 \times T - 0.41 \times T \times t - 0.32t^2 + 0.045T^2, \textbf{(10)}$$

Where T is temperature and t - extraction time

Table 3.5. Analysis of variance for the response surface model(quadratic)

Source	Sum of Squares	df	Mean Square	F-ratio	p-value
Model	72.4418586	5	14.4883717	111.435	<0.0001*
t-time	17.57881667	1	17.5788167	135.205	<0.0001*
T-temperature	53.88006667	1	53.8800667	414.412	<0.0001*
tT	0.680625	1	0.680625	5.23494	0.0560
tt	0.283123892	1	0.28312389	2.17761	0.1835
TT	0.005550082	1	0.00555008	0.04269	0.8422
Residual	0.910110632	7	0.1300158		
Lack of Fit	0.625710632	3	0.20857021	2.93348	0.1627**
Pure Error	0.2844	4	0.0711		
Cor Total	73.35196923	12			

* − significant values; ** − non-significant values

Effect of extraction conditions on TPC (Folin-Ciocalteu method) for *P. fruticosa* SFE-CO_2 residue:

The values of TPC ranged between 36.04 and 75.02 mg GAE/g of all pressurised liquid extracts under different extraction conditions with acetone and is presented in **Table 3.4**. The extracts obtained by pressurised liquid extraction at 60 ºC for 45min (75.02 mg GAE/g DW) and at 110 ºC for 30min (60.69 mg GAE/g) respectively showed the highest total phenolic contents. When the temperature is kept at 60 °C, increasing the extraction time from 15 min to 45 min generates the highest amount of TPC (from 56.48 to 75.02 mg GAE/g DW). A significant reduction can be found at high temperatures (above 100 °C); when the extraction is prolonged (45 min), raising the temperature from 60 to 120 °C decreases the TPC from 75.02 to 36.04 mg GAE/g DW. It indicates the degradation of phenolic compounds at a higher temperature. These response surface plots in **Figure 3.3** are of great benefit in making the extraction more efficient; it can be done by increasing the extraction time at lower temperatures or applying high temperature for a short extraction time. Nora *et al*, also studied the effect of temperature and extraction time on TPC content and saw an increase in TPC from 5.37mg GAE/g extract to 9.09 mg GAE/g extract at when temperature was increased from 30 to 110°C at 15 min extraction time[102].

Total phenolic content of the PLE extracts is expressed as milligrams of gallic acid equivalents per gram dry weight. According to ANOVA (Error! Reference source not found.) one effect, the quadratic v ariable of temperature, had a P-value <0.05. Thus, it is significantly different from zero at the 95.0% confidence level.

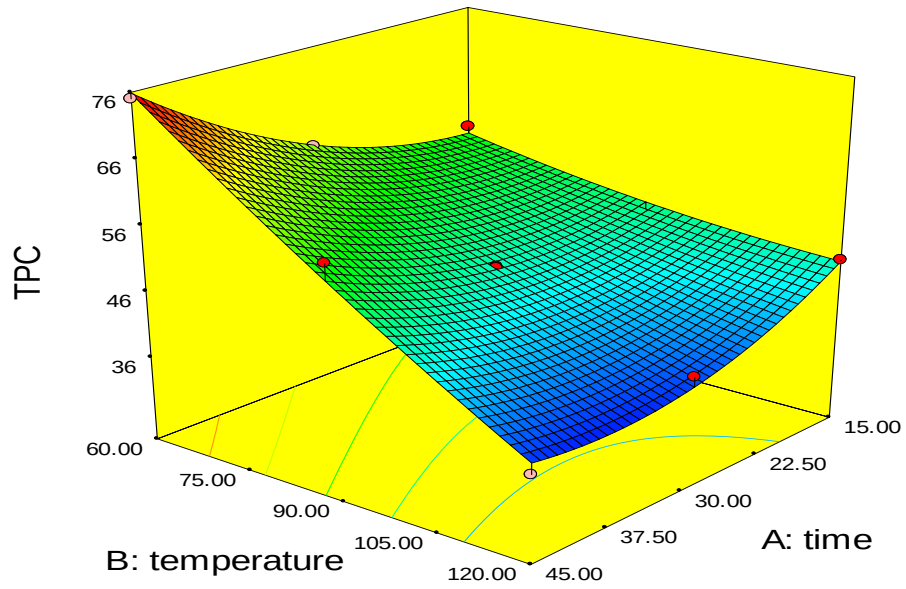


Figure 3.3. The response surface plots for the effects of PLE with acetone on the TPC of *P. fruticosa* from the residue after SC-CO_2

Table 3.6. Analysis of variance for the response surface model(quadratic)

Source	Sum of Squares	df	Mean Square	F-ratio	p-value
Model	1143.453526	5	228.690705	82.0423	< 0.0001*
t-time	38.66795521	1	38.6679552	13.872	0.0074
T-temperature	774.422565	1	774.422565	277.822	< 0.0001*
Tt	236.8736465	1	236.873646	84.9779	< 0.0001*
tt	62.66974017	1	62.6697402	22.4826	0.0021
TT	4.482308146	1	4.48230815	1.60802	0.2453
Residual	19.51231933	7	2.78747419		
Lack of Fit	15.90781466	3	5.30260489	5.88442	0.0599**
Pure Error	3.604504668	4	0.90112617		

* – significant values; ** – non-significant values

The determination coefficient is R^2=0.9832. An adequate precision of 33.601 indicates an adequate signal of the model. Thus, it can be used to navigate through the design space. Predicted and actual PLE Acetone TPC is also presented in **Appendix 2** in **Figure 3.13**. The dots of the predicted and actual values are close to 45° straight line; thus, it shows a normal distribution and confirms that the model is a perfect fit. The results from CCD can be used to compose the second-order polynomial model equation (**11**).

$$TPC = 48.25 + 2.54 \times t - 11.36 \times T - 7.70 \times T \times t + 4.76t^2 + 1.27T^2, \textbf{(11)}$$

Where T is temperature and t - extraction time

3.3.3.2 PLE Ethanol optimisation of *P. fruticosa* from PLE Acetone-residue:

Effect of time (t) and temperature (T) on extraction yield for *P. fruticosa* PLE Acetone-residue:

The residues obtained after acetone PLE optimisation were used for the PLE ethanol optimisation of *P. fruticosa*. The PLE extraction yield varies from 7.2 to 18.08 g/100 g DW. The highest yield with ethanol (18.08g/100 g DW) is obtained using 80°C temperature and extracting for 45 min. The application of CCD with extraction time (15,30,45 min) and temperature (40-80 ºC), for the experiment, produces the response surface design, which is presented in **Table 3.7**. Similar biorefining scheme was studied by Nora *et al*. on raspberry pomace in which the residues after SFE-CO2 were subjected to 50% ethanol optimization at 80 ºC, 10.3Mpa and 15min extraction time and the yield % varied from 21.6% to 25.5%. The yield% increased with the increase in the temperature and the extraction time[102]. However, in this study ethanol was used for optimisation and the yield % is comparatively lower than methanol. A similar trend can be seen in this study when the yield increases with increase in extraction time and temperature.

Table 3.7 Experimental design conditions and the response of each extract studied for PLE ethanol optimisation of *P. fruticosa* PLE Acetone-residue

			Variables		Response factors	
	Run	Block	Time (min)	Temperature ºC	Yield (g/ 100gDW)	TPC (mg GAE/ g)[b]
15	1	Block 1	30	80	14.9914	126.7239
2	2	Block 1	15	40	7.8282	72.7971
19[a]	3	Block 1	30	60	13.212	135.99
20[a]	4	Block 1	30	60	13.3181	124.2547
12	5	Block 1	45	60	17.2114	185.1409
4	6	Block 1	45	40	13.0897	131.364
21[a]	7	Block 1	30	60	13.3559	126.2071
11	8	Block 1	45	60	17.0953	183.4564
17[a]	9	Block 1	30	60	13.3725	125.103
13	10	Block 1	30	40	8.8603	75.9357
16	11	Block 1	30	80	15.4989	131.3298
5	12	Block 1	15	80	14.91104	86.9808
6	13	Block 1	15	80	14.9738	86.2786
10	14	Block 1	15	60	12.25	74.777
3	15	Block 1	45	40	13.1716	131.2991
7	16	Block 1	45	80	18.0437	172.6658
1	17	Block 1	15	40	7.2	66.3349
14	18	Block 1	30	40	8.8186	75.3311

9	19	Block 1	15	60	9.9465	78.9197
8	20	Block 1	45	80	18.0849	173.1984
18[a]	21	Block 1	30	60	13.2704	128.003
Optimal conditions			45	75	17.76	172.29

[a] Central point of experimental design; [b] mg gallic acid equivalents/g DW

These plots in **Figure 3.4** show the influence of two parameters on ethanol extraction of *P. fruticosa*. A more abundant yield of the extract is produced by increasing the extraction temperature and extraction time. When the temperature is kept at 60 °C, increasing the extraction time from 15 min to 45 min generates almost double the amount of yield (from 9.94 to 17.21 g/100 g DW). A better significant growth can be achieved at high temperatures (above 60 °C); when the extraction is prolonged (45 min), raising the temperature from 60 to 80 °C increases the extract yield from 17.21 to 18.08 g/100 g DW indicating that it is not a remarkable increase. When the time is kept constant at 15min, increasing the extraction temperature from 40 to 80 °C generates almost twice as large yield of extract (from 7.82 to 14.973 g/100 g DW). The central point of experimental design, which was considered at 60 °C for 30min gave a yield ranged from 13.21 to 13.37 g/100 g DW. Similar results can be replicated with lower yield and shorter extraction time. These response surface plots are of great benefit in making the extraction more efficient; it can be done by increasing the extraction time at lower temperatures or applying high temperature for a short extraction time.

Both independent variables (T and t) are significant in the evaluated central composite design (**Table 3.8**). Their importance can arrange them for the extract yield in the following order: extraction temperature > extraction time. ANOVA of the model shows that the model is significant according to the Student test (p<0.05); the lack of fit, however, is not significant.

The determination coefficient is R^2=0.9744. An adequate precision of 35.19 indicates an adequate signal of the model. Thus, it can be used to navigate through the design space. Predicted and actual PLE Ethanol extraction yields are also presented in **Appendix 3** in **Figure 3.14**. The dots of the predicted and actual values are close to 45° straight line; thus, it shows a normal distribution and confirms that the model is a perfect fit. The results from CCD can be used to compose the second-order polynomial model equation (**12**).

$$Yield\% = 13.17 + 2.47 \times t + 3.13 \times T - 0.62 \times T \times t + 1.12t^2 - 0.96T^2, \textbf{(12)}$$

Where T is temperature and t- extraction time.

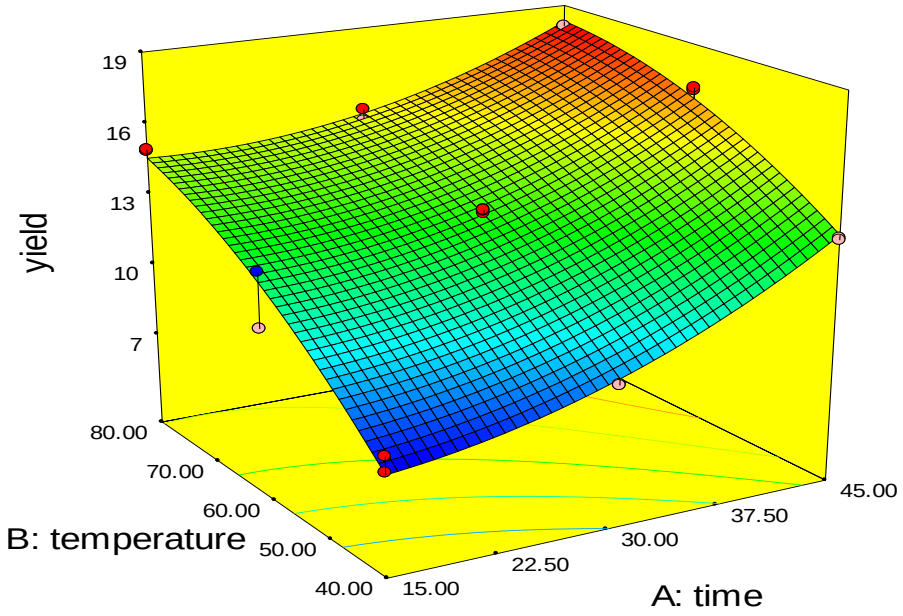


Figure 3.4. The response surface plots for the effects of PLE with ethanol on the yield% of *Potentilla fruticosa* from the residue after PLE-Acetone

Table 3.8 Analysis of variance for the response surface model(quadratic)

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Model	202.2292265	5	40.44584531	114.3407571	< 0.0001*
t-time	72.94950995	1	72.94950995	206.2289003	< 0.0001*
T-temperature	117.4084791	1	117.4084791	331.9147934	< 0.0001*
tT	3.111689204	1	3.111689204	8.796772494	0.0096
tt	6.136112141	1	6.136112141	17.34684249	0.0008
TT	4.535511881	1	4.535511881	12.82193161	0.0027
Residual	5.305961715	15	0.353730781		
Lack of Fit	2.295867073	3	0.765289024	3.050890216	0.0699
Pure Error	3.010094642	12	0.25084122		
Cor Total	207.5351883	20			

* − significant values; − non-significant values

Effect of extraction conditions on TPC (Folin-Ciocalteu method) *P. fruticosa* PLE Acetone-residue:

The values of TPC ranged between 66.33 and 185.14 mg GAE/g of all pressurised liquid extracts under different extraction conditions with ethanol and are presented in **Table 3.7**. Similar biorefining scheme was studied by Nora *et al*. on raspberry pomace in which the residues after SFE-CO2 were subjected to 50% ethanol optimization at 80 ºC, 10.3Mpa and 15min extraction time and the yield % varied from 21.6% to 25.5%. The yield% increased with the increase in the temperature and the extraction time[102]. However, in this study ethanol was used for optimisation and the yield % is comparatively lower than methanol. A similar trend can be seen in this study when the yield increases with increase in extraction time and temperature.

Table 3.7. The extracts obtained by pressurised liquid extraction at 60 ºC for 45min (185.14 mg GAE/g DW) and at 80 ºC for 45 min (173.19 mg GAE/g) respectively showed the highest total phenolic contents. When the temperature is kept at 60 °C, increasing the extraction time from 15 min to 45 min generates the highest amount of TPC (from 78.91 to 185.14 mg GAE/g DW). A significant reduction can be found at high temperatures (above 60 °C); when the extraction is prolonged (45 min), raising the temperature from 60 to 80 °C decreases the TPC from 185.14 to 172.66 mg GAE/g DW. It indicates the degradation of phenolic compounds at a higher temperature. These response surface plots in **Figure 3.5** are of great benefit in making the extraction more efficient; it can be done by increasing the extraction time at lower temperatures or applying high temperature for a short extraction time. There is a direct relation between the extraction parameters and the antioxidant capacity of the extracts, this is shown by Nora et al. on biorefining of raspberry pomace in which the TPC decreased from 38.95 to 31.75 mg GAE/g extract when the PLE temperature was raised from 30 to 70°C at same extraction time[102].

Total phenolic content of the PLE extracts is expressed as milligrams of gallic acid equivalents per gram. According to ANOVA (**Table 3.9**)one effect, the quadratic variable of temperature, had a P-value <0.05 thus, it is significantly different from zero at the 95.0% confidence level.

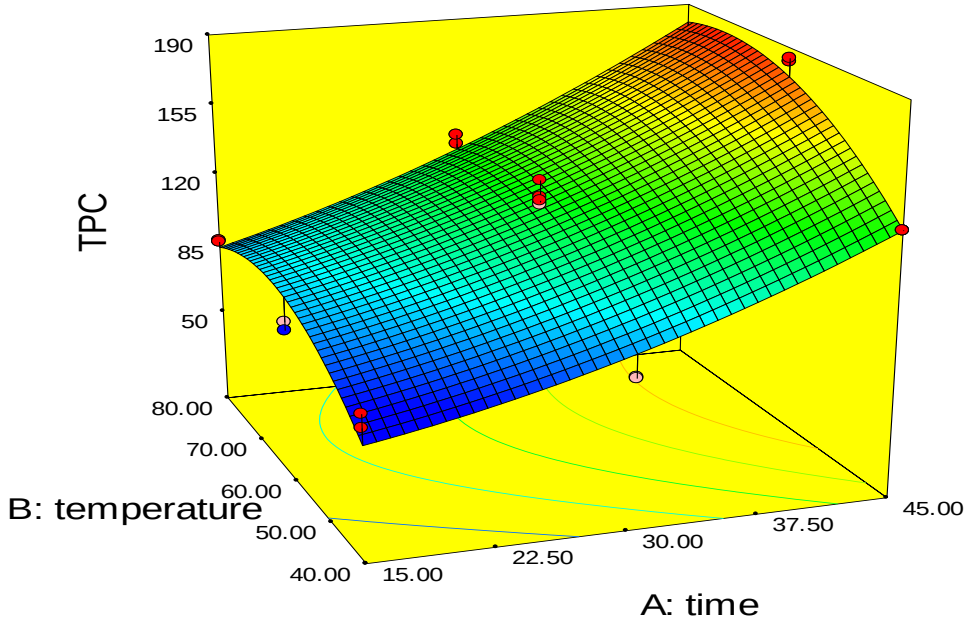


Figure 3.5 The response surface plots for the effects of PLE with Ethanol on the TPC of *P. fruticosa* from the residue after PLE-Acetone

Table 3.9 Analysis of variance for the response surface model(quadratic)

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Model	28288.54143	5	5657.708287	61.22614756	< 0.0001*
t-time	21763.19203	1	21763.19203	235.515219	< 0.0001*
T-temperature	4185.64271	1	4185.64271	45.29586277	< 0.0001*
tT	301.028504	1	301.028504	3.257646854	0.0912
tt	327.4034676	1	327.4034676	3.543069385	0.0793
TT	1967.291332	1	1967.291332	21.28948035	0.0003
Residual	1386.100999	15	92.40673328		
Lack of Fit	1254.605453	3	418.2018176	38.16419607	< 0.0001*
Pure Error	131.4955463	12	10.95796219		

* − significant values; ** − non-significant values

The determination coefficient is R^2=0.9533. An adequate precision of 23.84 indicates an adequate signal of the model. Thus, it can be used to navigate through the design space. Predicted and actual PLE ethanol extraction TPC are also presented in **Appendix 3** in **Figure 3.15**. The dots of the predicted and actual values are close to 45° straight line; thus, it shows a normal distribution and confirms that the model is a perfect fit. The results from CCD can be used to compose the second-order polynomial model equation (**13**).

$$TPC = 125.46 + 42.59 \times t + 18.68 \times T + 6.13 \times T \times t + 8.18t^2 - 20.06T^2, \textbf{(13)}$$

Where T is temperature and t- extraction time

3.3.3.3 PLE Water optimisation of *P. fruticosa* PLE-Ethanol residue:

Effect of time (t) and temperature (T) on the extraction yield of *P. fruticosa* from PLE-Ethanol residue

Using the PLE with water, the extract yield varies from 15.86 to 19.42 g/100 g DW. The highest yield with water (19.42 g/100 g DW) is obtained using 130°C temperature and extracting for 45 min, and the responses are presented in **Table 3.10.**

Table 3.10 Experimental design conditions and the response of each extract studied for PLE water optimisation of *P. fruticosa* PLE-Ethanol residue

		Variables		Response factor
Run	**Block**	**Time (min)**	**Temperature ^{o}C**	**Yield (%)**
1	Block 1	45	100	15.30
2	Block 1	45	100	16.42
3	Block 1	45	115	17.58
4	Block 1	45	115	18.87
5	Block 1	45	130	18.97
6	Block 1	45	130	19.86

Values represented as individual mean.

Here it shows the influence of temperature on water extraction of *P. fruticosa*. A more abundant yield of the extract is produced by increasing the extraction temperature while keeping the extraction time constant. When the time is kept constant at 45 min, increasing the extraction temperature from 100 to 130°C generates higher amounts of yield (from 16.42 to 19.86 g/100 g DW). Significant growth can be achieved at high temperatures (above 60 °C); when the extraction is prolonged for 45 min. Similar trend was observed on 3-step fractionation and optimization of buckwheat, the PLE-water extractions showed the maximum yield[103].

3.3.4 Pressurized liquid extraction of *H. odorata* SFE-CO_2 residue:

Table 3.11 PLE Yields at Optimal Conditions

	Optimal Conditions		Yield%	
	Time (min)	Temperature (^{o}C)	*P. fruticosa*	*H. odorata*
PLE-Ace	45	62	11.67±0.13	7.67±0.32
PLE-EtOH	45	75	17.76±0.38	15.27±0.75
PLE-H2O	45	130	19.42±0.62	18.60±0.61

Values represented as mean ± standard deviation.

The optimal conditions which were obtained for *P. fruticosa* were also used to carry out pressurised liquid extraction for *H. odorata* under same optimal conditions, and the results are presented in the

Error! Reference source not found.**,** the extract yield varies from 7.67 to 15.27 g/100 g DW under o ptimal conditions. The water extraction resulted in the highest yield % compared to other extraction solvents and conditions.

3.4 Enzyme-assisted extraction (EAE) of *P.fruticosa and H.odorata* SFE-CO_2 residues and PLE water residues

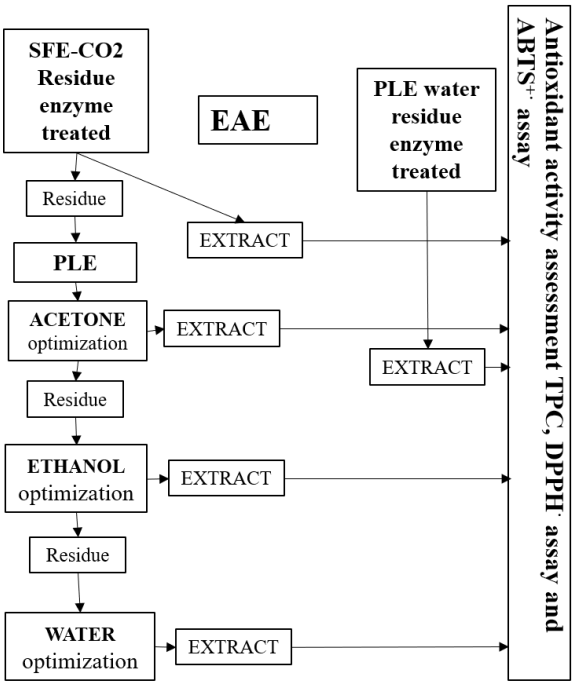


Figure 3.6 Enzyme-assisted extraction scheme

3.4.1 EAE of *P. fruticosa* and *H. odorata* PLE-water reside and SFE-CO_2 residue

Enzyme-assisted extractions of PLE-water residues and SFE-CO_2 residues of *P. fruticosa* and *H. odorata* was performed using enzyme Viscozyme L. The Resulting water-soluble supernatants were collected and their yield % was measured. The yield from 3-step PLE residues treated with enzyme were 7.25±0.58 and 5.80±0.16 for *P. fruticosa* and *H. odorata,* respectively. The yield % from SFE-CO_2 residues treated with enzymes were 15.60±0.52 and 13.72±0.30 for *P. fruticosa* and *H. odorata,* respectively and is presented in **Table 3.12**.

Table 3.12 Yield % of *P.fruticosa* and *H.odorata* PLE water residue and SFE-CO_2 residues treated with enzymes

Yield %	PLE water residue+ Enzyme	SFE-CO_2 + Enzyme
P. fruticosa	7.25±0.58	15.60±0.52
H. odorata	5.80±0.16	13.72±0.30

Values represented as mean ± standard deviation.

3.4.2 PLE of *P. fruticosa* and *H. odorata* for SCF-CO_2 defatted – Enzyme treated material:

The SFE-CO_2 defatted – Enzyme treated *P. fruticosa,* and *H. odorata* were subjected to 3-step PLE under optimal conditions which were used for SFE-CO_2 residues and the results are presented in the **Table 3.13,** the extract yield varies from 5.22 to 21.23 g/100 g DW under optimal conditions. The water extraction resulted in the highest yield % compared to other extraction solvents and conditions. A study was conducted by Vaida Kitryte *et al.,* on sea buckthorn pomace and seeds through 3-step fractionation using SFE-CO2, PLE-EtOH and EAE. The enzyme extraction increased the yield from 24% to 80% when compared to untreated residue materials and also indicated the recovery of glucose, fructose and maltose contents in the soluble fractions of EAE extracts, also indicated that presence of antioxidants in the soluble fractions of EAE[104]. Similar property is also reflected in this research by enzyme showing increased yield % during PLE water extractions.

Table 3.13 Enzyme treated PLE yields

	Optimal Conditions		Yield%	
Enzyme treated	**Time (min)**	**Temperature (°C)**	***P. fruticosa***	***H. odorata***
PLE-Ace	45	62	6.347 ±0.09	5.22±0.04
PLE-EtOH	45	75	14.936±0.17	14.87±0.08
PLE-H_2O	45	130	21.231±0.42	19.213±0.63

Values represented as mean ± standard deviation.

3.5 Comparison of yield % from SLEs and PLE techniques:

It is evident that temperature, pressure and time are the prominent factors which affects the yield % of the plant materials when the same solvents (hexane, acetone, ethanol and water) are used in different extraction techniques (SLE and PLE). It is represented in the **Figure 3.7** and **Figure 3.8**. When PLE (62°C and 45min) and SLE (40°C and 6hr) extractions with acetone for *P. fruticosa* was compared, the yield was almost double in which PLE had the yield of 11.67±0.13% and SLE had yield % of 6.06±0.14. It is a clear indication that higher yield is obtained when temperature and pressure is more with having less extraction time. The yield % was also more than double for water extractions with PLE (130°C and 45min) and SLE (100°C and 6hrs) for *P. fruticosa* having yield % of 19.42±0.62 and 8.31±0.0, respectively. However, when PLE (75°C and 45min) and SLE (60°C and 6hr) extractions with ethanol for *P. fruticosa* was compared, the increase in yield was not that significant in which PLE had the yield of 17.76±0.31% and SLE had yield % of 14.37±0.2, respectively. The effect of enzyme was only seen in water extraction and the yield was increased but not significantly.

When PLE (75°C and 45min) and SLE (60°C and 6hr) extractions with ethanol for *H. odorata* was compared, the yield was almost double in which PLE had the yield of 15.27±0.75% and SLE had yield % of 7.67±0.36. It is a clear indication that higher yield is obtained when temperature and pressure is more with having less extraction time. The yield % was also more than double for water extractions with PLE (130°C and 45min) and SLE (100°C and 6hrs) for *H. odorata* having yield % of 18.6±0.61 and 6.87±0.01, respectively. However, when PLE (62°C and 45min) and SLE (40°C

and 6hr) extractions with acetone for *H.odorata* was compared, the increase in yield was not that significant in which PLE had the yield of 7.67±0.32% and SLE had yield % of 6.84±0.10, respectively. The effect of enzyme was only seen in water extraction and the yield was increased but not significantly[104].

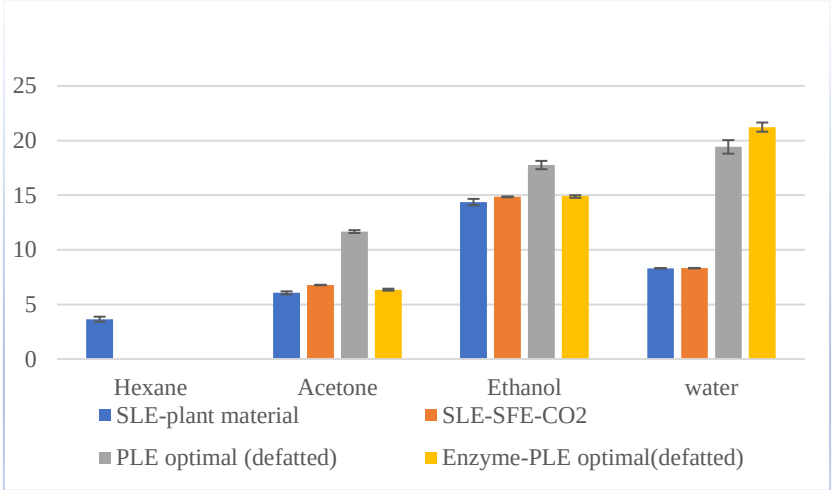


Figure 3.7 Yield % for *P. fruticosa* extracts under different methods

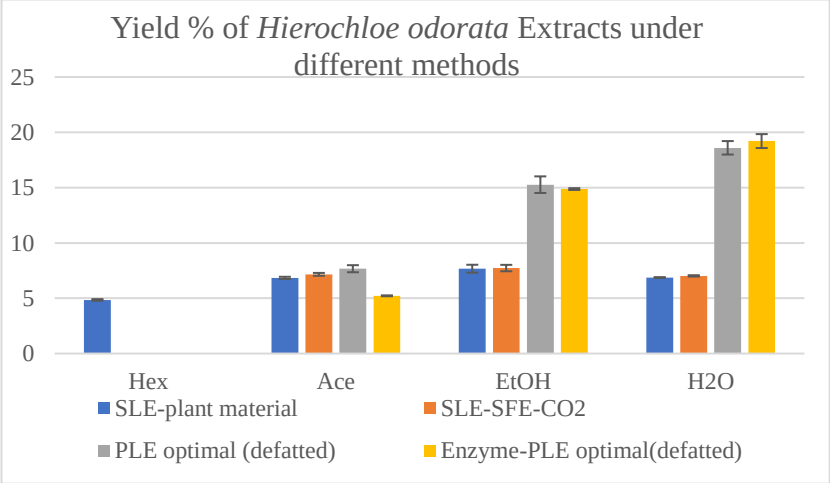


Figure 3.8 Yield % of *H. odorata* Extracts under different methods

3.6 *In vitro* antioxidant activity

3.6.1 Total phenolic content and Antioxidant activity for initial plant material and SLE

P. fruticosa and *H. odorata* were examined for their *in vitro* antioxidant activity. The TPC and antioxidant activity of *P. fruticosa* and *H. odorata* are shown in **Table 3.14** and **Table 3.15** .Total phenolic content of the initial plant material and extracts are expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 156.82±19.17 to 201.75±26.88 mg GAE/g DW of all initial plant materials, 1.58±0.08 to 115.45±3.07 mg GAE/g DW for SLE extracts, 13.42±0.28 to 191.42±0.38 mg GAE/g DW of SLE plant residues when subjected to solid-liquid

extractions (SLE) using different polarity solvents. The highest TPC was obtained in *P. fruticosa* initial plant material (201.75±26.88 mg GAE/g DW).

The values of ABTS•+ cation radical assay ranged between 159.81±9.39 and 250.55±56.08 mg TE/g DW of all initial plant materials and are expressed as milligrams of Trolox equivalents per gram dry weight, 1.87±0.26 to 93.09±9.00 mg TE/g DW for SLE extracts, 11.69±1.16 to 220.55±17.86 mg TE/g DW of SLE plant residues when subjected to solid-liquid extractions (SLE) using different polarity solvents. In ABTS•+ cation radical assay, the highest antioxidant activity was found in *P. fruticosa* initial plant material (250.55±56.08 mg TE/g DW).

The antioxidant capacity of the samples was also measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity. The values of DPPH• cation radical assay ranged between 113.84±3.61 and 161.59±17.73 mg TE/g DW of all initial plant materials, 2.26±0.30 to 139.44±16.04 mg TE/g DW for SLE plant residues, 3.98±0.21to 88.17±3.37 mg TE/g DW of SLE extracts. DPPH• radical scavenging results showed the highest antioxidant activity of *P. fruticosa* initial plant material (161.59±17.73 TE mg /g DW). In this case, initial material of *P. fruticosa* has a higher capacity to bind radicals (250.55±56.08 mg Trolox/g DW) than *H. odorata* (159.81±9.39 mg Trolox/g DW).

There is a significant difference in terms of antioxidant activity. It was observed that plant material has higher scavenging activity than SLE-residues and SLE-extracts. Thus, only a small part of compounds with antioxidant activity are extracted from the plant material using SLE of different polarity solvents by series of fractionation and is distributed among **SLE-Hex extract, SLE-Ace extract**, **SLE-EtOH extract** and **SLE-H2O extract**. It shows that the majority of the compounds is seen distributed in various extracts of SLE. **SLE-EtOH extract** of *P. fruticosa* showed the highest fraction of Phenolic compounds (115.45±3.07 mg GAE/g DW) and **SLE-Ace extract** of *H. odorata* showed the highest fraction of Phenolic compounds (61.61±4.19 mg GAE/g DW). These results also reflected in the antioxidant capacity of these extracts and also found that **SLE-EtOH extract** of *P. fruticosa* showed the highest ABTS•+ cation radical scavenging capacity (93.09±9.00 mg Trolox/g DW) and **SLE-Ace extract** of *H. odorata* showed the highest ABTS•+ cation radical scavenging capacity (65.55±3.84 mg Trolox/g DW). Also, the DPPH• radical scavenging results showed that **SLE-EtOH extract** of *P. fruticosa* showed the highest scavenging capacity (88.17±3.37mg Trolox/g DW) and **SLE-Ace extract** of *H. odorata* showed the highest radical scavenging capacity (50.70±1.20 mg Trolox/g DW). These results indicated that the majority of the phenolic and radical scavengers are present in ethanol extract of *P. fruticosa* and Acetone extract of *H. odorata.* Which are represented in **Table 3.15** and graphical representation of the Antioxidant activity of *P.fruticosa* and *H.odorata* SLE extracts and residues are presented in **Figure 3.16** and **Figure 3.17**, respectively in **Appendix 4**.

Michal Tomczyk *et al.* analysed the total phenolic content of *P. fruticosa* aerial parts extracted via water treated with ultrasonication for 45mins. The results showed that phenolic content was 116.3mg/g DW. However, in this study, the SLE-water extract showed Phenolic content of 31.75 mg/g DW. However, in this study, the plant material was subjected to a series of fractionation starting from Hexane, Acetone, Ethanol and water as solvents[20]. In a study conducted by Shan-Shan Wang *et al.* in which *P. fruticosa* leaves were extracted with 80% Acetone at 4ºC for 1hr gave a total

phenolic content of 144mg GAE/g of extract. However, in this study, when *P. fruticosa* blossoms were subjected to SLE with Acetone at 40ºC for 6hr gave a total phenolic content of 435.77 mg GAE/g of extract. It indicates that various factors, like time and temperature, play an essential role in obtaining phenolic compounds[5]. In a study conducted by G. Miliauskas where the shaker method for 2hr was applied on *P. fruticosa* to obtain methanol extracts, and the TPC was found to be 37.9mg GAE/g extract. However, similar polarity solvent ethanol was used in this research for TPC determination in which it was subject to the shaker method at 60 ºC for 6hr and 806.29mg GAE/g extract was obtained. Temperature, solvent and the type of method adapted plays a vital role in the recovery phenolic compounds[14]. Danmeng Yu *et al.* analysed ABTS•+ cation radical scavenging capacity of potentilla blossoms by extracting with 75% ethanol at 80ºC for 2hrs and obtained a range from 181 to 236 mg TE/g extract. However, in this study, when *P. fruticosa* was subjected to SLE ethanol extraction at 60ºC for 6hrs, the ABTS•+ radical capacity of the extract was found to be 658.19±19.79 mg TE/g extract[23].

In a study conducted by D. Bandoniene *et al.,* in which the aerial parts of H. *odorata* were subjected to Soxhlet extraction with acetone at 60ºC for 6hr and the TPC was measured to the obtained extracts and then it was found to be 22.00 mg GAE/g extract. However, in this study, the SLE-Acetone extract showed Total Phenolic content of 867 mg GAE/g extract[41].

Table 3.14. Total phenolic content and antioxidant capacity of *P. fruticosa* initial plant materials and SLE extracts and residues.

P. fruticosa			**Initial plant material**	**SLE-Hex**	**SLE-Ace**	**SLE-EtOH**	**SLE-H2O**
TPC mg GAE/g	**Extract**	**mg/g DW**	201.75±26.88^{a}	2.32±0.31^{b}	26.00±0.81^{c}	115.45±3.07^{d}	31.76±1.03^{e}
		mg/g extract	N/A	64.44±2.29^{a}	435.77±13.77^{b}	806.29±53.40^{c}	381.11±9.52^{d}
	Residue	**mg/g DW**	N/A	191.42±0.38^{a}	161.44±4.67^{b}	42.45±2.83^{c}	13.42±0.28^{d}
ABTS•+ mg TE/g	**Extract**	**mg/g DW**	250.55±56.08^{a}	1.87±0.26^{b}	85.76±2.53^{c}	93.09±9.00^{c}	28.064±4.59^{d}
		mg/g extract	N/A	43.43±11.88^{a}	422.29±5.14^{b}	658.19±19.79^{c}	350.27±17.14^{d}
	Residue	**mg/g DW**	N/A	220.55±17.86^{a}	113.69±9.67^{b}	37.32±1.20^{c}	18.33±1.04^{d}
DPPH• mg TE/g	**Extract**	**mg/g DW**	161.59±17.73^{a}	4.53±0.28^{b}	44.43±3.01^{c}	88.17±3.37^{d}	10.76±0.49^{e}
		mg/g extract	N/A	125.43±11.32^{a}	777.29±25.57^{b}	591.16±8.65^{c}	130.57±5.38^{a}
	Residue	**mg/g DW**	N/A	139.44±16.04^{a}	94.45±13.83^{b}	11.06±1.59^{c}	2.26±0.30^{c}

Table 3.15. Total phenolic content and antioxidant capacity of *H. odorata* initial plant materials and SLE extracts and residues.

H. odorata			**Initial plant material**	**SLE-Hex extract**	**SLE-Ace extract**	**SLE-EtOH extract**	**SLE-H2O extract**
TPC mg GAE/g	**Extract**	**mg/g DW**	156.82±19.17^{a}	1.58±0.08^{b}	61.61±4.19^{c}	46.94±3.26^{c}	15.92±0.81^{b}
		mg/g extract	N/A	32.45±0.82^{a}	867.98±38.17^{b}	551.98±30.48^{c}	229.65±5.68^{d}
	Residue	**mg/g DW**	N/A	153.08±2.91^{a}	84.55±2.51^{b}	45.17±2.93^{c}	24.45±2.14^{d}
ABTS•+ mg TE/g	**Extract**	**mg/g DW**	159.81±9.39^{a}	3.28±0.08^{b}	65.55±3.84^{c}	51.55±2.44^{d}	27.30±1.06^{e}
		mg/g extract	N/A	66.61±2.66^{a}	970.99±59.87^{b}	671.10±90.13^{c}	396.63±51.40^{d}
	Residue	**mg/g DW**	N/A	145.81±8.93^{a}	79.19±7.73^{b}	35.25±5.65^{c}	11.69±1.16^{d}
DPPH• mg TE/g	**Extract**	**mg/g DW**	113.84±3.61^{a}	3.98±0.21^{b}	50.70±1.20^{c}	37.73±2.72^{d}	11.09±0.81^{e}
		mg/g extract	N/A	86.42±7.29^{a}	763.58±49.08^{b}	465.55±4.12^{c}	165.30±10.64^{d}
	Residue	**mg/g DW**	N/A	94.68±5.53^{a}	70.17±2.40^{b}	20.84±2.29^{c}	7.15±0.34^{d}

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$)

3.6.2 Total phenolic content and Antioxidant activity of SFE-CO_2 Extracts, residues after SFE-CO2-SLE Extracts and SFE-CO2-SLE residue.

P. fruticosa and *H. odorata* residues after SFE-CO_2 extraction were subjected to SLE and was examined for their *in vitro* antioxidant activity. The TPC and antioxidant activity of *P. fruticosa* and *H. odorata* are shown in **Table 3.16** and **Table 3.17 .** Total phenolic content of the residues and extracts are expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 3.96±0.18 to 228.72±1.69 mg GAE/g DW of all SFE-SLE and SFE-CO_2 residues, 4.65±0.22 to139.02±12.42 mg GAE/g DW for SFE-SLE and SFE-CO_2 extracts, when subjected to solid-liquid extractions (SLE) using different polarity solvents. The highest total phenolic content was retained in *P. fruticosa* SFE-CO_2 residue (228.72±1.69 mg GAE/g DW) and SFE-CO_2 extracts showed a lower phenolic capacity of 5.65±0.74 and 4.65±0.22 mg GAE/g DW for *P. fruticosa* and *H. odorata* respectively.

The values of ABTS•+ cation radical assay ranged between 2.98±0.34 and 229.96±17.30 mg TE/g DW of all SFE-SLE and SFE-CO_2 residues when subjected to solid-liquid extractions (SLE) using different polarity solvents and are expressed as milligrams of Trolox equivalents per gram dry weight, 5.34±0.83 to 104.37±1.95 mg TE/g DW for SLE extracts. In ABTS•+ cation radical assay, the highest antioxidant activity was found in *P. fruticosa* SFE-CO_2 residue (229.96±17.30mg TE/g DW). The antioxidant capacity of the samples was also measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity. The values of DPPH• cation radical assay ranged between 2.175±0.154 to 179.88±8.92 mg TE/g DW for SFE-CO2-SLE plant residues, 5.92±0.45 to 96.44±5.56 mg TE/g DW of SFE-CO_2-SLE extracts. DPPH• radical scavenging results showed the highest antioxidant activity of *P. fruticosa* SFE-CO_2 residue (179.88±8.92 TE mg /g DW). It was observed that SFE-CO_2 residues had higher scavenging activity than SFE-SLE-residues and SLE-extracts. Thus, indicating that only a small part of compounds with antioxidant activity are extracted from the plant material using SFE-CO_2 extraction. When SFE-CO_2 residues were subjected to SLE using different polarity solvents by a series of fractionation and are distributed among **SFE-CO_2-SLE-Hex extract, SFE-CO_2-SLE-Ace extract**, **SFE-SLE-EtOH extract** and **SFE-CO_2-SLE-H2O extract**. It is shown that the majority of the compounds are seen distributed in various extracts of SFE-CO2-SLE. **SFE-CO_2-SLE-EtOH extract** of *P. fruticosa* showed the highest fraction of Phenolic compounds (139.02±12.42 mg GAE/g DW) and **SFE-CO_2-SLE-Ace extract** of *H. odorata* showed the highest fraction of Phenolic compounds (75.12±2.88 mg GAE/g DW). These results are also reflected in the antioxidant capacity of these extracts and also found that **SFE-CO_2-SLE-EtOH extract** of *P. fruticosa* showed the highest ABTS•+ cation radical scavenging capacity (113.12±3.57mg Trolox/g DW) and **SFE-CO_2-SLE-Ace extract** of *H. odorata* showed the highest ABTS•+ cation radical scavenging capacity (89.41±15.25mg Trolox/g DW). Also, the DPPH• radical scavenging results showed that **SFE-CO_2-SLE-EtOH extract** of *P. fruticosa* showed the highest scavenging capacity (96.44±5.56 mg Trolox/g DW) and **SFE-SLE-Ace extract** of *H. odorata* showed the highest radical scavenging capacity (51.21±6.84 mg Trolox/g DW). These results indicated that the majority of the phenolic and radical scavengers are present in ethanol extract of *P. fruticosa* and Acetone extract of *H. odorata.* Which are represented in **Table 3.16**.The graphical representation of the Antioxidant activity of *P. fruticosa* and *H. odorata* SFE-CO_2-SLE extracts and residues are presented in **Figure 3.18** and **Figure 3.19**, respectively in **Appendix 5**.

Table 3.16. Total phenolic content and antioxidant activity of *P. fruticosa* of SFE-CO_2 extracts and after SFE-CO_2-SLE extracts and residues.

P. fruticosa			**SFE-CO_2**	**SFE-SLE-Ace**	**SFE-SLE-EtOH**	**SFE-SLE-H2O**
TPC mg GAE/g	**Extract**	**mg/g DW**	5.65±0.74^{a}	39.45±0.95^{b}	139.02±12.42^{c}	14.06±1.04^{a}
		mg/g extract	231.80±13.67^{a}	585.81±31.66^{b}	944.95±25.12^{c}	168.80±7.00^{d}
	Residue	**mg/g DW**	228.72±1.69^{a}	171.71±8.05^{b}	28.37±3.07^{c}	5.36±0.33^{d}
ABTS•+ mg TE/g	**Extract**	**mg/g DW**	5.42±1.22^{a}	104.37±1.95^{b}	113.12±3.57^{c}	16.20±1.24^{d}
		mg/g extract	224.76±20.04^{a}	1511.11±31.11^{b}	744.11±21.51^{c}	197.66±52.25^{a}
	Residue	**mg/g DW**	229.96±17.30^{a}	122.92±4.83^{b}	19.32±3.05^{c}	2.98±0.34^{d}
DPPH• mg TE/g	**Extract**	**mg/g DW**	7.41±0.69^{a}	60.21±3.88^{b}	96.44±5.56^{c}	17.95±4.11^{d}
		mg/g extract	310.54±21.05^{a}	892.81±60.24^{b}	651.75±22.41^{c}	230.28±23.17^{d}
	Residue	**mg/g DW**	179.88±8.92^{a}	114.65±3.47^{b}	19.51±0.36^{c}	2.17±0.15^{d}

Table 3.17. Total phenolic content and antioxidant activity of *H. odorata* of SFE-CO_2 extracts and after SFE-CO2-SLE extracts and residues.

H. odorata			**SFE-CO_2**	**SFE-SLE-Ace**	**SFE-SLE-EtOH**	**SFE-SLE-H2O**
TPC mg GAE/g	**Extract**	**mg/g DW**	4.65±0.22^{a}	75.12±2.88^{b}	62.63±5.52^{c}	15.38±0.73^{d}
		mg/g extract	222.37±8.84^{c}	1059.63±22.81^{a}	814.22±30.97^{b}	218.34±18.14^{c}
	Residue	**mg/g DW**	140.39±2.89^{a}	71.53±2.38^{c}	4.62±0.24^{b}	3.96±0.18^{b}
ABTS•+ mg TE/g	**Extract**	**mg/g DW**	5.34±0.83^{a}	89.41±15.25^{b}	38.24±0.56^{c}	23.27±5.18^{d}
		mg/g extract	411.51±24.51^{abc}	1391.21±80.34^{d}	499.37±129.26^{ab}	329.44±35.22^{ac}
	Residue	**mg/g DW**	147.02±16.14^{a}	95.48±2.44^{b}	48.69±7.05^{c}	17.61±2.49^{d}
DPPH• mg TE/g	**Extract**	**mg/g DW**	5.92±0.45^{a}	51.21±6.84^{b}	30.67±3.34^{c}	20.41±0.89^{d}
		mg/g extract	291.65±41.86^{c}	782.43±13.11^{a}	442.48±32.41^{b}	293.89±16.07^{c}
	Residue	**mg/g DW**	99.77±19.69^{a}	77.89±4.27^{b}	12.72±1.01^{c}	2.31±0.121^{c}

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$).

3.6.3 Total phenolic content and Antioxidant activity of PLE Extracts under optimal conditions.

P. fruticosa and *H. odorata* residues after SFE-CO_2 extraction were subjected to PLE under optimal conditions and extracts were obtained, these extracts were examined for their *in vitro* antioxidant activity and are shown in **Table 3.18** and **Table 3.19.** Total phenolic content of the extracts is expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 36.78±2.72 to 172.79±3.29 mg GAE/g DW for PLE extracts, when subjected to Pressurised liquid extraction under optimal conditions using different polarity solvents. The highest total phenolic content was retained in *P. fruticosa* **PLE-EtOH extract** (172.79±3.29 mg GAE/g DW) and **PLE-H_2O** extracts showed a lower phenolic capacity of 36.78±2.72and 43.84±3.58 mg GAE/g DW for *P. fruticosa* and *H. odorata* respectively.

The values of ABTS•+ cation radical assay ranged between 60.21±1.19 to 184.47±10.88 mg TE/g DW for PLE extracts when subjected to Pressurized liquid extraction (PLE) using different polarity solvents and are expressed as milligrams of Trolox equivalents per gram dry weight. In ABTS•+ cation radical assay, the highest antioxidant activity was found in *P. fruticosa* **PLE-EtOH extract** (184.47±10.88 TE/g DW).

The antioxidant capacity of the samples was also measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity. The values of DPPH• cation radical assay ranged between 39.61±6.31to 173.36±32.93mg TE/g DW for PLE extracts when subjected to Pressurised liquid extraction (PLE). DPPH• radical scavenging results showed the highest antioxidant activity of *P. fruticosa* **PLE-EtOH extract** (173.36±32.93TE mg /g DW). The graphical representation of Antioxidant activity for *P. fruticosa* and *H. odorata* PLE extracts are presented in

Biorefining of *Cymbopogon nardus* was performed by Elodie Clain *et al*. by various extraction techniques like SFE-CO_2 and PLE, the residues after SF-CO_2 were subjected to 3-step fractionation similar to my studies in which they observed that type of solvent had an effect on isolation of phenolic and antioxidant capacity in which they found that PLE water extractions gave the highest phenolic content of 64.1±3.2 mg GAE/g extract and 443±25 mg TE/ g extract for DPPH• radical scavenging capacity and 702±47 mg TE/ g extract for ABTS•+ cation radical assay[105]. Similar trend of increase in antioxidant capacity was also seen in this research.

Table 3.18. Total phenolic content and Antioxidant activity of *P. fruticosa* PLE Extracts under optimal conditions

P. fruticosa	**Extract**	**PLE-Ace**	**PLE-EtOH extract**	**PLE-H2O**
TPC mg GAE/g	**mg/g DW**	77.43±0.37[a]	172.79±3.29[b]	43.84±3.58[c]
	mg/g extract	664.62±34.47[a]	969.92±18.56[b]	220.18±5.71[c]
ABTS•+mg TE/g	**mg/g DW**	166.77±7.56[a]	184.47±10.88[b]	74.95±7.18[c]
	mg/g extract	1402.35±78.94[a]	953.65±127.20[b]	365.57±7.34[c]
DPPH• mg TE/g	**mg/g DW**	85.62±5.80[a]	173.36±32.93[b]	50.21±2.32[c]
	mg/g extract	754.49±25.57[a]	740.30±18.16[a]	269.07±10.77[b]

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$).

Table 3.19. Total phenolic content and Antioxidant activity of *H. odorata* PLE Extracts under optimal conditions

H. odorata		**PLE-Ace**	**PLE-EtOH extract**	**PLE-H2O**
TPC mg GAE/g	**mg/g DW**	93.23±5.52[a]	69.97±5.10[b]	36.78±2.72[c]
	mg/g extract	1157.49±21.16[a]	413.99±22.86[b]	184.12±4.49[c]
ABTS•+ mg TE/g	**mg/g DW**	123.67±10.46[a]	102.65±4.86[b]	60.21±1.19[c]
	mg/g extract	1539.99±208.85[a]	700.08±70.98[b]	330.31±41.12[c]
DPPH• mg TE/g	**mg/g DW**	112.27±3.97[a]	96.47±5.69[b]	39.61±6.31[c]
	mg/g extract	1499.92±45.73[a]	670.87±46.84[b]	163.43±19.75[c]

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$).

3.6.4 Total phenolic content and Antioxidant activity of PLE-H_2O residues and SFE-CO_2 residues treated with enzymes.

P. fruticosa and *H. odorata* residues after PLE-H_2O extraction and SFE-CO_2 were subjected to Enzyme-assisted extraction, and the obtained supernatants were examined for their *in vitro* antioxidant activity. The TPC and antioxidant activity of *P. fruticosa* and *H. odorata* are shown in **Table 3.20.** Total phenolic content of these enzyme extracts is expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 4.95±0.22 to 12.69±2.35 mg GAE/g DW for all PLE-H_2O and SFE-CO_2 enzyme extracts, when subjected to EAE. The highest total phenolic content was retained in *P. fruticosa* **PLE-H_2O residue Enzyme extract** (12.69±2.35 mg GAE/g DW) and **SFE-CO_2 residue Enzyme extract** showed a lower phenolic capacity of 4.95±0.22and 11.60±1.74 mg GAE/g DW for *P. fruticosa* and *H. odorata* respectively. The values of ABTS•+ cation radical assay ranged between 8.96±1.19 and 24.44±2.40 mg TE/g DW of all PLE-H_2O and SFE-CO_2 enzyme extracts when subjected to Enzyme-assisted extractions (EAE) and are expressed as milligrams of Trolox equivalents per gram dry weight. In ABTS•+ cation radical assay, the highest antioxidant activity was found in *P. fruticosa* **PLE-H_2O residue +Enzyme treated extract** (24.44±2.40 mg TE/g DW). The TPC increased from 21.11 to 21.14mg GAE/g extract after treating blackcurrant pomace with Viscozyme enzyme on residues after SFE-CO_2 and before SFE-CO_2[106].

Table 3.20 Total phenolic content and Antioxidant activity of PLE-H_2O residues and SFE-CO2 residues treated with enzymes.

P. fruticosa		**PLE-H_2O residue Enzyme treated extract**	**SFE-CO_2 residue Enzyme treated extract**
TPC mg GAE/g	**mg/g DW**	12.69±2.35	11.60±1.74
	mg/g extract	41.11±1.52	73.65±2.62
ABTS•+ mg TE/g	**mg/g DW**	24.44±2.40	8.96±1.19
	mg/g extract	54.61±3.81	62.96±14.85
DPPH• mg TE/g	**mg/g DW**	15.20±0.34	22.18±1.78
	mg/g extract	83.34±0.91	129.21±11.32
H. odorata		**PLE-H_2O residue Enzyme treated extract**	**SFE-CO_2 residue Enzyme treated extract**
TPC mg GAE/g	**mg/g DW**	8.78±0.80	4.95±0.22
	mg/g extract	34.31±3.51	36.58±0.91
ABTS•+ mg TE/g	**mg/g DW**	13.10±0.99	9.29±0.23
	mg/g extract	42.61±5.60	68.86±17.89
DPPH• mg TE/g	**mg/g DW**	7.01±0.51	11.25±0.60
	mg/g extract	22.16±4.20	82.60±14.14

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey 's test, $p < 0.05$).

This indicates that effect of enzyme did not significantly increase the TPC content, similar results can also be seen in this study. The antioxidant capacity of the samples was also measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity. The values of DPPH• cation radical assay ranged between 7.01±0.51 to 22.18±1.78 mg TE/g DW for PLE-H_2O and SFE-CO_2 enzyme extracts. DPPH• radical scavenging results showed the highest antioxidant activity of *P. fruticosa* **SFE-CO_2 residue +Enzyme treated extracts** (22.18±1.78 TE mg /g DW). The graphical representation of the Antioxidant capacity of residues treated with enzyme of *P. fruticosa* and *H. odorata* extracts are in **Figure 3.9**

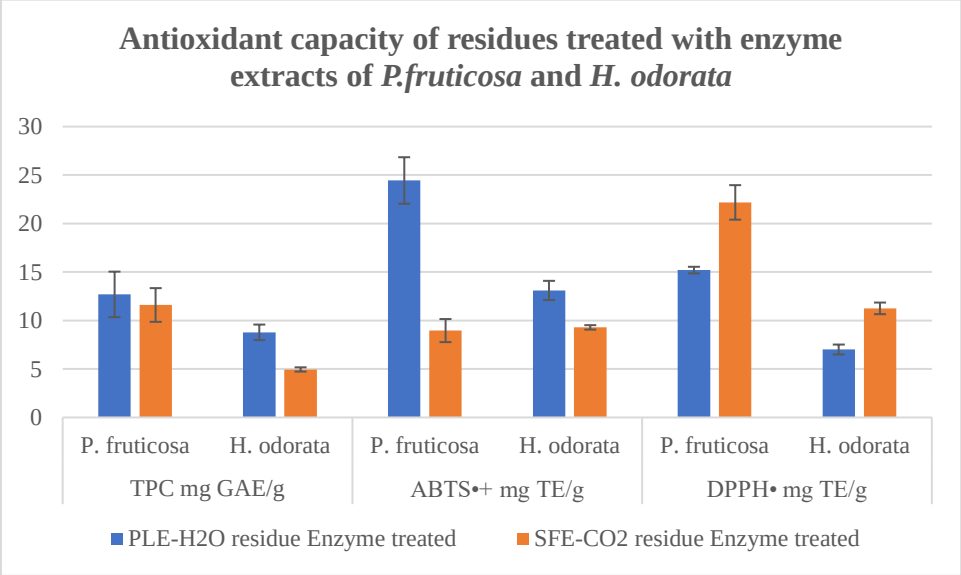


Figure 3.9 Antioxidant capacity of residues treated with enzyme of *P. fruticosa* and *H. odorata* extracts

3.6.5 Total phenolic content and Antioxidant activity of SFE-CO_2 residue-Enzyme treated PLE Extracts under optimal conditions.

P. fruticosa and *H. odorata* residues after SFE-CO_2 extraction were subjected to Enzyme-assisted extraction and the residues obtained after EAE were subjected to PLE under optimal conditions and extracts were obtained, these extracts were examined for their *in vitro* antioxidant activity and are shown in **Table 3.21**. Total phenolic content of the extracts is expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 37.89±2.68 to 144.86±2.77mg GAE/g DW for Enzyme + PLE extracts, when subjected to Pressurised liquid extraction under optimal conditions using different polarity solvents. The highest total phenolic content was retained in *P. fruticosa* **Enzyme+PLE-EtOH extract** (144.86±2.77 mg GAE/g DW) and **Enzyme+PLE-H_2O** extracts showed a lower phenolic capacity of 47.92±3.92 and 37.89±2.68 mg GAE/g DW for *P. fruticosa* and *H. odorata* respectively.

The values of ABTS•+ cation radical assay ranged between 62.18±1.23 to 155.10±9.15 mg TE/g DW for PLE extracts when subjected to Pressurised liquid extraction (PLE) using different polarity

solvents and are expressed as milligrams of Trolox equivalents per gram dry weight. In ABTS•+ cation radical assay, the highest antioxidant activity was found in *P. fruticosa* **Enzyme+PLE-EtOH extract** (155.10±9.15TE/g DW). The antioxidant capacity of the samples was also measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity. The values of DPPH• cation radical assay ranged between 42.91±8.21 to 145.77±27.69 mg TE/g DW for PLE extracts when subjected to Pressurised liquid extraction (PLE). DPPH• radical scavenging results showed the highest antioxidant activity of *P. fruticosa* **Enzyme +PLE-EtOH extract** (145.77±27.69TE mg /g DW). The Graphical representation of Antioxidant activity for *P. fruticosa* and *H. odorata* Enzyme treated SFE-CO_2-PLE extracts are in **Appendix 6** in **Figure 3.21** and **Figure 3.22**. Liza Laroze *et al*. studied the effect of enzymatic hydrolysis on antioxidant extraction in raspberry solid wastes. Raspberry wastes were treated with cellulase, hemicellulase and pectinase enzymes and showed best recovery of polyphenols and phenolic compounds. EAE with an water ethanol mixture for 18 hr at 50°C drastically increased the phenolic content to 35% and antioxidant capacity to 50% and 15% for DPPH and ABTS, respectively[107]. It was also evident in my research that the antioxidant capacity also increased when enzyme treated plant residues were subjected to 3-step PLE fractionation.

Table 3.21 Total phenolic content and Antioxidant activity of SFE-CO_2 residue-Enzyme treated PLE Extracts under optimal conditions.

P. fruticosa	**extract**	**SFE-CO_2 residue EAE- PLE-ace extract**	**SFE-CO_2 residue EAE -PLE-EtOH**	**SFE-CO_2 residue EAE-PLE-H2O**
TPC mg GAE/g	**mg/g DW**	41.85±0.47^{a}	144.86±2.77^{b}	47.92±3.92^{c}
	mg/g extract	513.22±21.65^{a}	786.20±15.22^{b}	212.91±4.55^{c}
ABTS•+mg TE/g	**mg/g DW**	89.81±2.65^{a}	155.10±9.15^{b}	81.96±7.88^{a}
	mg/g extract	1255.87±55.11^{a}	854.14±77.15^{b}	390.15±21.88^{c}
DPPH• mg TE/g	**mg/g DW**	46.32±3.07^{b}	145.77±27.69^{a}	54.90±2.54^{b}
	mg/g extract	685.41±31.24^{a}	671.45±21.78^{a}	199.57±31.22^{b}
H. odorata	**extract**	**SFE-CO_2 residue EAE- PLE-ace**	**SFE-CO_2 residue EAE -PLE-EtOH**	**SFE-CO_2 residue EAE-PLE-H2O**
TPC mg GAE/g	**mg/g DW**	63.42±3.75^{a}	68.12±4.97^{a}	37.89±2.68^{b}
	mg/g extract	1011.10±34.57^{a}	378.22±39.10^{b}	244.41±5.1^{c}
ABTS•+mg TE/g	**mg/g DW**	84.12±7.11^{a}	99.94±4.73^{b}	62.18±1.23^{c}
	mg/g extract	1387.41±141.66^{a}	651.66±85.01^{b}	278.56±49.78^{c}
DPPH• mg TE/g	**mg/g DW**	76.66±2.70^{a}	91.73±5.50^{b}	42.91±8.21^{c}
	mg/g extract	1352.45±87.65^{a}	590.18±65.10^{b}	145.11±23.60^{c}

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey ‘s test, $p < 0.05$).

3.7 Chemical characterisation of *P. fruticosa* and *H. odorata*

3.7.1 Analysis by GCxGC/TOF MS

Gas chromatography-time of flight mass spectrometry preliminary analysis of the SFE-CO_2 extract of *P. fruticosa* and *H. odorata* were analysed. Thirty-nine compounds were characterised and identified in *P. fruticosa* SFE-CO_2 extracts by comparison of the mass spectra of the elements with the pubchem, NIST library, and comparing their calculated retention indexes with those available in the literature. All 39 compounds are listed in **Table 3.22** with their retention time, name, calculated retention index, retention index from the reference and area. The difference between Retention Index obtained from analysis and the RI from reference literature were within the range of ±20 and the rest of the **Nineteen** identified compounds which had higher area % and more than the range of ±20 are presented in **Annex 8** in **Table 3.24**. The compounds which was >1% in their area were 2-pentylfuran (2.7074), phenol (2.0942) and Myrcene (1.1732). From Annex it was Hexanoic acid (4.3124) and coumarin (2.7762) in area %. 2-pentylfuran is volatile heterocyclic compound used as food additive and has a flavour of reverted soybean oil and formed by auto oxidation of linolenic acid[108]. Myrcene is acyclic monoterpene which occurs in the essential oils like lemon grass, hop, bay and used as fragrances in cosmetic industry, and has proven antimutagenic properties[109]. Hexanoic acid also known as caproic acid, it is a carboxylic acid naturally found in oil and several plant sources. It is used as hexanoate flavour substance to reconstitute the lost flavours of fruits and vegetables lost during processing, also in perfume industry[110]

Table 3.22 Results of the GC-MS analysis of *P. fruticosa* SFE-CO_2 extract.

R.T. (s)	Name	RI Calculated	RI Reference*	Database	Area %
475.7	1-Methylcyclopentanol	809.12	796	Pherobase	0.1602
483.8	Hexanal	814.93	801	adams	0.1425
586.5	Pentanoic acid	888.66	938-888	pubchem	0.0838
621.8	Heptanal	914	899-900	pherobase	0.0475
729.1	Benzaldehyde	991.03	961-996	pherobase	0.0067
734.5	Myrcene	994.9	991-994	pherobase	1.1732
739.3	Phenol	998.35	1002	pubchem	2.0942
740.1	2-pentylfuran	998.92	991	pherobase	2.7074
756.7	Hemimellitene	1011.1	996	pubchem	0.1205
763.4	2,4-Heptadienal	1016.1	1003	pubchem	0.0606
786.7	2-Hexenoic acid	1033.3	1042-1047	pubchem	0.3020
793.6	1,1'-Oxydipropan-2-ol	1038.4	1018-989	pubchem	0.0491
796.3	o-Cymene	1040.4	1020-1011	pubchem	0.0687
847.8	Levulinic acid	1078.4	1065.8	pubchem	0.0220
850.5	gamma-Caprolactone	1080.4	1081	pubchem	0.1362
855.2	Heptanoic acid	1083.8	1083-1071	NIST	0.6074

859	3,5-Octadien-2-one	1086.6	1072-1057	pubchem	0.1572
871.9	m-Cresol	1096.2	1084-1075	pherobase	0.0591
877.3	Undecane	1100.2	1100	adams	0.0304
899	Nonanal	1117.1	1108-1098	pherobase	0.1059
907	2-ethylhexanoic acid	1123.3	1129	pherobase	0.0975
976.6	2-Nonenal	1177.5	1162-1155	pubchem	0.03854
982.9	Octanoic acid	1182.4	1171	adams	0.5701
1028.6	Decanal	1219.1	1209	pubchem	0.09831
1100.2	Nonanoic acid	1278.3	1270	adams	0.3597
1140.7	3-Methyl-4-isopropylphenol	1312.5	1290	pubchem	0.02508
1143.9	Anethole	1315.3	1300	pubchem	0.01309
1145.9	Methyl (E)-4-decenoate	1317	1290	chemspider	0.06833
1152.4	Carvacrol	1322.7	1329	pubchem	0.04476
1161.5	Methyl decanoate	1330.7	1325	adams	0.0117
1185.4	Paroxypropione	1351.7	1349	chem spider	0.1004
1211.9	Decanoic acid	1374.9	1366	Adams	0.0924
1232.7	cis-3-tetradecene	1393.2	1396-1384	pubchem	0.2312
1239.5	Tetradecane	1399.1	1400	pubchem	0.0832
1250.6	Patchoulane	1409.4	1393	chem spider	0.0287
1334.5	Humulene	1487.6	1487	pubchem	0.10968
1346.8	alpha-Curcumene	1499.1	1483	pherobase	0.4536
1347.3	Caryophyleine-(I3)	1499.5	1499	chemspider	0.3368
1361.6	Geranyl isobutyrate	1513.7	1514	Pherobase	0.0506

* Retention index according to available literature.

Twenty-six compounds were characterised and identified in *H. odorata* SFE-CO_2 extracts by comparison of the mass spectra of the elements with PubChem, NIST library and comparing their calculated retention indexes with those available in the literature. All 26 compounds are listed in **Table 3.23.** with their retention time, name, calculated retention index, retention index from the reference and area. The difference between Retention Index obtained from analysis and the RI from reference literature were within the range of ±20 and the rest of the **Eighteen** identified compounds which had higher area % and more than the range of ±20 are presented in **Annex 8** in **Table 3.25**. The compounds which was >1% in their area were beta-ionone (18.57) and cuparene (5.1309). From Annex it was coumarin (18.67) in area %. Yoshitaka Ueyama *et al.* studied the volatile constituents of ethanol extracts from *H. odorata* and showed that aerial part of oil contains 24.9% of coumarin analysed by gas chromatography and in this research, it is also proved to contain 18.67% of coumarin. Yoshitaka also showed the presence of few minor compounds like hex-3-enoic acid, cis hex 3-enoic acid, trans-hex-2-enoic acid and Massoia lactone (1.40%)[111]. However, in this study Massoia lactone has relatively lower percentage of 0.0018%, due to fact that it was a non-polar extract and

also did not contains hexanoic acids. Beta-ionone is a ionone used as flavouring substance and has a role of antioxidant and it is a major volatile component of green tea usually occurring in many plant essential oils[112]. Cuparene belongs to the class of sesquiterpenoids, and known to be isoprenoid lipid molecule[113]. Cuparenes have also proven to have antibacterial and antifungal properties against bacteria like *Bacillus subtilis* and fungus *Cladosporium herbarum*[114]. Coumarins belongs to class of benzopyrone and is found in many plants. Coumarins shows a variety of biological activity like antimicrobial, antiviral, anti-inflammatory, antidiabetic, antioxidant, and enzyme inhibitory activity and also proven to be toxic in nature[90].

Table 3.23 Results of the GC-MS analysis of *H. odorata* SFE-CO_2 extract.

R.T. (s)	Name	RI Calculated	RI Reference*	Reference	Area %
611.6	Ethyl pentanoate	906.68	898	pherobase	0.0026
661.5	Dimethyl sulfone	942.5	931-909	pubchem	0.0118
740.7	2-Pentylfuran	999.35	993-972	pubchem	0.0139
800.6	limonene	1043.5	1039-1031	pherobase	0.0022
839.3	Benzeneacetaldehyde	1072.1	1061-1002	pubchem	0.0008
899.6	Nonanal	1117.5	1108-1098	pherobase	0.0020
930.9	Phenylethyl Alcohol	1141.9	1122-1110	pubchem	0.0034
977.1	Octanoic acid	1177.9	1203-1150	pubchem	0.0026
1006.9	Diethylene Glycol Monobutyl Ether	1201.2	1193-1167.8	pubchem	0.0037
1084	ethyl phenylacetate	1264.9	1244	pherobase	0.0006
1126.2	2,4,6,8-tetramethylundecene	1299.8	1287	chemspider	0.0031
1143.3	Anethole	1314.7	1300-1255	pubchem	0.0785
1151.6	alpha-Isosafrole	1322	1336-1308	pubchem	0.0013
1171.4	1-methylnaphthalene	1339.4	1330-221	pubchem	0.0003
1182.2	Triacetin	1348.9	1348-1285	pubchem	0.0253
1250.9	alpha-Bourbonene	1409.7	1400-1367	pubchem	0.0003
1260.4	4-methoxyphenylacetone	1418.5	1411-1374	pubchem	0.0018
1263.6	Methyleugenol	1421.5	1403	Adams RP	0.0028
1307.2	Nerylacetone	1462.2	1445-1412	pubchem	0.0028
1350.3	Cuparene	1502.5	1498-1502	pherobase	5.1309
1356.8	beta-ionone	1508.9	1494-1484	pherobase	18.570
1361.8	Massoia lactone	1513.9	1499-1443	pubchem	0.0018
1401	trans-calamenene	1552.7	1579-1507	pubchem	0.0007
1446.2	Ethyl dodecanoate	1597.5	1593-1595	pherobase	0.0330
1498.8	Dill apiole	1652.8	1642-1682	pubchem	0.0035
1798.1	Ethyl hexadecanoate	1999.5	1983-1993	pherobase	0.0315

* Retention index according to available literature.

3.7.2 Analysis by UPLC/ESI-QTOF-MS

The identification of chemical compounds from *P. fruticosa* and *H. odorata* from SFE-CO_2, PLE-Ace, PLE-EtOH, PLE-H_2O extracts was based on characteristics of m/z detected under ESI Positive and negative ionization mode and identified by METLIN databases. The compounds from *P. fruticosa* are represented in **Table 3.26** and **Table 3.27**, positive and negative ionization modes respectively. The compounds from *H. odorata* are represented in **Table 3.28** and **Table 3.29**, positive and negative ionization modes respectively in **Appendix 9**.

The PLE-Ace, PLE-EtOH, PLE-H2O extracts from *P. fruticosa* contains several compounds both in the positive and negative ionization modes. The compounds which were detected in positive mode were, Quercetin, Rutin, Quercitrin, Quercetin 3-galacturonide and Kaempferol rhamnoside from PLE- Ace extracts. Quercetin and Quercetin 3-galacturonide both were detected in PLE-EtOH and PLE-H2O extracts. The compounds which are detected in negative mode were, Catechin, Ellagic acid and Quercetin 3-galacturonide in PLE-Ace, EtOH, H2O extracts. Quercitrin and Quercetin 7-(6"-galloylglucoside) in PLE-Ace, EtOH extracts and Rutin in PLE acetone extract. Miliauskas *et al*, detected potential antioxidant compounds from *P. fruticosa* using RP-HPLC technique from ethanol, butanol and water fractions and showed the presence of several compounds, which is similar to the compounds found in this study and some of the compounds which are common are Catechin, Ellagic acid, Quercitrin, Quercetin and Rutin[15]. Phytochemical profile of *P. fruticosa* was studied by several other authors also, indicate the presence of Catechin, Ellagic acid and Rutin[5][22][23]. Wei Liu et al, detected the presence of Quercetin, Rutin and Kaempferol in *P. fruticosa*, obtained from various regions of china using RP-HPLC technique[21]. The presence of these bioactive compounds is studied in various fruits, vegetable and plants and is associated with strong antioxidant activity and radical scavenging activity which by enlarge gives a beneficial effect on human health[115]. The compounds are also proven to be chemo preventive in nature by protecting against DNA damage[116].

The PLE-Ace, PLE-EtOH, PLE-H2O extracts from *H. odorata* contains several compounds both in the positive and negative ionization modes. The compounds which were detected in positive mode were, Coumarin in SFE-CO_2, PLE-Ace, EtOH extracts and 7,8-Dihydroxycoumarin was found in PLE-Ace, EtOH, H_2O extracts. However, m-Coumaric acid was only detected in PLE-EtOH extract. The compounds which are detected in negative mode were, 7,8-Dihydroxycoumarin was found in SFE-CO_2, PLE-Ace, EtOH, extracts and 3-Hydroxycoumarin in PLE-Ace, EtOH extracts. In 1991, Yoshitaka *et al*. identified the presence of coumarin and several other compounds using HPLC in ethanol extracts of *H. odorata*, which can also be seen in this study[28]. Pukalskas *et al*. detected the presence of 5,8-dihydroxycoumarin radical scavenger which was responsible for the antioxidant activity of *H. odorata*[36].However, in this study 7,8-dihydroxycoumarin was detected which is the structural analogue of dihydroxycoumarin. Coumarins in general possess anti-inflammatory, anticoagulant activity, antibacterial and antiviral activity[117]. The most important property of these coumarins are the presence of high radical scavenging and antioxidant activity[118].

Conclusions:

1. The chemical composition was determined for *P. fruticosa* and *H. odorata*. The protein content was found to be 10.54% and 14.31% for *P. fruticosa* and *H. odorata* respectively. The oil content by Soxhlet-Hex extraction technique gave 2.68 and 3.79 % for *P. fruticosa* and *H. odorata* respectively. The ash content of 4.10 and 5.45% was found in *P. fruticosa* and *H. odorata* respectively. Water content of 9.59 and 7.21% was determined in *P. fruticosa* and *H. odorata* respectively.
2. *P. fruticosa* and *H. odorata* polar extracts were obtained using pilot scale supercritical carbon dioxide extraction resulting with the yield of 2.46 and 2.10% respectively.
3. The solid-liquid extractions with different polarity solvents starting from hexane, acetone, ethanol and finally water on plant materials gave a yield of 3.65, 6.06, 14.37 and 8.31 %, respectively for *P. fruticosa*. For *H. odorata* the yield was 4.85, 6.84, 7.67 and 6.87 %, respectively. The defatted residues of *P. fruticosa* and *H. odorata* after SFE-CO_2 when SLE was carried out with solvents acetone, ethanol and water gave a yield of 6.78, 14.85 and 8.32% respectively for *P. fruticosa*. For *H. odorata* the yield was 7.16, 7.73 and 7.02 %, respectively. However, SFE-CO2 extraction had little influence on the yields obtained through the SLE extraction when same solvents are used.

4. The effect of temperature and extraction time on the 3- step pressurized liquid extraction applied to *P. fruticosa* and *H. odorata* SFE-CO2 defatted material using acetone, ethanol and water was investigated. The extraction condition was optimized using the response surface methodology to maximize the yield and total phenolic content of *P. fruticosa*. The following optimum extraction conditions were suggested by the model for *P. fruticosa*: 62ºC and 45min for acetone optimization. Acetone extract obtained under optimal conditions showed the yield of 11.67% and TPC content of 77.43 mg GAE/g DW. 75ºC and 45min for ethanol optimization. Ethanol extract obtained under optimal conditions showed the yield of 17.76% and TPC content of 172.29 mg GAE/g DW. 130ºC and 45min for water optimization. Water extract obtained under optimal conditions showed the yield of 19.42% and TPC content of 43.84 mg GAE/g DW. Using the same optimal conditions for *H. odorata* PLE extractions were performed with acetone, ethanol and water as solvents. The yield and TPC of the extracts were as follow (7.67, 15.27 and 18.60) % respectively, TPC of (93.23, 69.97 and 36.78) mg GAE/g DW respectively.

5. Enzyme-assisted extraction was applied to *P. fruticosa* and *H. odorata* residues after SFE-CO_2 and PLE optimized water extraction for recovery of polar fractions, the resulting fractions gave a yield % of 7.25 and 5.80 for *P. fruticosa* and *H. odorata* residues after PLE optimized water extraction. Residues after SFE-CO_2 gave a yield % of 15.60 and 13.72 for *P. fruticosa* and *H. odorata* respectively with Enzyme-assisted extraction. The residues after SFE-CO_2 treated with enzymes were subjected to PLE with acetone, ethanol and water at the same optimal conditions obtained and the resulting extracts gave a yield % of (6.34, 14.94, 21.23), respectively for *P. fruticosa* and (5.22, 14.87, 19.21) % respectively for *H. odorata*. The TPC of (41.85, 144.86, 47.92), mg GAE/g DW respectively for *P. fruticosa* and TPC of (63.42, 68.12, 37.89), mg GAE/g DW respectively for *H. odorata.*

6. The total phenolic content of *P. fruticosa* and *H. odorata* initial plant material was 201.75 and 156.82 mg GAE/g DW, respectively. The radical scavenging capacity of *P. fruticosa* and *H. odorata* initial plant material was 250.55 and 159.81 mg TE/g DW for $ABTS^{\bullet+}$ assay, and 161.59 and 113.84 mg TE/g DW for $DPPH^{\bullet}$ assay, respectively. The solid liquid extractions using hexane, acetone, ethanol and water resulted in the extracts having TPC ranging from lower to a higher of 1.58 to 115.45 mg GAE/g DW for *P. fruticosa* and *H. odorata*. The $ABTS^{\bullet+}$ assay ranged from lower to a higher of 43.43 to 970.99 mg TE/g extract for *P. fruticosa* and *H. odorata.* $DPPH^{\bullet}$ assay ranged from lower to a higher of 86.42 to 777.29 mg TE/g extract for *P. fruticosa* and *H. odorata.* The SFE-CO_2 extracts gave an TPC value of 5.65 and 4.65 mg GAE/g DW for *P. fruticosa* and *H. odorata*, respectively. The $ABTS^{\bullet+}$ values were 224.76 and 411.56 mg TE/g extract for *P. fruticosa* and *H. odorata* respectively. The $DPPH^{\bullet}$ values were 310.54 and 291.65 mg TE/g extract for *P. fruticosa* and *H. odorata* respectively. The Pressurised liquid extractions using acetone, ethanol and water resulted in the extracts having TPC values ranged from 36.78 to 172.79 mg GAE/g DW for both *P. fruticosa* and *H. odorata.* The $ABTS^{\bullet+}$ assay ranged from lower to a higher value of 330.31 to 1539.99 mg TE/g extract for both *P. fruticosa* and *H. odorata.* The $DPPH^{\bullet}$ assay ranged from lower to a higher value of 163.43 to 1499.92 mg TE/g extract for both *P. fruticosa* and *H. odorata.* The enzyme assisted extracts had the TPC values ranging from 4.95 to 12.69 mg GAE/g DW for both *P. fruticosa* and *H. odorata.* The $ABTS^{\bullet+}$ assay ranged from lower to a higher value of 42.61 to 68.86 mg TE/g extract for both *P. fruticosa* and *H. odorata.* The $DPPH^{\bullet}$ assay ranged from lower to a higher value of 22.16 to 129.21 mg TE/g extract for both *P. fruticosa* and *H. odorata.*

7. GCxGC/ TOF MS method was performed to determine volatile compounds in *P. fruticosa* and *H. odorata* SFE-CO2 extracts. The major volatile compounds from *P. fruticosa* identified were 2-pentylfuran (2.7074), phenol (2.0942), Myrcene (1.1732), Hexanoic acid (4.3124) and coumarin (2.7762) in area %. The major volatile compounds from *H. odorata* identified were beta-ionone (18.57), cuparene (5.1309) and coumarin (18.67) in area %.

8. UPLC/ESI-QTOF-MS/MS was applied for the determination of phytochemical composition of *P. fruticosa* and *H. odorata* PLE extracts obtained under optimal conditions and SFE-CO2 extracts. Potential phenolic and antioxidant compounds like Quercetin, Rutin, Quercitrin, Quercetin 3-galacturonide, Kaempferol rhamnoside, Catechin, Ellagic acid and Quercetin 7-(6"-galloylglucoside) were identified in *P. fruticosa.* 7,8-Dihydroxycoumarin, Coumarin and m-Coumaric acid were the main compounds identified in *H. odorata* which have potential antioxidant activity.

This work demonstrates the application of biorefining concept of *P. fruticosa* and *H. odorata* in order to obtain high-value compounds. The extracts obtained contains bioactive compounds and would be beneficial in pharmaceutical applications.

List of References

[1] W. J. Pu, D. W. Li, and D. M. Wang, "*P. fruticosa* extracts as antimicrobials in post-harvest storage of fruits," vol. 34, no. 1, p. 712100, 2014.

[2] G. Miliauskas, T. A. van Beek, P. R. Venskutonis, J. P. H. Linssen, P. de Waard, and E. J. Sudhölter, "Antioxidant activity of *P. fruticosa*," *J. Sci. Food Agric.*, vol. 84, no. 15, pp. 1997–2009, Dec. 2004.

[3] Y. Zhao, G. Cai, X. Hong, L. Shan, and X. Xiao, "Anti-hepatitis B virus activities of triterpenoid saponin compound from *Potentilla anserine* L .," vol. 15, pp. 253–258, 2008.

[4] M. Tomczyk and K. Peter, "Potentilla — A review of its phytochemical and pharmacological profile," vol. 122, pp. 184–204, 2009.

[5] S. Wang, D. Wang, W. Pu, and D. Li, "Phytochemical profiles , antioxidant and antimicrobial activities of three Potentilla species," pp. 1–11, 2013.

[6] A. Pukalskas, Isolation , identification and activity of natural antioxidants from sweet grass (*Hierochloe odorata*), costmary (*Chrysanthemum balsamita*) and horehound (*Marrubium vulgare*), cultivated in Lithuania Audrius Pukalskas. .

[7] L. W. Mitich, "Weed Science Society of America Intriguing World of Weeds Cinquefoils (Potentilla spp .) -The Five Finger Weeds '," vol. 9, no. 4, pp. 857–861, 2015.

[8] *Rosaceae Potentilla fruticosa*. International Plant Names Index.

[9] M. Blamey and C. Grey-Wilson, *The illustrated flora of Britain and northern Europe*. Hodder & Stoughton, 1989.

[10] "Image/JPEG:Potentillafruticosa."[Online].Available: http://www.efloras.org/object_page.aspx?object_id=91367&flora_id=800. [Accessed: 20-May-2019].

[11] G. M. Fedoseeva, "Phenolic compounds ofPotentilla fruticosa," *Chem. Nat. Compd.*, vol. 15, no. 4, p. 501, Jul. 1979.

[12] B. T. V. Ganenko, "Chemical composition of Potentilla frutiocsa(flavonoids)," *Tep. Apx.*, vol. 60, no. 8, pp. 27–30, 1988.

[13] T. V. Ganenko, "CHEMICAL COMPOSITION OF Potentilla fruticosa (TRITERPENOIDS)," vol. 6, no. i, p. 727512, 1990.

[14] G. Miliauskas, P. R. Venskutonis, and T. A. Van Beek, "Screening of radical scavenging activity of some medicinal and aromatic plant extracts," vol. 85, pp. 231–237, 2004.

[15] G. Miliauskas, T. A. Van Beek, P. R. Venskutonis, J. P. H. Linssen, P. De Waard, and E. J. R. Sudhölter, "Antioxidant activity of Potentilla fruticosa," *J. Sci. Food Agric.*, vol. 84, no. 15, pp. 1997–2009, 2004.

[16] "CSID:4444362,http://www.chemspider.com/Chemical-Structure.4444362.html (accessed 11:36, May 25, 2019)."

[17] "CSID:4444962,http://www.chemspider.com/Chemical-Structure.4444962.html (accessed 11:38, May 25, 2019)."

[18] "CSID:8711,http://www.chemspider.com/Chemical-Structure.8711.html(accessed 11:41, May 25, 2019)."

[19] “CSID:4445149,http://www.chemspider.com/Chemical-Structure.4445149.html (accessed 11:42, May 25, 2019).”

[20] T. A. Activity, “Variation in Total Polyphenolics Contents of Aerial Parts of,” pp. 4639–4651, 2010.

[21] W. Liu, D. Wang, J. Liu, D. Li, and D. Yin, “Quality Evaluation of Potentilla fruticosa L . by High Performance Liquid Chromatography Fingerprinting Associated with Chemometric Methods,” pp. 1–20, 2016.

[22] Z. Luo, S. Wang, and D. Wang, “Phenolic profiles and antioxidant capacities of crude extracts and subsequent fractions from Potentilla fruticosa L . leaves,” vol. 6419, no. September, 2015.

[23] D. Yu, W. Pu, D. Li, D. Wang, Q. Liu, and Y. Wang, “Phenolic Compounds and Antioxidant Activity of Different Organs of Potentilla fruticosa L . from Two Main Production Areas of China,” pp. 1140–1148, 2016.

[24] Z. Liu, Z. Luo, C. Jia, D. Wang, and D. Li, “Synergistic Effects of Potentilla fruticosa L . Leaves Combined with Green Tea Polyphenols in a Variety of Oxidation Systems,” vol. 81, no. 5, pp. 1091–1101, 2016.

[25] K. Leszczy and P. Jakoniuk, “Antimicrobial activity of Potentilla species,” vol. 79, pp. 592–594, 2008.

[26] G. Miliauskas, “MEAT Evaluation of antioxidative properties of Geranium macrorrhizum and Potentilla fruticosa extracts in Dutch style fermented sausages,” vol. 77, pp. 703–708, 2007.

[27] W. E. T. Al, “the Occurrence and Distribution of Hierochloe,” vol. 60, no. 6, pp. 358–365, 1960.

[28] S. Ueyama, Y., Arai, T., Hashimoto, “Volatile Constituents of Ethanol Extracts of Hierochloe odorata,” vol. 6, pp. 63–68, 1991.

[29] M. Krasińska and Z. A. Krasiński, *European bison : the nature monograph*. Springer, 2013.

[30] M. Krasińska and Z. A. Krasiński, “Food and Use of the Environment,” in *European Bison*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2013, pp. 157–179.

[31] “Native American sweetgrass: its meaning and use - Traditional Native Healing.” [Online]. Available: http://traditionalnativehealing.com/native-american-sweetgrass-its-meaning-and-use. [Accessed: 21-May-2019].

[32] “NativeTech: Native American Uses for Sweetgrass.” [Online]. Available: http://www.nativetech.org/plants/sweetgrass.html. [Accessed: 21-May-2019].

[33] “Plant Guide SWEETGRASS Hierochloe odorata (L.) Beauv. Plant Symbol = HIOD.”

[34] M. Officinalis, “Biosynthesis of the coumarins. tracer studies on coumarin formation in,” vol. 38, no. 5498, pp. 1957–1958, 1960.

[35] R. O. F. Glycosides, T. H. E. Formation, O. F. Coumarin, and H. Odorata, “Biosynthesis of the coumarins 111.,” vol. 40, no. 6801, 1962.

[36] J. O. P. H. L. Inssen, A. L. V. A. N. V Eldhuizen, and Æ. D. E. D. E. G. Root, “Identification of Radical Scavengers in Sweet Grass (Hierochloe odorata),” pp. 2914–2919, 2002.

[37] W. Łuczaj, A. Stankiewicz-kranc, E. Milewska, W. Roszkowska-jakimiec, and E. Skrzydlewska, “Effect of sweet grass extract against oxidative stress in rat liver and serum,” vol. 50, pp. 135–140, 2012.

[38] B. Szachowicz-petelska, “Effect of sweet grass (Hierochloe odorata) on the physico-chemical properties of liver cell membranes from rats intoxicated with ethanol Izabela Dobrzy nska,” vol. 5, pp. 247–253, 2013.

[39] J. A. Hart, “THE ETHNOBOTANY OF THE FLATHEAD INDIANS OF WESTERN MONTANA,” *Botanical Museum Leaflets, Harvard University*, vol. 27. Harvard University Herbaria, pp. 261–307.

[40] R. Plant, C. L. Cantrell, A. M. P. Jones, and A. Ali, “Isolation and Identi fi cation of Mosquito (Aedes aegypti) Biting- Deterrent Compounds from the Native American Ethnobotanical Remedy Plant Hierochloe¨odorata (Sweetgrass),” 2016.

[41] D. Bandoniene, A. Pukalskas, P. R. Venskutonis, and D. Gruzdiene, “Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil,” vol. 33, 2000.

[42] A. Zainuddin, J. Pokorny, and R. Venskutonis, “Antioxidant activity of sweetgrass (Hierochlo odorata Wahlnb .) extract in lard and rapeseed oil emulsions,” vol. 46, no. 1, pp. 15–17, 2002.

[43] J. Damašius, P. R. Venskutonis, R. Ferracane, and V. Fogliano, “Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat,” vol. 126, pp. 149–156, 2011.

[44] A. Pukalskas and P. R. Venskutonis, “Redox Properties of Novel Antioxidant 5 , 8-Dihydroxycoumarin : Implications for its Prooxidant Cytotoxicity,” 2005.

[45] J. R. Lazutka, A. Pukalskas, and P. R. Venskutonis, “Evaluation of the Biological Activity of Naturally Occurring 5,8-Dihydroxycoumarin,” pp. 4419–4436, 2013.

[46] G. Romanik, E. Gilgenast, A. Przyjazny, and M. Kami, “Techniques of preparing plant material for chromatographic separation and analysis,” vol. 70, pp. 253–261, 2007.

[47] D. P. Xu *et al.*, “Natural antioxidants in foods and medicinal plants: Extraction, assessment and resources,” *Int. J. Mol. Sci.*, vol. 18, no. 1, pp. 20–31, 2017.

[48] C. M. Galanakis, G. Laboratories, V. Goulas, S. Tsakona, and G. Manganaris, “International Journal of Food Properties A Knowledge Base for The Recovery of Natural Phenols with Different Solvents,” no. November 2015, 2011.

[49] C. M. Galanakis, “Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications,” *Trends Food Sci. Technol.*, vol. 26, no. 2, pp. 68–87, 2012.

[50] F. J. Barba, Z. Zhu, M. Koubaa, A. S. Sant’Ana, and V. Orlien, “Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review,” *Trends Food Sci. Technol.*, vol. 49, pp. 96–109, 2016.

[51] M. Herrero, A. Cifuentes, and E. Iban, “Food Chemistry Sub- and supercritical fluid extraction of functional ingredients from different natural sources : Plants , food-by-products , algae and microalgae A review,” vol. 98, pp. 136–148, 2006.

[52] C. G. Pereira and M. A. A. Meireles, “Supercritical Fluid Extraction of Bioactive Compounds : Fundamentals , Applications and Economic Perspectives,” pp. 340–372, 2010.

[53] M. McHugh and V. Krukonis, *Supercritical Fluid Extraction: Principles and Practic*. 2013.

[54] G. Brunner, *Gas Extraction: An Introdution to Fundamentas of Supercritical Fluids and the*

Application to Separation Process 1 Ed. 1994.

[55] A. Janghel, S. Deo, P. Raut, D. Bhosle, and C. Verma, "Supercritical Fluid Extraction (SFE) Techniques as an Innovative Green Technologies for the Effective Extraction of the Active Phytopharmaceuticals Supercritical Fluid Extraction (SFE) Techniques as an Innovative Green Technologies for the Effective E," no. June, 2015.

[56] L. Laboureur, M. Ollero, and D. Touboul, "Lipidomics by Supercritical Fluid Chromatography," pp. 13868–13884, 2015.

[57] G. N. Sapkale, S. M. Patil, U. S. Surwase, and P. K. Bhatbhage, "- A REVIEW SUPERCRITICAL FLUID EXTRACTION," vol. 8, no. 2, pp. 729–743, 2010.

[58] L. Henrique, C. Carlson, A. Bolzan, and F. Machado, "Separation of d -limonene from supercritical CO 2 by means of membranes," vol. 34, pp. 143–147, 2005.

[59] A. Capuzzo, M. E. Maffei, and A. Occhipinti, "Supercritical Fluid Extraction of Plant Flavors and Fragrances," pp. 7194–7238, 2013.

[60] S. Machmudah, A. Shotipruk, M. Goto, M. Sasaki, and T. Hirose, "Extraction of astaxanthin from Haematococcus pluvialis using supercritical CO2 and ethanol as entrainer," *Ind. Eng. Chem. Res.*, vol. 45, no. 10, pp. 3652–3657, 2006.

[61] A. Laitinen, "Supercritical fluid extraction of organic compounds from solids and aqueous solutions Supercritical fluid extraction of organic compounds from solids and," 1999.

[62] J. W. King, "Modern Supercritical Fluid Technology for Food Applications," *Annu. Rev. Food Sci. Technol.*, vol. 5, no. 1, pp. 215–238, 2013.

[63] E. Reverchon and F. Senatore, "Isolation of rosemary oil: Comparison between hydrodistillation and supercritical CO2 extraction," *Flavour Fragr. J.*, vol. 7, no. 4, pp. 227–230, 1992.

[64] P. Ramírez, M. R. García-Risco, S. Santoyo, F. J. Señoráns, E. Ibáñez, and G. Reglero, "Isolation of functional ingredients from rosemary by preparative-supercritical fluid chromatography (Prep-SFC)," *J. Pharm. Biomed. Anal.*, vol. 41, pp. 1606–1613, 2006.

[65] K. L. Fernando Montanés , Owen J. Catchpole, Stephen Tallon, Kevin Mitchell, "Semi-preparative supercritical chromatography scale plant for polyunsaturated fatty acids purification," *J. Supercrit. Fluids*, pp. 46–54, 2013.

[66] M. R. García-Risco, G. Vicente, G. Reglero, and T. Fornari, "Fractionation of thyme (Thymus vulgaris L.) by supercritical fluid extraction and chromatography," *J. Supercrit. Fluids*, vol. 55, no. 3, pp. 949–954, Jan. 2011.

[67] H. Sun, X. Ge, Y. Lv, and A. Wang, "Application of accelerated solvent extraction in the analysis of organic contaminants , bioactive and nutritional compounds in food and feed," *J. Chromatogr. A*, vol. 1237, pp. 1–23, 2012.

[68] A. Mustafa and C. Turner, "Analytica Chimica Acta Pressurized liquid extraction as a green approach in food and herbal plants extraction : A review," *Anal. Chim. Acta*, vol. 703, no. 1, pp. 8–18, 2011.

[69] C. Pronyk and G. Mazza, "Design and scale-up of pressurized fluid extractors for food and bioproducts," *J. Food Eng.*, vol. 95, no. 2, pp. 215–226, 2009.

[70] H. Giergielewicz-możajska, Ł. Dąbrowski, and J. Namieśnik, "Accelerated Solvent Extraction

(ASE) in the analysis of environmental solid samples - Some aspects of theory and practice Accelerated Solvent Extraction (ASE) in the Analysis of Environmental Solid Samples — Some Aspects of Theory and Practice," no. September 2018, 2001.

[71] S. De Koning and H. Janssen, "Modern Methods of Sample Preparation for GC Analysis Modern Methods of Sample Preparation for GC Analysis," no. May 2014, 2009.

[72] J. F. Osorio-tobón and M. A. A. Meireles, "Recent Applications of Pressurized Fluid Extraction : Curcuminoids Extraction Recent Applications of Pressurized Fluid Extraction : Curcuminoids Extraction with Pressurized Liquids," no. October 2015, 2013.

[73] L. Ramos, E. M. Kristenson, and U. A. T. Brinkman, "Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis," *J. Chromatogr. A*, vol. 975, no. 1, pp. 3–29, 2002.

[74] M. Plaza and C. Turner, "Trends in Analytical Chemistry Pressurized hot water extraction of bioactives," *Trends Anal. Chem.*, vol. 71, pp. 39–54, 2015.

[75] L. U. K. E. R. H. Oward, "Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red AND," 2003.

[76] H. Kamali, E. Khodaverdi, F. Hadizadeh, and S. H. Ghaziaskar, "Optimization of phenolic and flavonoid content and antioxidants capacity of pressurized liquid extraction from Dracocephalum kotschyi via circumscribed central composite," *J. Supercrit. Fluids*, vol. 107, pp. 307–314, Jan. 2016.

[77] E. Golmakani, A. Mohammadi, T. A. Sani, and H. Kamali, "The Journal of Supercritical Fluids Phenolic and flavonoid content and antioxidants capacity of pressurized liquid extraction and perculation method from roots of Scutellaria pinnatifida A. Hamilt. subsp alpina (Bornm) Rech. f," *J. Supercrit. Fluids*, vol. 95, pp. 318–324, 2014.

[78] I. Rodríguez-Meizoso *et al.*, "Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization," *J. Pharm. Biomed. Anal.*, vol. 41, pp. 1560–1565, 2006.

[79] J. Azmir *et al.*, "Techniques for extraction of bioactive compounds from plant materials : A review," *J. Food Eng.*, vol. 117, no. 4, pp. 426–436, 2013.

[80] M. Pinelo, A. Arnous, and A. S. Meyer, "Upgrading of grape skins : Significance of structural components and extraction techniques for phenol release," vol. 17, 2006.

[81] A. S. Meyer, "Enzyme technology for precision functional food ingredient processes," vol. 1190, pp. 126–132, 2010.

[82] M. Puri, D. Sharma, and C. J. Barrow, "Enzyme-assisted extraction of bioactives from plants," *Trends Biotechnol.*, vol. 30, no. 1, pp. 37–44, 2012.

[83] A. S. Meyer, S. M. Jepsen, and N. S. Sørensen, "Enzymatic Release of Antioxidants for Human Low-Density Lipoprotein from Grape Pomace," 1998.

[84] A. Landbo and A. S. Meyer, "Enzyme-Assisted Extraction of Antioxidative Phenols from Black Currant Juice Press Residues (Ribes nigrum)," pp. 3169–3177, 2001.

[85] S. M. Choudhari and L. Ananthanarayan, "Enzyme aided extraction of lycopene from tomato tissues."

[86] M. Vergara-Barberán, M. J. Lerma-García, J. M. Herrero-Martínez, and E. F. Simó-Alfonso,

"Use of an enzyme-assisted method to improve protein extraction from olive leaves," *Food Chem.*, vol. 169, pp. 28–33, Feb. 2015.

[87] S. Latif and F. Anwar, "Aqueous enzymatic sesame oil and protein extraction," *Food Chem.*, vol. 125, pp. 679–684, 2011.

[88] K. A. K. Alia, K. A. S. Harma, H. A. P. R. S. Ingh, and B. I. S. Ingh, "Effects of Extraction Methods on Phenolic Contents and Antioxidant Activity in Aerial Parts of Potentilla atrosanguinea Lodd . and Quantification of Its Phenolic Constituents by RP-HPLC †," pp. 10129–10134, 2008.

[89] E. Iban *et al.*, "Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants," 2003.

[90] H. Herve´martial, P. Poumale, R. Hamm, Y. Zang, Y. Shiono, and V. Kuete, "8. Coumarins and Related Compounds from the Medicinal Plants of Africa," 2013.

[91] T. E. Journal, "Opinion of the Scientific Panel on Food Additives , Flavourings , Processing Aids and Materials in Contacts with Food (AFC) on a request from the Commission related to Coumarin Question number EFSA-Q-2003-118," pp. 1–36, 2004.

[92] "CFR - Code of Federal Regulations Title 21."

[93] D. Grigonis, P. R. Venskutonis, B. Sivik, M. Sandahl, and C. S. Eskilsson, "Comparison of different extraction techniques for isolation of antioxidants from sweet grass (Hierochlo ¨ e odorata)," vol. 33, pp. 223–233, 2005.

[94] D. Kim, S. Weon, and C. Y. Lee, "Antioxidant capacity of phenolic phytochemicals from various cultivars of plums," vol. 81, pp. 321–326, 2003.

[95] S. Pastoriza, C. Delgado-Andrade, A. Haro, and J. A. Rufián-Henares, "Analytical Methods A physiologic approach to test the global antioxidant response of foods. The GAR method," 2011.

[96] A. Activity, A. An, I. Abts, and C. D. Assay, "ANTIOXIDANT ACTIVITY APPLYING AN IMPROVED ABTS RADICAL," vol. 26, no. 98, pp. 1231–1237, 1999.

[97] M. E. Cuvelier and C. Berset, "Use of a Free Radical Method to Evaluate Antioxidant Activity," vol. 30, pp. 25–30, 1995.

[98] S. Uysal and A. Aktumsek, "A phytochemical study on Potentilla anatolica : An endemic Turkish plant," *Ind. Crop. Prod.*, vol. 76, pp. 1001–1007, 2015.

[99] P. Macek and Æ. J. Leps, "of the stoloniferous plant Potentilla palustris," pp. 419–435, 2008.

[100] D. Grigonis, P. R. Venskutonis, B. Sivik, M. Sandahl, and C. S. Eskilsson, "Comparison of different extraction techniques for isolation of antioxidants from sweet grass (Hierochloë odorata)," *J. Supercrit. Fluids*, vol. 33, no. 3, pp. 223–233, 2005.

[101] V. Kraujaliene, A. Pukalskas, P. Kraujalis, and P. R. Venskutonis, "Biorefining of Bergenia crassifolia L. roots and leaves by high pressure extraction methods and evaluation of antioxidant properties and main phytochemicals in extracts and plant material," *Ind. Crops Prod.*, vol. 89, pp. 390–398, 2016.

[102] N. Kryževičiūtė, P. Kraujalis, and P. R. Venskutonis, "Optimization of high pressure extraction processes for the separation of raspberry pomace into lipophilic and hydrophilic fractions," *J. Supercrit. Fluids*, vol. 108, pp. 61–68, Feb. 2016.

[103] I. Mackėla, T. Andriekus, and P. R. Venskutonis, “Biorefining of buckwheat (Fagopyrum esculentum) hulls by using supercritical fluid, Soxhlet, pressurized liquid and enzyme-assisted extraction methods,” *J. Food Eng.*, vol. 213, pp. 38–46, Nov. 2017.

[104] V. Kitrytė, D. Povilaitis, V. Kraujalienė, V. Šulniūtė, A. Pukalskas, and P. R. Venskutonis, “Fractionation of sea buckthorn pomace and seeds into valuable components by using high pressure and enzyme-assisted extraction methods,” *LWT - Food Sci. Technol.*, vol. 85, pp. 534–538, 2017.

[105] E. Clain *et al.*, “Biorefining of Cymbopogon nardus from Reunion Island into essential oil and antioxidant fractions by conventional and high pressure extraction methods,” *Ind. Crops Prod.*, vol. 126, no. April, pp. 158–167, 2018.

[106] H. I. O. Basegmez *et al.*, “Biorefining of blackcurrant pomace into high value functional ingredients using supercritical CO 2 , pressurized liquid and enzyme assisted extractions,” *J. Supercrit. Fluids*, vol. 124, pp. 10–19, Jun. 2017.

[107] L. Laroze, C. Soto, · María, and E. Zúñiga, “Electronic Journal of Biotechnology Phenolic antioxidants extraction from raspberry wastes assisted by-enzymes,” 2010.

[108] R. G. KRISHNAMURTHY, T. H. SMOUSE, B. D. MOOKHERJEE, B. R. REDDY, and S. S. CHANG, “Identification of 2-Pentyl Furan in Fats and Oils and Its Relationship to the Reversion Flavor of Soybean Oil,” *J. Food Sci.*, vol. 32, no. 4, pp. 372–374, 1967.

[109] A. C. A. X. De-Oliveira, L. F. Ribeiro-Pinto, and F. J. R. Paumgartten, “In vitro inhibition of CYP2B1 monooxygenase by β-myrcene and other monoterpenoid compounds,” *Toxicol. Lett.*, vol. 92, no. 1, pp. 39–46, 1997.

[110] A. Šalić *et al.*, “Bioproduction of Food Additives Hexanal and Hexanoic Acid in a Microreactor,” *Appl Biochem Biotechnol*, vol. 171, pp. 2273–2284, 2013.

[111] L. Kryl, “Volatile Constituents of Ethanol Extracts of Hierochloe odorata,” vol. 6, pp. 63–68, 1991.

[112] T. Yamanishi, M. Nose, and Y. Nakatani, “Agricultural and Biological Chemistry Studies on the Flavor of Green Tea,” 2014.

[113] C. Fuganti and S. Serra, “Cuparene Sesquiterpenes: Synthesis of (+)-3-Hydroxycuparene and (+)-Cuparene,” 1999.

[114] N. Kazue Ishikawa, Y. Fukushi, K. Yamaji, S. Tahara, and K. Takahashi, “Antimicrobial Cuparene-Type Sesquiterpenes, Enokipodins C and D, from a Mycelial Culture of Flammulina velutipes Scheme 1 Scheme 2,” *J. Nat. Prod*, vol. 64, pp. 932–934, 2001.

[115] S. R. Abbas, S. M. Sabir, S. D. Ahmad, A. A. Boligon, and M. L. Athayde, “Phenolic profile, antioxidant potential and DNA damage protecting activity of sugarcane (Saccharum officinarum),” *Food Chem.*, vol. 147, pp. 10–16, Mar. 2014.

[116] Y. Cai, Q. Luo, M. Sun, and H. Corke, “Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer,” *Life Sci.*, vol. 74, no. 17, pp. 2157–2184, Mar. 2004.

[117] K. N. Venugopala, V. Rashmi, and B. Odhav, “Review on natural coumarin lead compounds for their pharmacological activity,” *Biomed Res. Int.*, vol. 2013, no. Table 1, 2013.

[118] M. Foti, M. Piattelli, M. Tiziana Baratta, and G. Ruberto, “Flavonoids, Coumarins, and

Cinnamic Acids as Antioxidants in a Micellar System. Structure-Activity Relationship ," 1996.

Appendices

Appendix 1. Solid-liquid extraction (SLE)

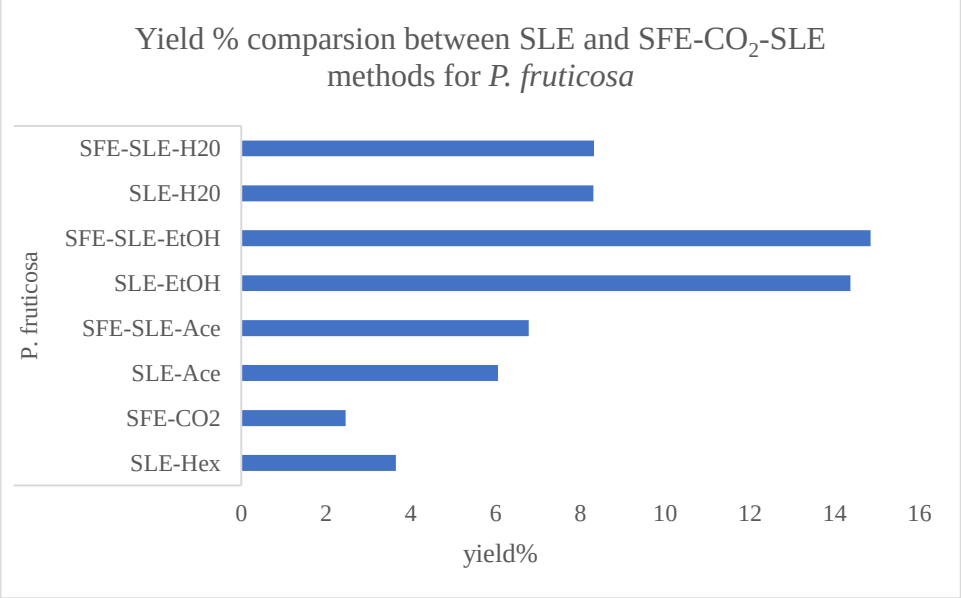


Figure 3.10 Yield % comparison between SLE and SFE-CO_2-SLE methods for *P. fruticosa*

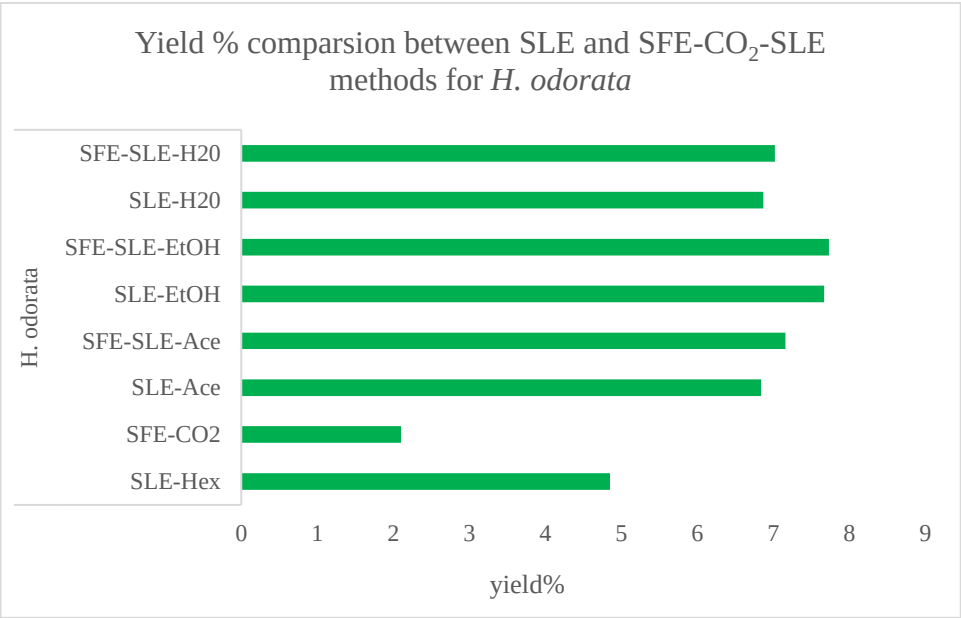


Figure 3.11 Yield % comparison between SLE and SFE-CO_2-SLE methods for *H. odorata*

Appendix 2. PLE Acetone optimisation of P. fruticosa SFE-CO2 residue

Effect of time and temperature on the extraction yield of *P. fruticosa* SFE-CO_2 residue

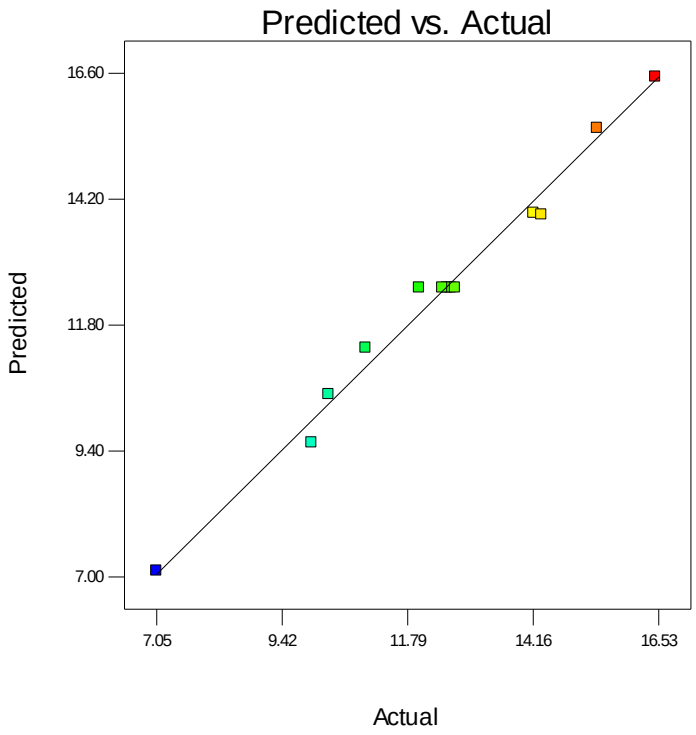


Figure 3.12 Predicted vs Actual yields of PLE Acetone optimisation for *P. fruticosa*

Effect of time and temperature on TPC of *P. fruticosa* SFE-CO_2 residue

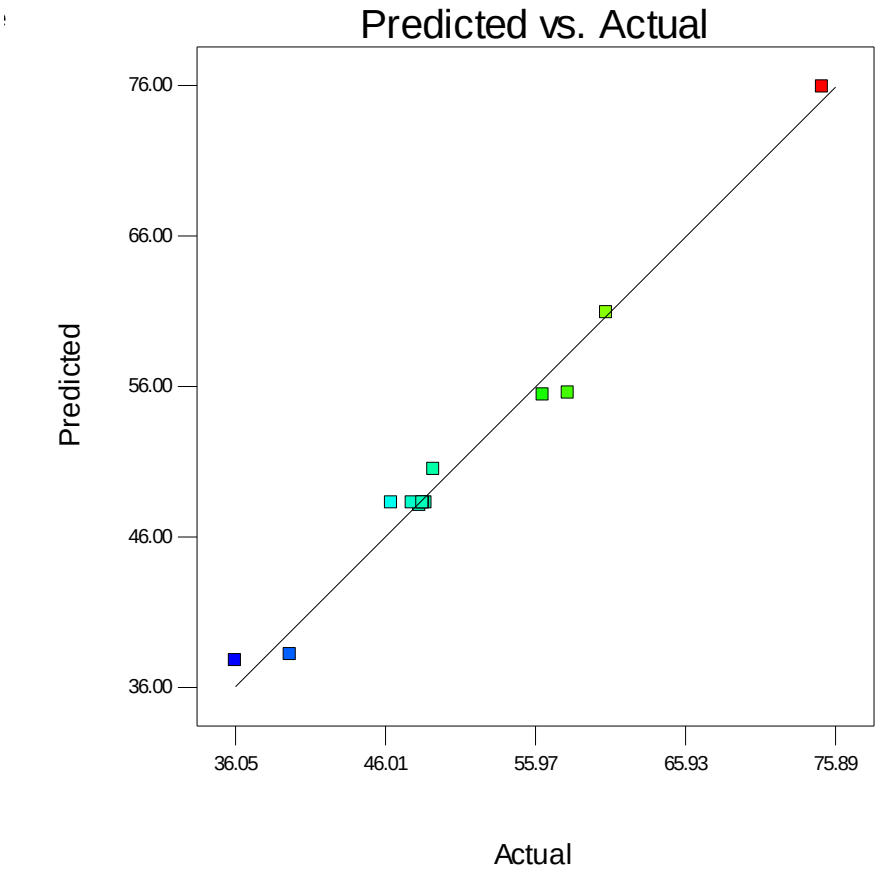


Figure 3.13 Predicted vs Actual TPC of PLE Acetone optimisation for *P. fruticosa*

Appendix 3. PLE Ethanol optimisation of P.fruticosa PLE Acetone-residue

Effect of time and temperature on extraction yield for *P. fruticosa* PLE Acetone-residue:

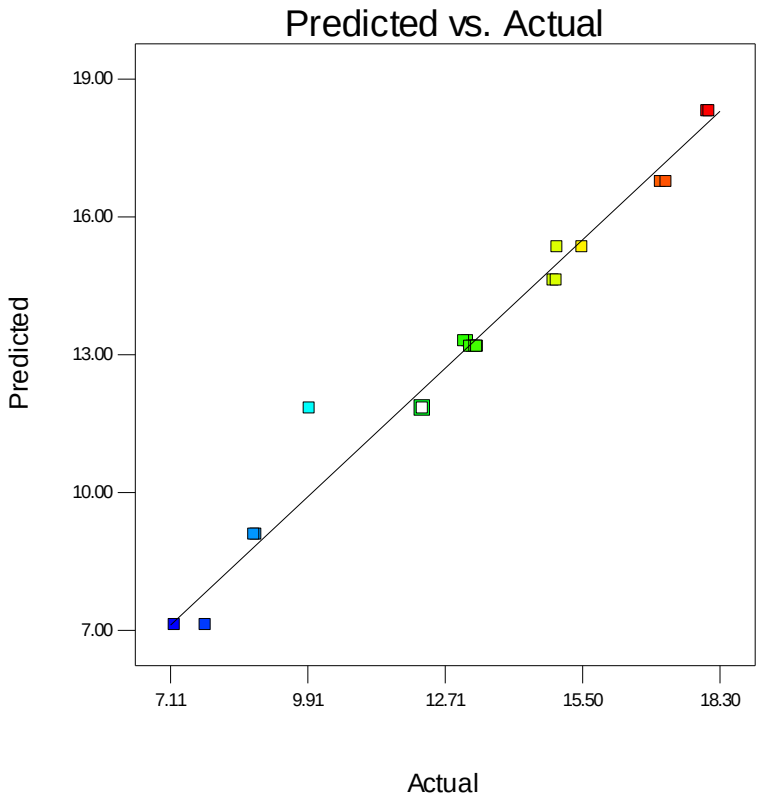


Figure 3.14 Predicted vs Actual yields of PLE ethanol optimisation for *P. fruticosa*

Effect of time and temperature on TPC of *P. fruticosa* SFE-CO_2 residue

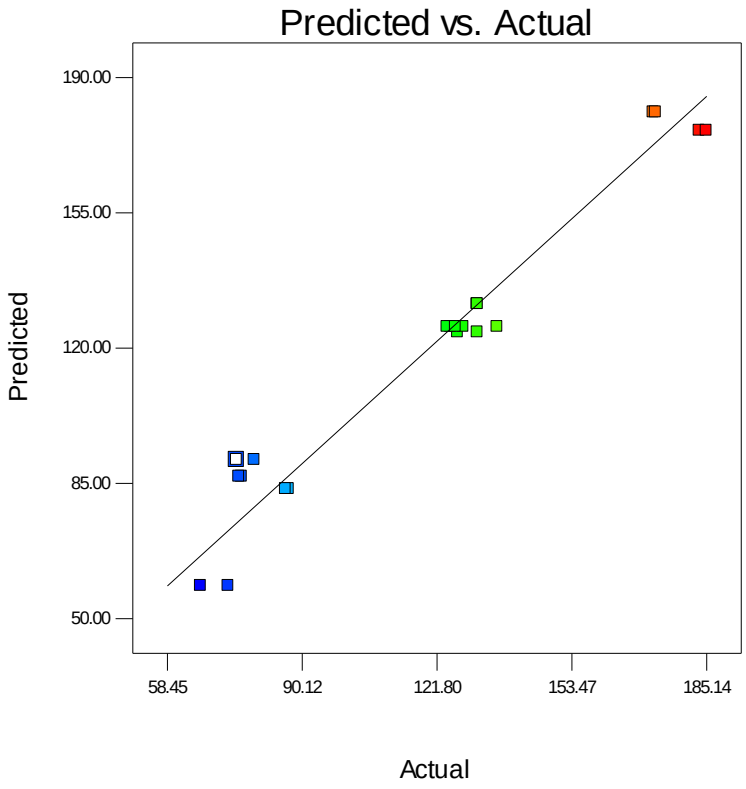


Figure 3.15 Predicted vs Actual TPC of PLE Ethanol optimisation for *P. fruticosa*

Appendix 4. Total phenolic content and Antioxidant activity for P. fruticosa and H. odorata SLE extracts and residue materials

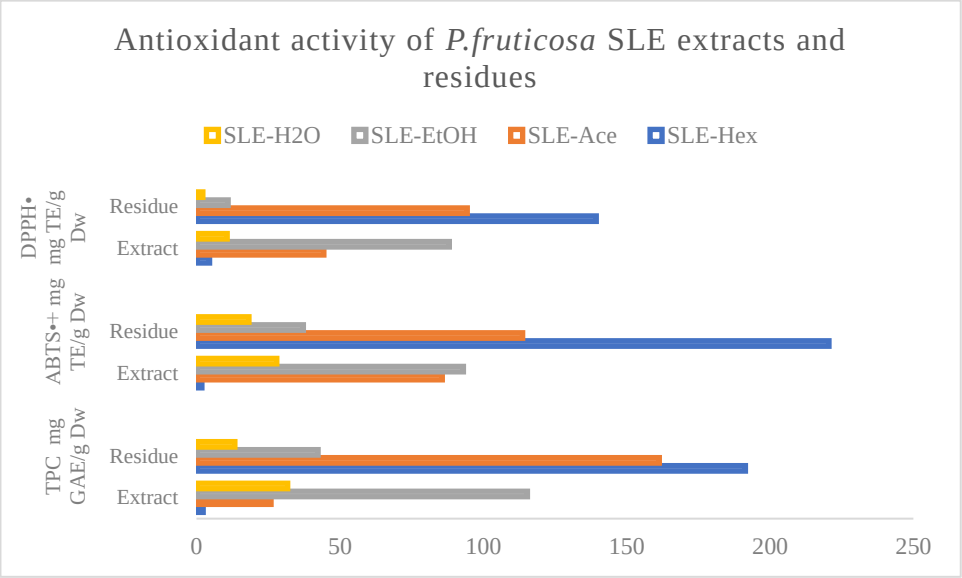


Figure 3.16 Antioxidant activity of *P. fruticosa* SLE extracts and residues

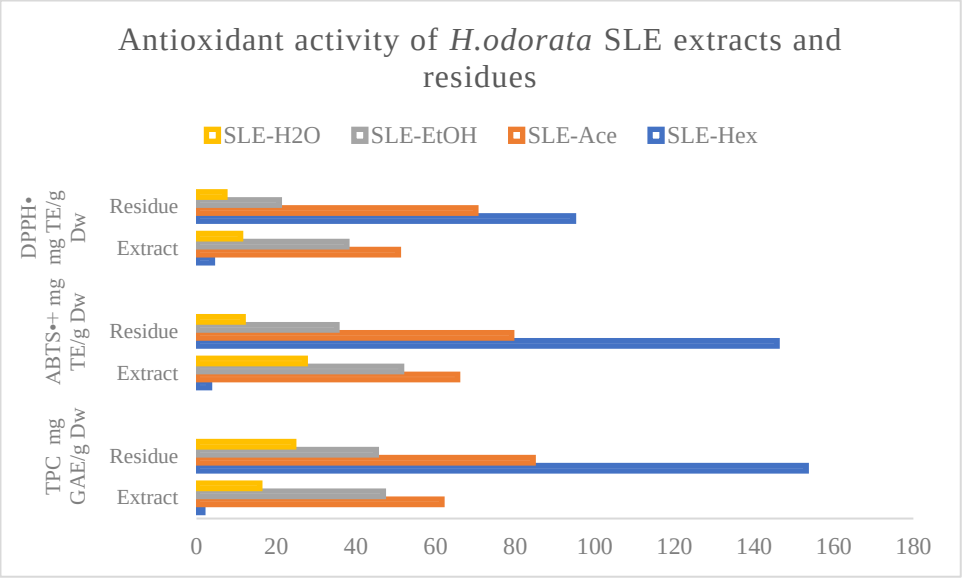


Figure 3.17 Antioxidant activity of *H. odorata* SLE extracts and residues

Appendix 5. Total phenolic content and Antioxidant activity for P. fruticosa and H. odorata SFE-CO2-SLE extracts and residue materials

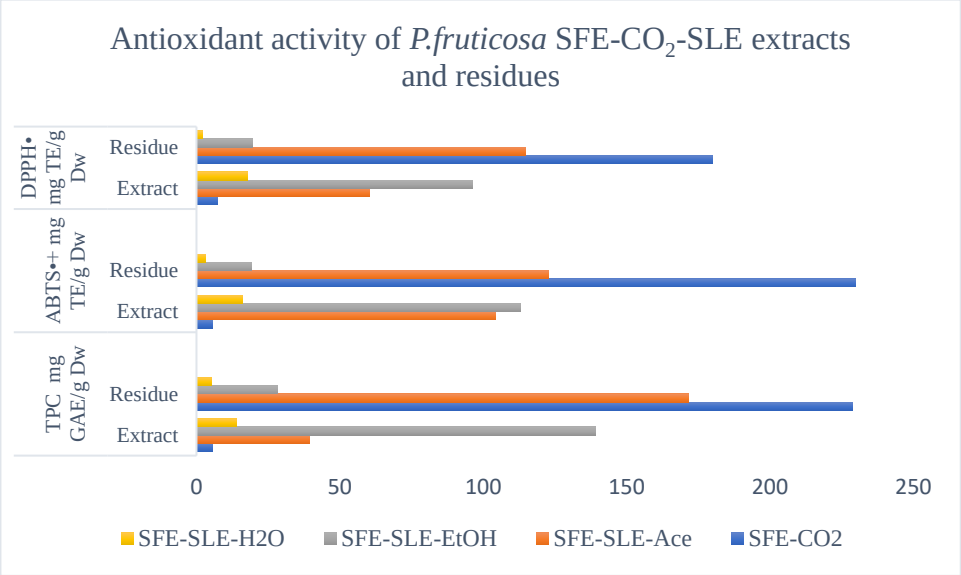


Figure 3.18 Antioxidant activity of *P. fruticosa* SFE-CO_2-SLE extracts and residues

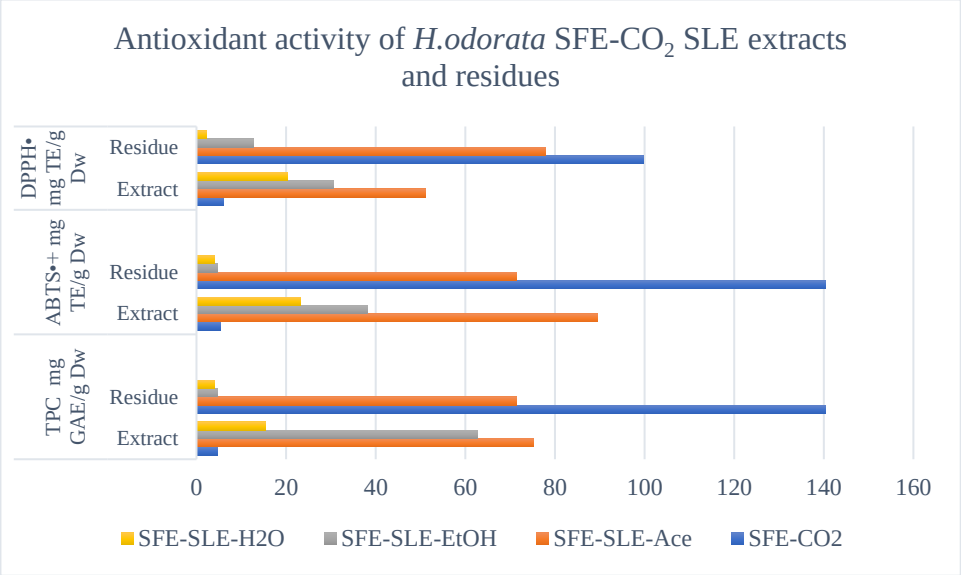


Figure 3.19 Antioxidant activity of *H. odorata* SFE-CO_2 SLE extracts and residues

Appendix 6. Total phenolic content and Antioxidant activity of P. fruticosa and H. odorata SFE-CO2 residue PLE Extracts under optimal conditions.

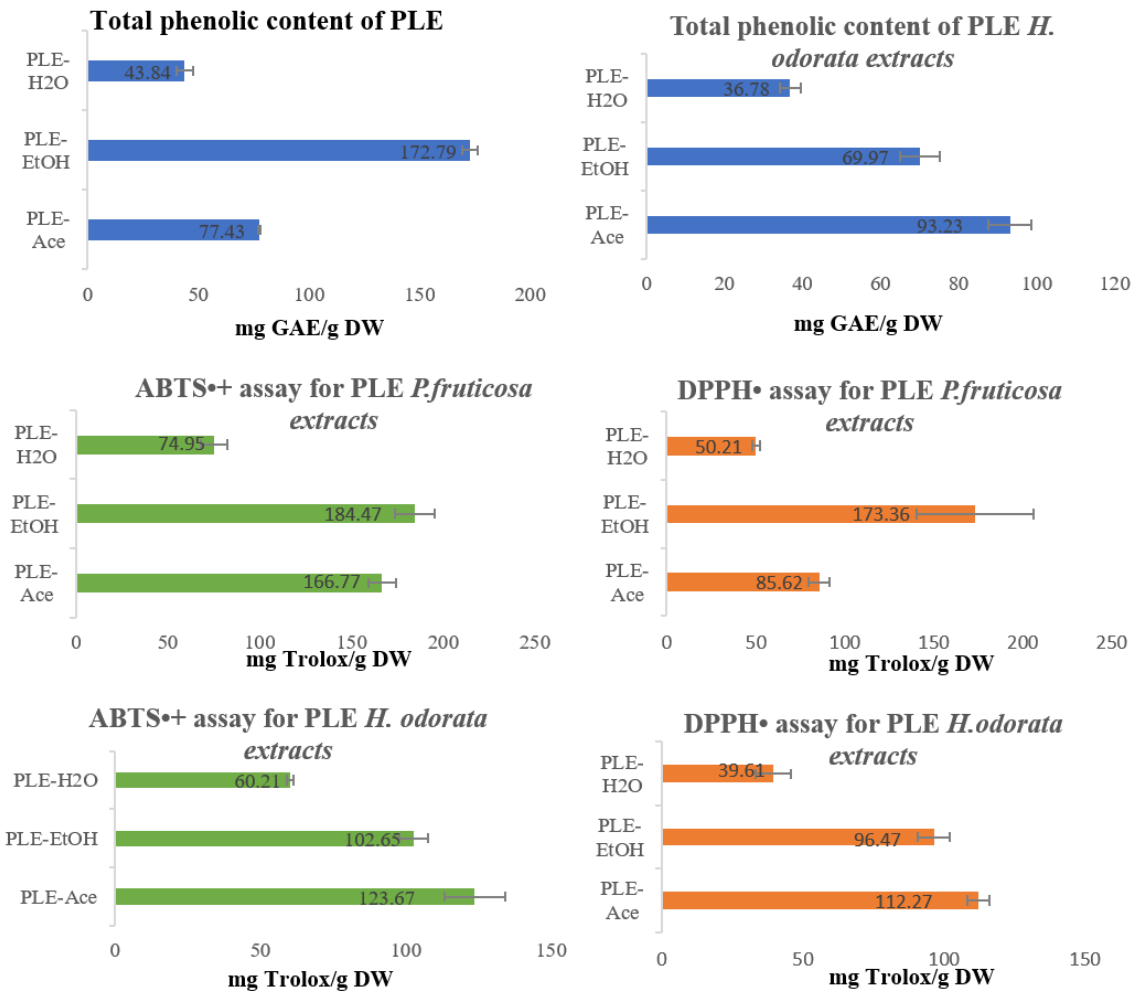


Figure 3.20 Antioxidant activity of PLE optimisation from *P. fruticosa* and *H. odorata* extracts

Appendix 7. Total phenolic content and Antioxidant activity of P. fruticosa and H. odorata SFE-CO2 residue-Enzyme treated PLE Extracts under optimal conditions.

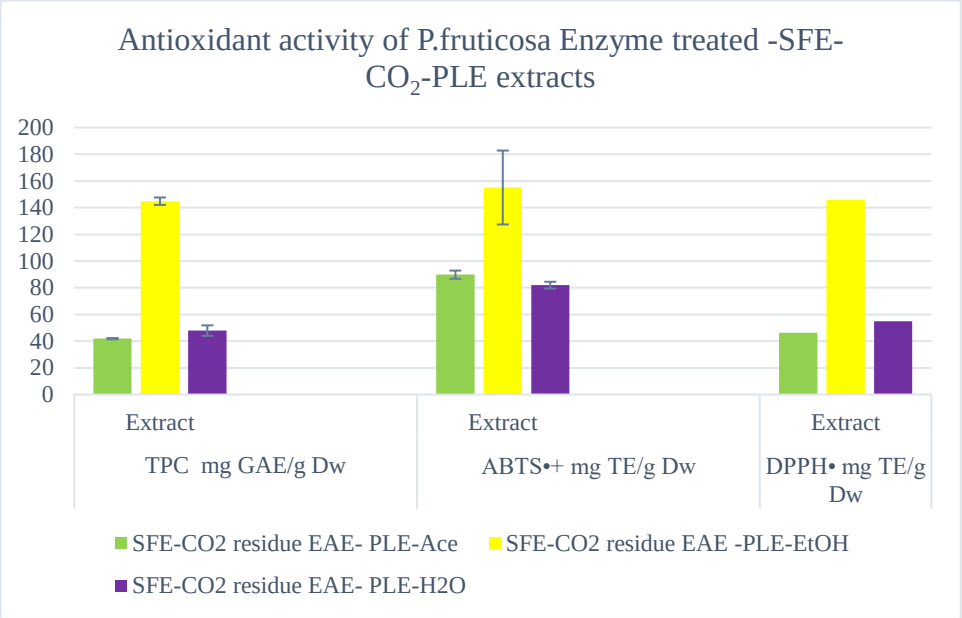


Figure 3.21 Antioxidant activity of *P. fruticosa* Enzyme treated -SFE-CO_2-PLE extracts

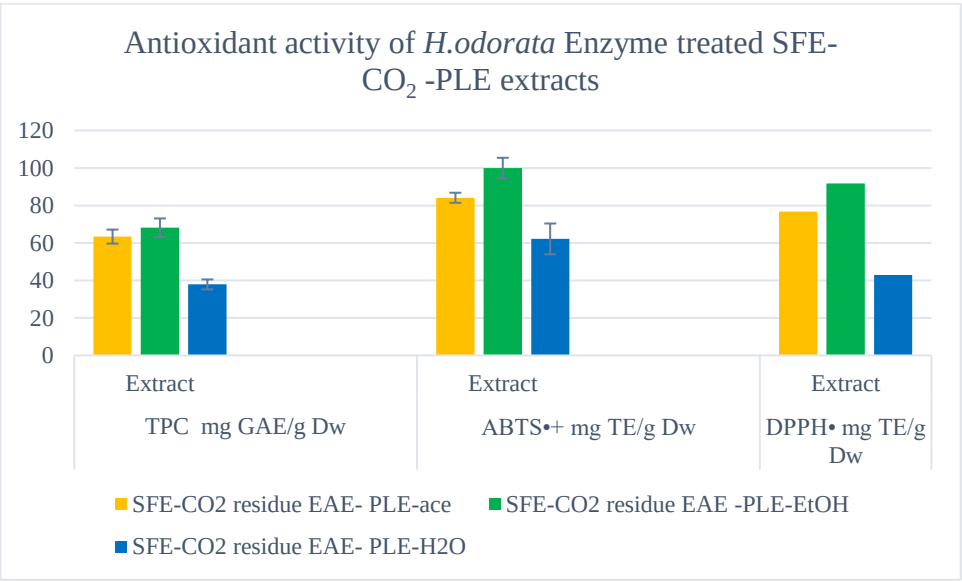


Figure 3.22 Antioxidant activity of *H. odorata* Enzyme treated SFE-CO_2 -PLE extracts

Appendix 8. GCxGC/TOF MS analysis results from P. fruticosa and H. odorata

Table 3.24 Compounds from GC-MS analysis of *Potentilla fruticosa* SFE-CO_2 extract.

R.T. (s)	Name	Retention Index	RI Reference*	Reference	Area %
464.3	Butanediol<2,3->	800.93	769-768	Pherobase	0.0190
744.4	Hexanoic acid	1002.1	973	Pubchem	4.3124
755.2	Hexanoic acid	1010	973	Pubchem	0.0363
843.7	2-Octenal	1075.4	1047	Pubchem	0.0219
854.9	Diethylene glycol monoethyl ether	1083.6	999	Pubchem	0.59969
969.7	Nonadienal <(2E,6Z)->	1172.1	1154	Adams	0.0493
1095.2	(-)-Carvone	1274.1	1229-1223	Pubchem	0.22259
1121.5	4-Methoxybenzaldehyde	1295.9	1262	Pubchem	0.03772
1174.9	2,4-Decadienal	1342.5	1288-1291	Pubchem	0.03068
1230.9	gamma-Nonanolactone	1391.6	1325-1324	Pubchem	0.23123
1306.5	Sesquisabinene	1461.5	1437-1446	Pubchem	0.59
1343.5	Coumarin	1496	1428-1429	Pubchem	2.7762
1400.5	trans-calamenene	1552.2	1508-1505	Pubchem	0.04152
1409.8	trans-calamenene	1561.4	1508-1505	Pubchem	0.08119
1478.5	á-Elemenone	1631.4	1601-1596	Pubchem	0.14116
1506.7	Cedrol	1661.1	1604-1596	Pubchem	0.1216
1562.9	Acorenone B	1721.6	1655-1640	Pubchem	0.21767
1631.6	Nonadecane	1798.3	1900	Pubchem	0.03758
1788.4	Dibutyl phthalate	1987.5	1957	Pubchem	0.06454

* Retention index according to available literature.

Table 3.25 Compounds from GC-MS analysis of *H. odorata* SFE-CO_2 extract.

R.T. (s)	Name	Retention Index	RI Reference*	Reference	Area %
425.8	Methyl 1-methylcyclopropyl ketone	773.3	730	chemspider	0.010027
477.1	2,3-Butanediol	810.12	769	pherobase	0.0013
531.4	Isovaleric acid	849.1	900-812	pubchem	0.018355
541.4	isovaleric acid	856.28	834	pherobase	0.0060
604	2,6-Dimethylpyridine	901.22	882-859.2	pubchem	0.00051475
655.9	Butyrolactone	938.48	914-864	pubchem	0.039527
796.5	o-Cymene	1040.5	1020-1011	pubchem	0.0012151
816.2	2,2,6-Trimethylcyclohexanone	1055.1	1035-1008	pubchem	0.0017963
863.7	2-Acetylpyrrole	1090.1	1064-1021	pubchem	0.00039539
874.4	2-Pyrrolidinone	1098	1045-1048	pubchem	0.0072719
1090.5	Cyclohexyl Isothiocyanate	1270.2	1231	pubchem	0.0072049
1095	(-)-Carvone	1274	1242-1243	pherobase	0.0303
1121.9	4-Methoxybenzaldehyde	1296.2	1252	pubchem	0.013298
1175.6	2-Methoxy-4-vinylphenol	1343.1	1313	pubchem	0.0018029
1218.4	Eugenol	1380.6	1356-1351	pherobase	0.0046
1288.8	Vanillin	1445	1420-1391	pherobase	0.0076
1358.2	Coumarin	1510.3	1428-1429	Adams RP	18.67
1478.7	á-Elemenone	1631.6	1601	pubchem	0.049435

* Retention index according to available literature.

Appendix 9. Analysis by UPLC/ESI-QTOF-MS

Table 3.26 Compounds found in *P. fruticosa* extracts positive ionisation mode using UPLC

Time (min)	Meas. m/z	Δppm	Suggested formula	Name+		SFE-CO2	PLE-ACE	PLE-EtOH	PLE-H2O
***P.fruticosa* +ve**									
0.3-0.4	104.107	0	$C_5H_{14}NO$	Choline	M+H				*
2.3-2.4	118.0862	0	$C_5H_{12}NO_2$	L-Valine	M+H				*
2.3-2.4	143.1064	1	C_8H15O_2	2-Octenoic acid	M+H		*		
0.3-0.4	145.0493	1	$C_6H_9O_4$	3-hexenedioic acid	M+H			*	
1.7-1.8	163.0388	1	$C_9H_7O_3$	3-Hydroxycoumarin	M+H				*
0.3-0.4	163.0599	1	$C_6H_{11}O_5$	Hydroxyadipic acid	M+H			*	
0.3-0.4	203.0523		$C_4H_7N_6O_4$	unknown	M+H		*	*	
7.0-7.1	254.2475	1	$C_{16}H_{32}NO$	Palmitoleamide	M+H	*	*	*	
7.8-7.8	256.2633	0	$C_{16}H_{34}NO$	Palmitic amide	M+H		*	*	
0.3-0.4	258.1099	0	$C_{12}H_{18}O_6$	UNKNOWN	M+H				*
7.3-7.4	280.2633	0	$C_{18}H_{34}NO$	Linoleamide	M+H	*	*	*	
7.9-7.9	282.2793	0	$C_{16}H_{34}N_4$	Oleamide	M+H	*	*	*	
8.6-8.7	284.2945	1	$C_{18}H_{38}NO$	Stearamide	M+H			*	
1.7-1.8	291.0861	0	$C_{15}H_{15}O_6$	(±)-Catechin	M+H		*	*	
1.7-1.8	293.0289		$C_{13}H_9O_8$	unknown	M+H				*
2.5-2.6	303.0496	0	$C_{15}H_{11}O_7$	Quercetin	M+H		*	*	*
3.1-3.2	317.0652	1	$C_{16}H_{13}O_7$	Isorhamnetin	M+H		*		
3.3-3.4	317.0653	6	$C_{16}H_{13}O_7$	dehypoxanthine futalosine	M+H		*		
0.3-0.4	325.1125	1	$C_{12}H_{21}O_{10}$	D-Fructofuranose dianhydride	M+H			*	

5.1-5.1	334.2011	0	$C_{19}H_{28}NO_4$	alpha-Eucaine	M+H	*		
1.6-1.8	355.102	1	$C_{16}H_{19}O_9$	Chlorogenic Acid	M+H		*	*
0.3-0.4	360.1497	9	$C_{12}H_{26}NO_{11}$	unknown	M+H		*	
8.3-8.4	371.101		$C_{20}H_{13}N_5O_3$	unknown	M+H		*	
2.3-2.4	435.0918	0	$C_{20}H_{19}O_{11}$	Avicularin	M+H	*	*	
3.3-3.4	449.1075	0	$C_{21}H_{21}O_{11}$	Quercitrin	M+H	*		
2.3-2.4	465.1024	0	$C_{21}H_{21}O_{12}$	Myrtillin	M+H	*	*	
2.3-2.4	479.082		$C_{21}H_{19}O_{13}$	Quercetin 3-galacturonide	M+H	*	*	*
3.1-3.3	479.1179	1	$C_{22}H_{23}O_{12}$	Petunidin 3-galactoside	M+H		*	*
3.1-3.1	487.1228	1	$C_{24}H_{23}O_{11}$	Epigallocatechin gallate	M+H		*	
1.6-1.8	579.1493	0	$C_{30}H_{27}O_{12}$	Procyanidin	M+H		*	
1.6-1.6	579.1494	0	$C_{30}H_{27}O_{12}$	Kaempferol rhamnosides	M+H	*		
8.3-8.4	593.2755	0	$C_{35}H_{37}N_4O_5$	Pheophorbide a	M+H	*	*	
3.1-3.3	595.1441	0	$C_{30}H_{27}O_{13}$	Hyacinthin	M+H		*	
2.3-2.4	611.1598	1	$C_{27}H_{31}O_{16}$	Rutin	M+H	*		
3.1-3.1	616.2646		$C_{46}H_{34}NO$	unknown	M+H		*	
3.3-3.4	630.2801		$C_{32}H_{32}N_{13}O_2$	unknown	M+H	*		
3.3-3.3	630.2804		$C_{34}H_{34}N_{10}O_3$	unknown	M+H	*		
1.6-1.8	867.213	0	$C_{45}H_{39}O_{18}$	Cinnamtannin	M+H		*	

*Indicates the presence of particular compound in their respective extract, + suggested name from METLIN database

Table 3.27 Compounds found in *P. fruticosa* extracts negative ionisation mode using UPLC

Time(min) P.fruticosa -ve	Meas. m/z	Δppm	Suggested formula	Name+		SFE-CO2	PLE-ACE	PLE-EtOH	PLE-H2O
0.4-0.5	133.0142	0	$C_4H_5O_5$	Malic acid	M-H				*
1.9-2.0	165.0193	0	$C_8H_5O_4$	Benzoquinoneacetic acid	M-H		*	*	
1.0-1.0	169.0142	0	$C_7H_5O_5$	Gallic acid	M-H				*
0.3-0.4	179.056	0	$C_6H_{11}O_6$	α-D-Glucose	M-H		*	*	*
0.7-0.8	191.0198	0	$C_6H_7O_7$	Citric acid	M-H				*
0.4-0.4	191.0562	0	$C_7H_{11}O_6$	Quinic acid	M-H			*	*
0.3-0.4	195.0511	0	$C_6H_{11}O_7$	Gulonic acid	M-H				*
0.3-0.4	215.0325		$C_8H_3N_6O_2$	unknown	M-H		*	*	
0.9-0.9	217.0354		$C_7H_3N_7O_2$	unknown	M-H				*
0.3-0.4	269.0877		$C_8H_{11}N_7O_4$	unknown	M-H		*		
1.7-1.8	289.0718	0	$C_{15}H_{13}O_6$	(±)-Catechin	M-H		*	*	*
1.8-1.9	291.0148		$C_{13}H_7O_8$	unknown	M-H				*
2.3-2.4	300.9987	0	$C_{14}H_5O_8$	Ellagic acid	M-H		*	*	*
2.5-2.6	319.0456	1	$C_{15}H_{11}O_8$	Dihydromyricetin	M-H			*	
1.9-2.0	327.1083	0	$C_{15}H_{19}O_8$	Ethyl vanillin glucoside	M-H		*	*	
3.9-4.0	329.2336	0	$C_{18}H_{33}O_5$	trihydroxy octadecenoic acid	M-H		*		
0.4-0.4	341.1086	0	$C_{12}H_{21}O_{11}$	Sucrose	M-H		*	*	
1.7-1.8	353.0877	0	$C_{16}H_{17}O_9$	Chlorogenic Acid	M-H			*	*
0.4-0.4	377.0854		$C_{13}H_7N_{13}O_2$	unknown	M-H			*	

0.3-0.4	387.1143		$C_{13}H_{23}O_{13}$	unknown	M-H	*	*	*
2.6-2.7	433.0771	0	$C_{20}H_{17}O_{11}$	Avicularin	M-H	*		
2.5-2.6	433.078	0	$C_{20}H_{17}O_{11}$	Tricetin 3'-xyloside	M-H		*	
3.3-3.4	447.0938	1	$C_{21}H_{19}O_{11}$	Quercitrin	M-H	*	*	
2.6-2.7	451.1035	0	$C_{24}H_{19}O_{9}$	Cinchonain	M-H	*		
2.3-2.3	463.088	0	$C_{21}H_{19}O_{12}$	Myrtillin	M-H	*	*	*
6.3-6.3	471.3483	0	$C_{30}H_{47}O_{4}$	Maslinic Acid	M-H	*		
2.3-2.4	477.0674	0	$C_{21}H_{17}O_{13}$	Quercetin 3-galacturonide	M-H	*	*	*
3.2-3.2	477.104	0	$C_{22}H_{21}O_{12}$	Petunidin 3-galactoside	M-H	*	*	*
0.6-0.6	481.0622		$C_{20}H_{17}O_{14}$	unknown	M-H			*
1.6-1.7	483.0784	0	$C_{20}H_{19}O_{14}$	Hamamelitannin	M-H		*	
3.1-3.1	485.1094	0	$C_{24}H_{21}O_{11}$	Epigallocatechin 3-O-(3,5-di-O-methylgallate)	M-H		*	
5.7-5.8	485.3276	0	$C_{30}H_{45}O_{5}$	Quillaic acid	M-H	*		
5.0-5.1	487.3431	0	$C_{30}H_{47}O_{5}$	bayogenin	M-H	*		
3.2-3.3	491.0833	0	$C2_{2}H_{19}O_{13}$	6-Methoxyluteolin 7-glucuronide	M-H			*
6.3-6.3	517.3537	0	$C_{31}H_{49}O_{6}$	unknown	M-H	*		
3.1-3.1	521.0856		$C_{26}H_{13}N_{6}O_{7}$	unknown	M-H		*	
5.7-5.8	531.3332	0	$C_{31}H_{47}O_{7}$	1α,25-dihydroxy-22-oxavitamin D3 3-hemiglutarate	M-H	*		
5.0-5.1	533.3489		$C_{30}H_{43}N_{7}O_{2}$	unknown	M-H	*		
2.2-2.3	576.1272		$C_{28}H_{12}N_{14}O_{2}$	unknown	M-H	*		
1.9-2.0	577.1347	0	$C_{30}H_{25}O_{12}$	Procyanidin B1	M-H	*	*	*
1.7-1.8	579.1507	0	$C_{30}H_{27}O_{12}$	Prunin 6"-p-coumarate	M-H	*	*	

3.2-3.2	593.1308	1	$C_{30}H_{25}O_{13}$	Hyacinthin	M-H	*	*	*
2.3-2.3	609.1458	0	$C_{27}H_{29}O_{16}$	Rutin	M-H	*		
2.2-2.3	609.1458		$C_{39}H_{15}N_9$	unknown	M-H		*	
3.1-3.1	614.2514		$C_{36}H_{38}O_9$	unknown	M-H	*	*	
2.2-2.3	615.0988	0	$C_{28}H_{23}O_{16}$	Quercetin 7-(6"-galloylglucoside)	M-H	*	*	
3.3-3.4	628.267		$C_{37}H_{40}O_9$	unknown	M-H	*		
1.9-2.0	635.0886		$C_{40}H_{15}N_2O_7$	unknown	M-H		*	
1.9-2.0	635.0889	0	$C_{27}H_{23}O_{18}$	Gallotannin	M-H	*		
1.7-1.8	643.1667	0	$C_{44}H_{23}N_2O_4$	unknown	M-H		*	
3.1-3.1	720.1593		$C_{38}H_{28}N_2O_{13}$	unknown	M-H	*	*	
1.9-2.0	865.198		$C_{46}H_{33}N_4O_{14}$	unknown	M-H	*		
6.3-6.3	943.7025		$C_{60}H_{95}O_8$	unknown	M-H	*		

*Indicates the presence of particular compound in their respective extract, + suggested name from METLIN database

Table 3.28 Compounds found in *H.odorata* extracts positive ionisation mode using UPLC

Time(min)	Meas. m/z	Δ ppm	Suggested formula	Name+		SFE-CO2	PLE-ACE	PLE-EtOH	PLE-H2O
H.odorata +ve									
0.3-0.4	116.0706	0	$C_5H_{10}NO_2$	L-Proline	M+H			*	*
2.8-3.0	147.0439	1	$C_9H_7O_2$	Coumarin	M+H	*	*	*	
2.1-2.1	165.0544	1	$C_9H_9O_3$	m-Coumaric acid	M+H			*	
1.4-1.5	179.0337	1	$C_9H_7O_4$	7,8-Dihydroxycoumarin	M+H		*	*	*
4.0-4.1	181.1221	1	$C_{11}H_{17}O_2$	Jasmolone	M+H	*			
2.5-2.6	197.1171	0	$C_{11}H_{17}O_3$	2,6-Dimethoxy-4-propylphenol	M+H	*			
2.5-2.6	197.1171	0	$C_{11}H_{17}O_3$	Hexyl 2-furoate	M+H		*		
0.3-0.4	203.0526		$C_4H_7N_6O_4$	unknown	M+H		*		
1.6-1.7	217.1336	0	$C_{13}H_{17}N_2O$	Tetrahydroharmine	M+H		*	*	
7.0-7.1	254.2476	0	$C_{16}H_{32}NO$	Palmitoleamide	M+H	*	*	*	
7.7-7.8	256.2634	0	$C_{16}H_{34}NO$	Palmitic amide	M+H	*	*	*	
7.5-7.6	279.2316	0	$C_{18}H_{31}O_2$	Linolenic Acid	M+H	*			
7.2-7.4	280.2631	1	$C_{18}H_{34}NO$	Linoleamide	M+H	*		*	
7.9-8.0	282.2794	0	$C_{18}H_{36}NO$	Oleamide	M+H	*	*	*	
7.2-7.4	323.2574	2	$C_{20}H_{35}O_3$	5-HETrE	M+H	*			
2.1-2.1	344.134	0	$C_{15}H_{22}NO_8$	b-D-Glucopyranosiduronic acid	M+H			*	
1.4-1.5	358.1129	0	$C_{15}H_{20}NO_9$	cyclo-Dopa 5-O-glucoside	M+H		*	*	*
2.1-2.1	535.291	1	$C_{29}H_{43}O_9$	Helveticoside	M+H			*	
2.7-2.7	563.3227	0	$C_{32}H_{43}N_4O_5$	Carpipramine maleate	M+H		*		
2.7-2.8	577.3383	2	$C_{32}H_{49}O_9$	Oleandrin	M+H		*	*	
8.4-8.5	593.2756	0	$C_{35}H_{37}N_4O_5$	Pheophorbide a	M+H		*	*	

8.9-9.0	628.1947	$C_{48}H_{24}N_2$	unknown	M+H		*		
8.9-9.0	628.1948	$C_{48}H_{24}N_2$	unknown	M+H			*	
8.9-9.0	628.1949	$C_{48}H_{24}N_2$	unknown	M+H	*			

*Indicates the presence of particular compound in their respective extract, + suggested name from METLIN database

Table 3.29 Compounds found in *H.odorata* extracts negative ionisation mode using UPLC

Time(min) **H.odorata - ve**	**Meas. m/z**	**Δ ppm**	**Suggested formula**	**Name+**		**SFE-CO2**	**PLE-ACE**	**PLE-EtOH**	**PLE-H2O**
0.3-0.6	133.0141	0	$C_4H_5O_5$	Malic acid	M-H			*	*
2.4-2.5	161.0244	0	$C_9H_5O_3$	3 Hydroxycoumarin	M-H		*	*	
2.6-2.7	165.0557	0	$C_9H_9O_3$	Tropic acid	M-H		*	*	
1.9-2.0	177.0192	0	$C_9H_5O_4$	7,8-Dihydroxycoumarin	M-H	*	*	*	
0.3-0.4	179.0559	1	$C_6H_{11}O_6$	α-D-Glucose	M-H		*	*	
0.7-0.9	191.0197	0	$C_6H_7O_7$	Citric acid	M-H				*
0.3-0.5	191.056	0	$C_7H_{11}O_6$	Quinic acid	M-H			*	*
1.6-1.7	203.0826	0	$C_{11}H_{11}N_2O_2$	L-Tryptophan	M-H			*	
0.6-0.7	217.0353		$C_8H_9O_7$	unknown	M-H				*
0.7-0.9	235.0458		$C_7H_5N_7O_3$	unknown	M-H				*
8.6-8.7	255.2331	0	$C_{16}H_{31}O_2$	Palmitic acid	M-H	*			
1.6-1.7	261.1243	0	$C_{14}H_{17}N_2O_3$	L-prolyl-L-phenylalanine	M-H			*	
7.6-7.6	277.2175	0	$C_{18}H_{29}O_2$	α-Linolenic Acid	M-H	*	*		
5.7-5.9	293.2124	0	$C_{18}H_{29}O_3$	alpha-kamlolenic acid	M-H	*			

4.9-5.0	311.223	0	$C_{18}H_{31}O_4$	9E-Octadecenedioic acid	M-H	*			
4.9-5.0	313.0718	0	$C_{17}H_{13}O_6$	Odoratin	M-H	*			
7.4-7.5	321.2436	0	$C_{20}H_{33}O_3$	(±)5-HETrE	M-H	*			
1.7-1.8	325.0926	0	$C_{15}H_{17}O_8$	trans-β-D-Glucosyl-2-hydroxycinnamate	M-H		*	*	*
1.6-1.7	339.0719	0	$C_{15}H_{15}O_9$	Sinapoyl malate	M-H		*	*	*
0.3-0.4	341.1086		$C_{25}H_{13}N_2$	unknown	M-H		*		
0.3-0.5	341.1086	0	$C_{12}H_{21}O_{11}$	Sucrose	M-H			*	*
8.6-8.7	355.1584		$C_{24}H_{21}NO_2$	unknown	M-H	*			
0.3-0.5	377.0852		$C_{14}H_{13}N_6O_7$	unknown	M-H			*	
7.6-7.6	377.1429		$C_{26}H_{19}NO_2$	unknown	M-H		*		
0.3-0.5	387.1141		$C_{13}H_{23}O_{13}$	unknown	M-H			*	
0.3-0.4	387.1144		$C_{11}H_{11}N_{14}O_3$	unknown	M-H		*		
0.3-0.6	387.1144		$C_{13}H_{23}O_{13}$	unknown	M-H				*
0.3-0.6	475.1303		$C1_6H_{27}O_{16}$	unknown	M-H				*
1.9-2.0	483.1143	0	$C_{21}H_{23}O_{13}$	Diospyrin	M-H			*	
1.3-1.4	501.1244		$C_{20}H_{19}N_7O9$	unknown	M-H			*	
1.6-1.7	501.1259		$C_{37}H_{15}N_3$	unknown	M-H			*	
7.2-7.3	585.3071		$C_{33}H_{45}O_9$	unknown	M-H		*		
8.3-8.4	591.2618	0	$C_{35}H_{35}N_4O_5$	Pheophorbide a	M-H		*		
7.2-7.3	631.312		$C_{47}H_{39}N_2$	unknown	M-H		*		
2.1-2.2	651.1924	1	$C_{30}H_{35}O_{16}$	Cladrastin 7-O-laminaribioside	M-H		*	*	*
1.5-1.6	679.1512	0	$C_{30}H_{31}O_{18}$	Palargonidin 3-(6"-malonylsophoroside)	M-H		*	*	*

*Indicates the presence of particular compound in their respective extract, + suggested name from METLIN database