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Isolation and identification of Phenolic compounds by HPLC and Electrospray Ionization Mass Spectrometry of *Svensonia Hyderobadensis* – A Rare Medicinal Plant Taxon

***Linga Rao M**

Savithramma N

Department of Botany, S. V. University, Tirupati, A.P, India.

Corresponding Authors:
M. Linga Rao
Research Scholar
Department of Botany,
Sri Venkateswara University,
Tirupati – 517 502.
Andhra Pradesh, INDIA.
Email: matti2010rao@gmail.com

Abstract:
The impetus for developing analytical methods for phenolic compounds in natural products has proved to be multifaceted. Hence the present study intended to isolate phenolic compounds from leaves of *Svensonia hyderobadensis* by using 70% acetone and poly vinyl poly pyrrolidone (PVPP); and characterized by U.V. Visible spectrometry, High performance liquid chromatography/ electrospray ionization mass spectrometry. Total 82 phenolic compounds were obtained at both positive and negative ion modes of LCMS. Among the isolated phenols, 19 phenolic compounds have been identified based on their retention time and m/z values. Namely, Caffeic acid, Rhamnetin hexoside, 1-Caffeoyl-5-feruloylquinic acid, 9-COA, Oleuropein, P-Coumaroyl hexosa, Kaempferol-3-O-glucose, Carnosic acid, Coumaroyl 5-O-caffeoyl quinic, Oleuropein aglycon, 3, 9-di-C DOA glucoside, Luteolin-3-O-glucoronide, Quercetin-3-O-glucoside, Rhamnetin hexosyl pentoside, 6-Rhamnopyranosyl oleoside, 5-pyranopelargonidin-3-glucoside, Ligstroside, 4-Malonyl-3, 5-diCQA, Rhamnetin-O-rutinoside. This study illustrate the rich array of phenolic compounds of leaves of *Svensonia hyderobadensis* could be utility as health beneficial bioactive compounds.

Keywords: *Svensonia hyderobadensis*, leaves, phenolic compounds, liquid chromatography, Electro Spray Ionization mass spectrometry

Full Length Original Research Paper

Introduction

Plants are sessile organisms that cannot evade their environment and have thus evolved all sorts of plastic mechanisms to deal with a wide range of potential threats. Among those mechanisms, the expansion of secondary metabolites with defence, communication and protection roles is now considered to be particularly important. The range of secondary metabolites including phenols, amines, indoles, alkaloids and sulphonates may act as reductant substrates of peroxidases [1]. Researchers have become increasingly interested in dietary phenolic compounds because of free radical scavenging activity and other potential beneficial effects on human health associated with their consumption [2]. Many activities have been reported for most of the phenolic compounds from plants they act as anti-oxidant, anti-inflammatory, anti-viral and anti carcinogenic agents [3].

Phenolic compounds are one of the most diverse groups of phytochemicals that are universally distributed in fruits, vegetable and herbs. Approximately 8000 phenolic compounds have been isolated from natural resources [4]. Polyphenols in nature generally occur as conjugates of sugar, usually o-glycosides, phenolic acids contain two distinctive carbon frame works, the hydroxyl cinnamic and hydroxyl benzoic structures [5]. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring make the difference and establish the variety [6].

The *Svensonia hyderobadensis* a delicate shrub belonging to the family Verbenaceae, it is commonly called as "Adavichiki" in Telugu language and listed under rare and also recorded highly threatened taxon in Sri Lanka [7]. The plant is used to treat hepatotoxic diseases [8] and has also been studied for biological synthesis of silver nanoparticles and antimicrobial activity [9, 10], phytochemistry [11], pharmacognostical studies [12]. The macroscopical studies revealed that the plant average is 0.5 to 2.0 M in length. Stem is green in colour, hard, branched, branchlets 4-angular, pubesent. Leaves are 6-8 cm long, 2-4 cm in broad. Opposite phyllotaxy, elliptic-ovate to obovate, coarsely serrate, acute, base rounded to decurrent, charataceous, lateral nerves 6 pairs. Flowers are pink-purple in colour, small, terminal spikes, bracts linear-lanceolate, scarious. Calyx tubular, unequally 5-toothed, 5-ribbed, splitting along 2 longer-teeth. Corolla salver-form, slightly widened above, obscurely 2-lipped, lobes 5. Stamens 4, inserted at the dilated portion of corolla-tube, included, filaments hairy. Ovary bicarpellary, bilocular; ovule 1 per locule, basal, stigma bilobed. Fruits of 2 oblong, 1-seeded pyrenes. Flowering and fruiting season is November-February [13]. The present study aimed to expand the compositional database of on phenolic compounds by isolation and characterization of *Svensonia hyderobadensis* leaves.

Material and methods

Extraction of polyphenols from leaf

The fully matured healthy leaves of *Svensonia hyderobadensis* were collected from Mamanduru forest area of Chittoor District of Andhra Pradesh, India during December 2011. The materials were washed thoroughly and shade dried.

30 g of leaf powder reduced in a mortar and consequently extracted with 500 mL of dichloromethane by ultra-sonication for about 30 min and shaken by vortex for 30 min to remove hydrophilic compounds. Dilapidated powder were extracted with 500 mL of acetone/water (70:30, v/v) by sonication for 15 min and shaking for more 15 min to extract polyphenols. 150 mL of each leaf water extract (pH adjusted to 4.0) was mixed with 5 g of PVPP (30 mg/mL) for 15 min of shaking for adsorption of phenolic compounds to PVPP (Sample-1). The remaining PVPP was re-extracted twice again with 200 mL of fresh extraction solvent for the same period time that was used before. The combined extracts were evaporated at room temperature by rotary evaporation to remove the organic solvent (acetone) (Sample-2).

HPLC-ESI-MS/MS analysis

The qualitative study of the phenolic compounds in all samples was performed by HPLC coupled on-line with electrospray ionization (ESI) mass spectrometry. The HPLC system (Agilent 1100 series) consisted of a low-pressure quaternary pump (Agilent 1100 series) and an auto-sampler. A quadropole ion trap mass spectrometer (Agilent 1100) equipped with an ESI source in the positive and negative ion mode and Xcalibur software Version 1.4 (Finningan) were used for data acquision and processing.

Results and discussion

Phenolic compounds

In this study over 82 (Positive mode-45 and Negative mode-37) phenolic compounds were extracted from lyophilized leaf samples of

Svensonia hyderobadensis. Among these 19 phenolic compounds were identified by comparing retention times, UV-Vis, HPLC and MS spectral data with those of literature data. The identification of these compounds was summarized in **Table-1** and **Fig-4**. UV-Visible spectrometry was a valuable tool for identifying the class of phenolic compounds. The extracted sample solution has given maximum height of the peak at 262 nm from the range of 254 nm to 300 nm. According to Aaby *et al*. [14] most of the phenolic compounds absorb wavelength at 260 nm giving a non specific chromatogram. All phenolic compounds exhibit intense absorption in the UV region of the spectrum and those that are absorb colour strongly in the visible region as well. Each class of phenolic compounds has distinctive absorption characteristics. Phenols and phenolic acids show spectral maxima in the range between 250-290 nm, cinnamic acid derivatives have principal maxima in the range 290-330 nm, flavones and flavonols exhibit absorption bands of approximately the same intensity at about 250 and 350 nm, chalcones and aurones have an absorption peak of great intensity above 350 nm and a much less intense band at 250 nm, anthocyanins and betacyanins showed rather similar absorption in visible region 475-560 nm and 535-545 nm, respectively and a subsidiary peak at about 270-275 nm [6, 15, 16].

High performance liquid chromatography (HPLC) detection has given 22 peaks (sample-1 and sample-2) at various retention times starting from 5 min to 140 min. maximum number of peaks obtained with first 70 min with Sample-1 and 40-120 min with Sample-2 respectively. Among the eluted peaks 2nd and 3rd showed more height (715.441 mV and 994.009 mV) with large area 26646.2 mV.s and 122238.482 mV.s of sample-1 and sample-2 respectively **(Fig-1 a-b)**. Acidification of the mobile phase allows the best separation of phenols, because the hydroxyl groups are kept in their acidic form thereby increasing their retention on the column and decreasing peak broadening caused by formation of deprotonated form [17]. Liquid chromatography, electrospray ionization – Tandem mass spectrometry (LC-ESI-MS) has been given MS fragmentation data for structural characterization of the extracts phenolic compounds. Identification has been carried out based on their Pseudomolecular (M-H) ions and m/z values of mass chromatography **(Fig-2 a-b; Fig-3 a-b)**. The combination of HPLC and MS as an identification method provided a highly selective method for the characterization of the analytes. ESI operated in negative mode, which is known as a soft and highly efficient ionization method, proved to be an excellent tool for the identification of flavonoid glycosides by providing information on the glycoside molecular masses due to their prominent $(M-H)^-$ ions and fragmentation products of the aglycone arising from Retro-Diels-Alder reactions [18, 19, 20]. ESI-MS parameters were optimized for the components and the solvent system, respectively to maximum ionization efficiency fragmentation of phenolic acid yield product ions more efficiently at lower collision energies. While flavonols and particularly their aglycones needed higher collision energies to obtain diagnostically relevant product ions [21].

The fragment ions m/z 179 is unique to caffeic acid was observed. This is produced as a result of the cleavage of the phenolic ring of the precursor ion at m/z 133 at different sites and other fragment ions m/z 113, 101 and 71 also unique to caffeic acid [22]. Identification of caffeic acid was achieved by comparing the ESI-MS spectra with

those of pure standard. While it generated in negative ion mode gave the deprotonated molecule at m/z 135 ($M-H^-$) owing to the loss of CO_2, reported from *Olea europara* by Savarese *et al.* [17]. The fragment ions m/z 165 was observed as Rhamnetin. Similar fragment was observed in Jocote [21].

Glucoronide is derivative of flavonoids could be detected in leaf extract of S. hyderobadensis. This compound eluting at 12.15 min was identified as luteolin-3-O-glucoronide, subsequent collision induced fragmentation of luteolin-3-O-glucoronide showed the loss of a glucoroonic acid (m/z 176) and produced the predominant fragment at m/z 461 corresponding to deprotonated luteolin-3-O-glucoronoide. Similar fragmentation of the compound was reported by Justesen [19] in analyzing thyme extract.

The identification of Oleuropein was corroborated by detection of the molecular ion at m/z 275 and its aglycone fragment at m/z 377. Similar results were reported from olive pulp. The mass spectrum of m/z 275 was formed by the lose of 162 Da and another intense peak at m/z 377 indicative of the elimination of another hexose unit [23]. These two main fragments correspond to Oleuropein and its aglycone respectively and together they support the hypothesis of a hexose derivative of the Oleuropein structure [24]. The fragments m/z 191, m/z 215 and m/z 623 were observed as 1-Caffeoyl-5-ferruoylquinic acid, 9-COA and Rhamnetin-O-rutinoside respectively. Similar fragments were reported by Havattum and Ekeberg [18].

As far as the role of plant phenolics an internal physiological regulator or chemical messengers within the intact plant is concerned, some information is available. Hydroxycinnamic acids, particularly p-coumaric acid and ferulic acid, are found in the insoluble or cell wall fraction also as esters. These pools of wall-bound acids act as a reservoir of phenylpropanoid units for lignin biosynthesis or even that they represent the beginnings of lignifications itself. In addition, by radical dimerization of ferulates, polysaccharide-polysaccharide cross-linking is effected: feruloylation occurs on the arabinose or galactose side chains of pectic polysaccharides. A possible role of feruloyl pectin may be in the regulation of cell expansion, possibly through coupling reactions leading to the production of diferulate [25, 26, 27]. Some intriguing effects of plant phenolics are the ones associated with the growth hormone auxin (Indole Acetic Acid). Monohydroxy B-ring flavonoids are suggested as cofactors of peroxidase functioning as an IAA oxidase that destroys the hormone, where dihydroxy B-ring forms act as inhibitors of the IAA degrading activity [28].

Plant material phenolics affecting seed germination and dormancy, and these substances, found in both seed coats and embryos, have been identified as phenolic acids, hydroxycinnamic acids, and coumarins. In this connection, non-germinating seeds of *Melilotus alba* were found to possess a large amount of free coumarin, while in rapidly germinating seeds Coumarinic acid and β-glucoside were more prevalent. Another naturally occurring phenolic compound inhibiting the germination of seeds of the same or of other species is ferulic acid: pure solution of ferulic acid gave strong inhibition of *Raphanus sativus* seed germination at concentration of 10^{-4} M. It has been suggested that phenolics may be active as germination inhibitors by inhibiting the transport of amino acids and the formation of proteins in the seeds [29, 30].

Several studies have indicated a high degree of compartmentation of phenolic compounds and of the enzymes involved in their biosynthesis. Phenolics usually accumulate in the central vacuoles of guard cells and epidermal cells as well as subepidermal cells of leaves and shoots. Furthermore, some phenolics are found covalently linked to plant cell wall; others occur in waxes or on the external surfaces of plant organs. Some findings suggest that a deposition of flavonoids in nuclei of certain tree species: it has been suggested that a flavonoid-DNA complex provides a mutual protection against oxidative damage [31, 32].

Leaves of *Svensonia hyderobadensis* is rich source of phenolic compounds. Moreover, these phenols could be used as natural antioxidants substituting the synthetic antioxidants in food, cosmetic and pharmaceutical industries.

Table 1: Phenolic compounds identified in *Svensonia hyderobadensis*

S. No.	Pseudomolecular Ion m/z values	Empirical molecular formula	Compound
1.	179	$C_9H_8O_4$	Caffeic acid
2.	165		Rhamnetin hexoside
3.	191		1-Caffeoyl-5-feruloylquinic acid
4.	215		9-COA
5.	275		Oleuropein
6.	325		P-Coumaroyl hexosa
7.	327		Kaempferol-3-O-glucose
8.	331	$C_{20}H_{28}O_4$	Carnosic acid
9.	354		Coumaroyl 5-O-caffeoyl quinic
10.	377		Oleuropein aglycon
11.	407		3, 9-di-C DOA glucoside
12.	461	$C_{21}H_{17}O_{12}$	Luteolin-3-O-glucoronide
13.	463	$C_{21}H_{19}O_{12}$	Quercetin-3-O-glucoside
14.	459		Rhamnetin hexosyl pentoside
15.	491		6-Rhamnopyranosyl oleoside
16.	501		5-pyranopelargonidin-3-glucoside
17.	523		Ligstroside
18.	556		4-Malonyl-3, 5-diCQA
19.	623		Rhamnetin-O-rutinoside

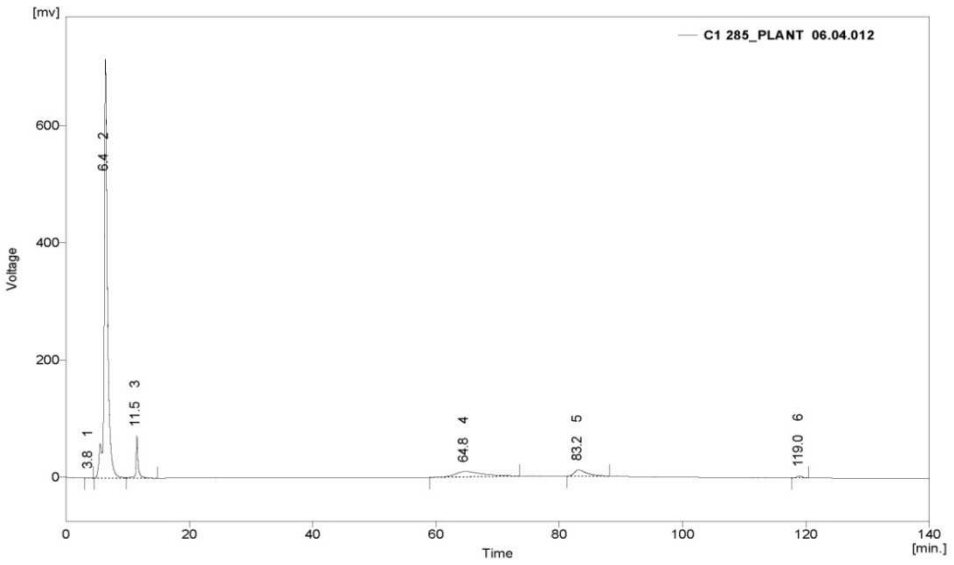


Fig: 1(a) HPLC chromatogram (monitored at 285 nm) of phenolic extract of *Svensonia hyderobadensis* (Sample-1)

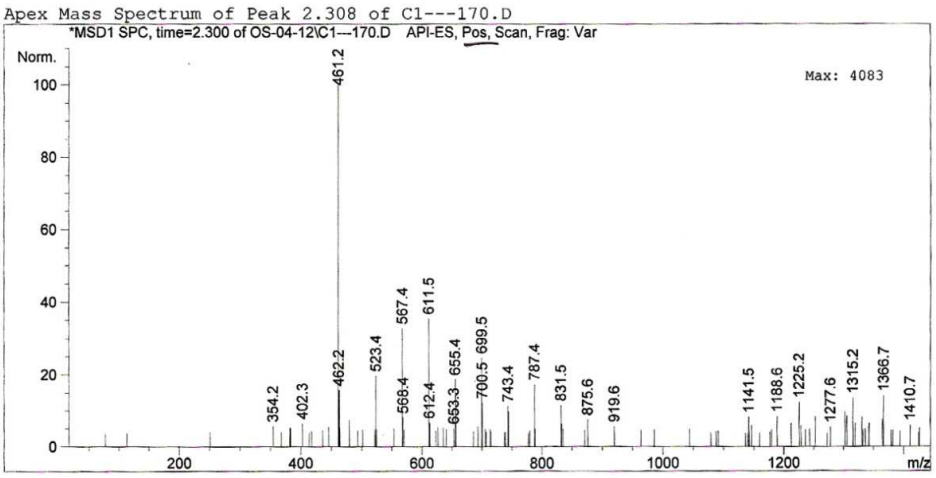


Fig: 2(a) Mass spectra obtained in positive ion mode fragmentation of Phenolic extract of *Svensonia hyderobadensis* (Sample-1)

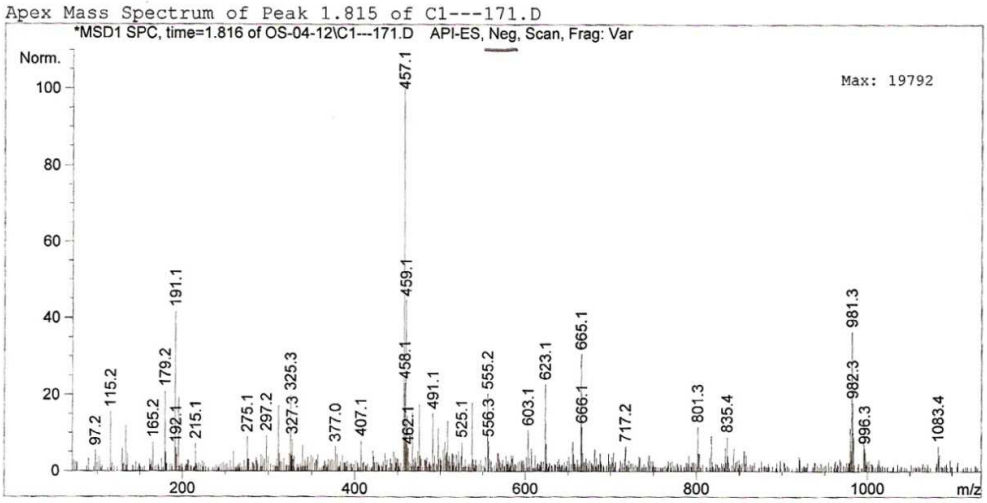


Fig: 2(b) Mass spectra obtained in negative ion mode fragmentation of Phenolic extract of *Svensonia hyderobadensis* (Sample-1)

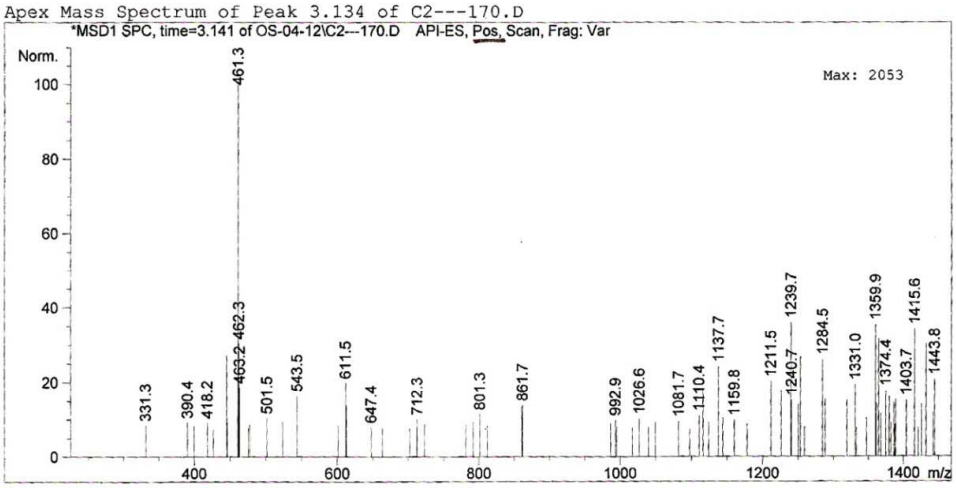


Fig: 3(a) Mass spectra obtained in positive ion mode fragmentation of Phenolic extract of *Svensonia hyderobadensis* (Sample-2)

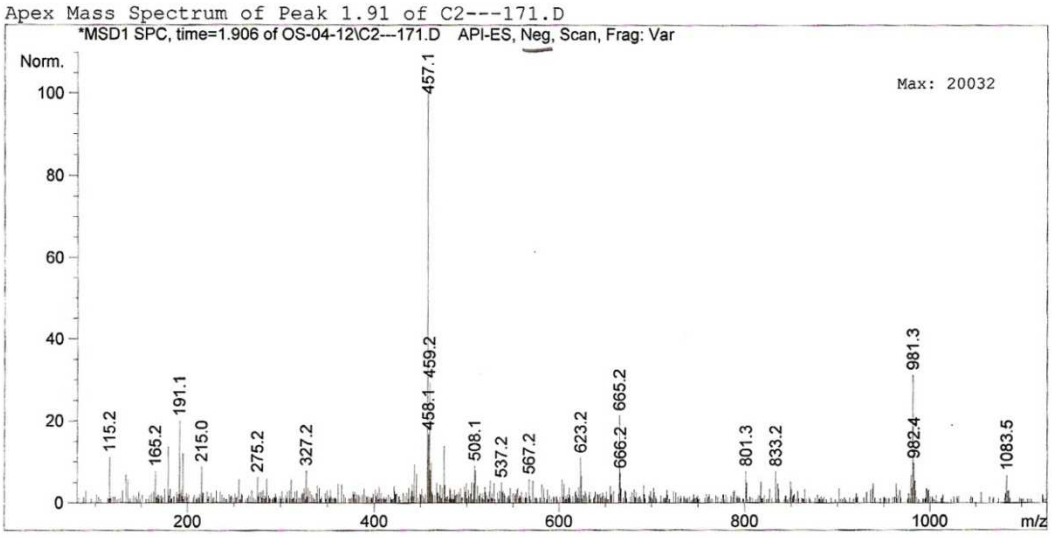
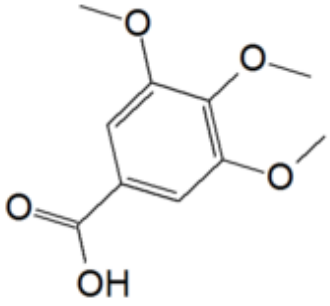
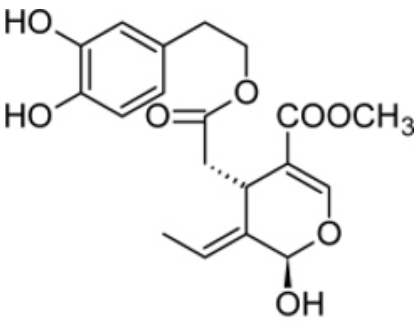


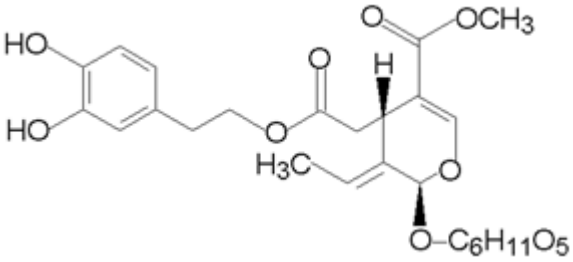
Fig: 3(b) Mass spectra obtained in negative ion mode fragmentation of Phenolic extract of *Svensonia hyderobadensis* (Sample-2)



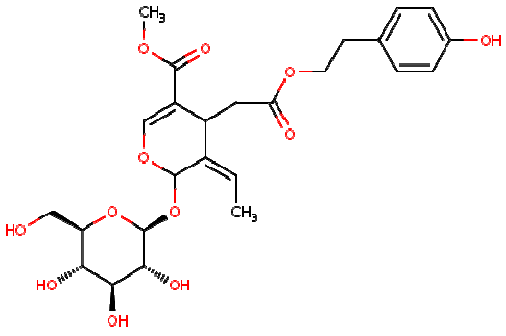
Caffeic acid



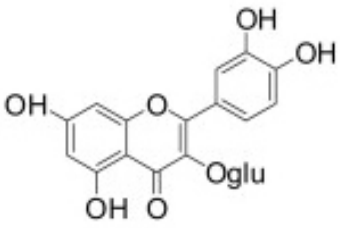
Oleuropein aglycon



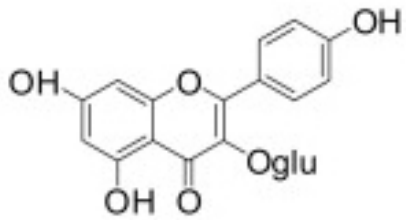
Oleuropein



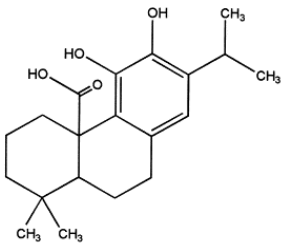
Ligstroside



Quercetin-3-O-Glucoside



Kaempferol-3-O-glucoside



Carnosic Acid

Fig-4: Chemical structures of phenolic compound detected in *Svensonia hyderobadensis*

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