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# Resveratrol and other phenolic compounds from wild grape *Vitis vinifera.* ssp *sylvestris*

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**Key words:** Antioxidant capacity, Gel permeation chromatography, Resveratrol, Phenolic, Wild grape

**Abstract**

Resveratrol is a stilbene polyphenol which is widely considered for its several therapeutic effects. Grapes are the main resource of resveratrol. Wild grapes, as the proper source of phenolic compounds, have been grossly underestimated. Consequently, phenolic compounds and resveratrol of wild grape (*Vitis vinifera.* ssp *sylvestris*) in Guilan province were studied. The results revealed that wild grape contains high concentration of phenolic compounds, especially resveratrol. After identification of resveratrol, the separation and purification continued through four stages stepwise as reflux extraction, protein precipitation, remove of lipids and gel permeation chromatography on sepahdex column. Subsequently, the FT-IR, MASS spectra and melting point were recorded and monitored to ensure the identity of the purified material. The obtained amount of resveratrol after extraction was 0.014%. After purification process, the content of resveratrol in the dried product reached 0.1% which, as a result, indicates an efficient purification method. Moreover, wild grapes have the usage capacity for natural antioxidants production in pharmaceutical industry.

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**Introduction**

Resveratrol (3, 5, 4´-trihydroxystilbene) is a natural polyphenolic compound present in some plant species including mulberries, peanuts, grapes, grapevines, legumes and raspberry. It is assumed to be produced in plants under several stresses caused by fungal attack, drought, and ultraviolet irradiation. It has been first isolated from the *Veratrum grandiflorum* in 1940 and then from *Polygonum cuspidatum* root extracts which are used in traditional folk-medicines in Japan and China (Baur and Sinclair, 2006). This compound is attracting increasing attention due to its activity against many diseases, including heart disorders and cancer, and because of its low in vivo toxicity. It was reported that resveratrol has anti-inflammatory, anti-mutagenic and anticarcinogenesis properties. The studies have focused on trans-resveratrol due to its various physiological functions in consumers.

Some beneficial effects of resveratrol on human health are given in the following (Fig. 1). The desire for scientific study on resveratrol has steadily increased in recent years as is evident in the 4375 published studies reported in PubMed and more than 13,000 ones in Chemical Abstracts databases (Fig. 2).

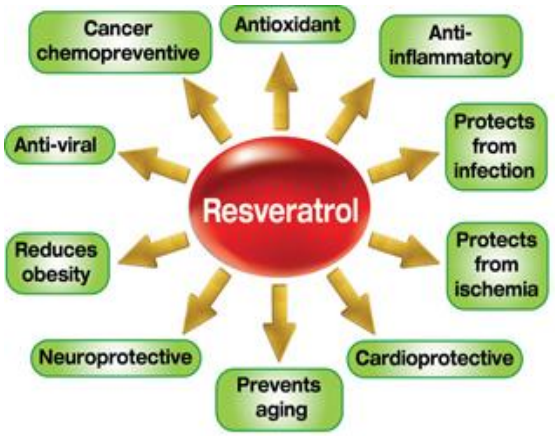


**Fig. 1.** Beneficial effects of resveratrol on human health.

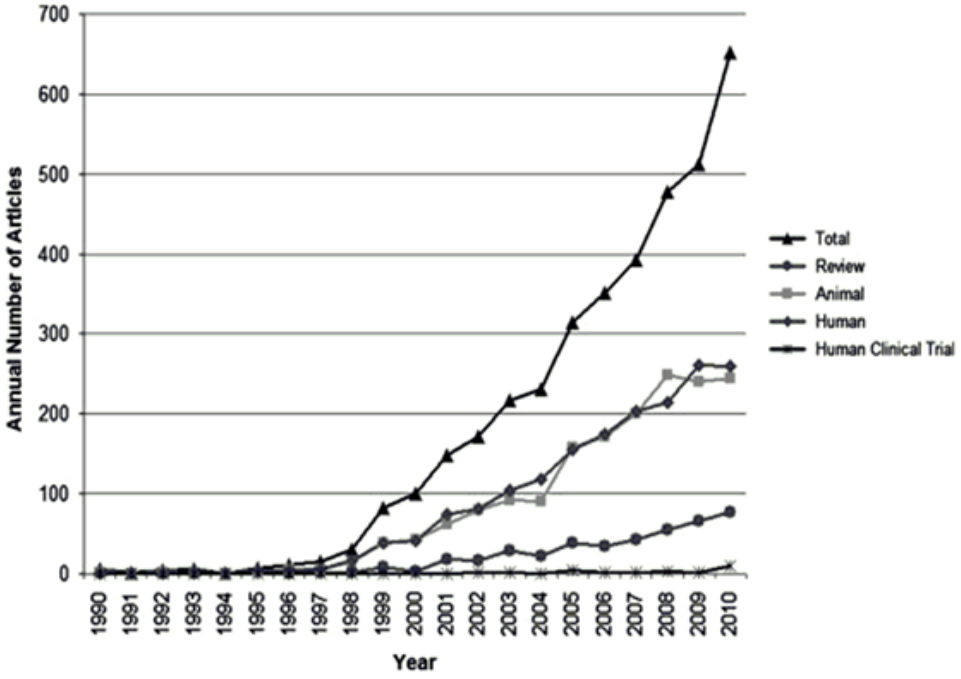


**Fig. 2.** Annual count of resveratrol articles indexed on PubMed (Smoliga *et al.*, 2011).

Red grape is a primary dietary source of resveratrol. Iran used to be ranked as the 8th largest grape producing after China, USA, Spain, France, Turkey, Australia and Argentina respectively and its share from the world grape production quantity is around 3% ((Seccia *et al.*, 2015).

Additionally, Iran is one of the most important centers of origin of *Vitis* species and several wild grape species that occur widely in their native habitats such as *Vitis labrosca* and *Vitis vinifera* ssp. *sylvestris* in the northern regions of Iran which are not consumed by their native population (Mozaffarian, 2005).

Determination of resveratrol content requires a reliable separation method. Among currently used methods, the high performance liquid chromatography (HPLC) coupled with various detectors, gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis (CE) are mostly the beneficial techniques (Fan *et al.*, 2011). Hence for resveratrol purification, Conventional methods entail extraction by heating under reflux, followed by filtration, concentration and purification. Other scholars have suggested molecular imprinted polymers, supercritical fluid extraction, enzymic hydrolysis technology and high-speed counter-current chromatography. However, these mentioned new technologies are simple but expensive and time-consuming. In some studies, scholars used macroporous resin adsorption and reversed-phase liquid chromatography to purify resveratrol (Xiong *et al.*, 2014; Zhang *et al.*, 2009). In other studies, stilbenes were purified in grape, peanut and *Polygonum cillinerve* (Nakai) by high-speed counter-current chromatography (HSCCC) with a suitable quaternary solvent system (Chi *et al.*, 2014; Chu *et al.*, 2005; Wei *et al.*, 2014). The separation and purification of resveratrol in wine grape residue with aqueous two phase extraction method were studied (He *et al.*, 2012).

Hence, this study was stablished to evaluate *Vitis vinifera* ssp. *sylvestris* growing in Guilan province in order to introduce new potential source of resveratrol, phenolic compounds and antioxidants.

**Materials and methods**

*Plant material and chemicals*

Plant material was collected from Rudsar forest (37°8′ 15″N, 50°17′ 17″E) in 2016. Sample fruits were dried in hot air oven with 40°C for 72 hours. Trans-resveratrol standard (purity> 97%) was purchased from Sigma-Aldrich Chemical Co. All materials and reagents were products of E-Merck and Sigma-Aldrich Chemical Co.

*Sample extraction*

One gram of specimen was ground up and then 3 mL methanol: acetic acid (85:15 v:v) was added and shook on a shaker at 100 rpm overnight at room temperature. Sample was incubated for 24h at 4°C. Then, mixtures were centrifuged at 10000 rpm for 15min and supernatant fluids were stored at – 20°C until analysis (Bakhshi and Arakawa, 2006).

*Total phenolic content*

Total phenolic content (TPC) was measured using the Folin-Ciocalteu colorimetric procedure based on the formation of a blue molybdenum-tungsten complex. Gallic acid was used as standard (Guerrero *et al.*, 2011). Samples were diluted accordingly: 50µL of the methanolic extract was mixed with 150µL of distilled water (DI) in a test tube followed by addition of 1mL of 10% Folin–Ciocalteu reagent and kept for 6min; then 800 µL of 7.5% sodium carbonate solution was added to the test tubes. Samples were kept for 90min at room temperature in darkness (Lister *et al.*, 1994). The absorbance was measured at 760nm with UV-VIS spectrophotometer (PG Instrument +80 Leicester, UK). Results were expressed as mg gallic acid equivalent in 1gr dry sample (GAE/ 1g DW).

*Total flavonoid content*

Total flavonoid content (TFC) of sample was determined based on the aluminum chloride colorimetric procedure, with some modifications (Park *et al.*, 2008). First, sample was diluted by fifth. In a 2ml test tube, 100µL of extract, 1750µL of DI, 75µL of $NaNO_2$ (0.5M) and 75µL of $AlCl_3.6H_2O$ (0.3M) were mixed. 500µL of NaOH (1M) was added after 5min. The solution was shacked well and absorbance of the mixture was determined at 506nm. Catechin standard procedure of 0 to 120mg/l was prepared as standard curve. Flavonoid content was expressed as mg catechin equivalent per 1g dry weight (CAT/1g DW).

*Antioxidant capacity*

Antioxidant capacity (AOX) was evaluated by DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay using the method described by Brand-Williams and coworkers, with some modifications (Brand-Williams *et al.*, 1995). 50µL of the extracted sample was added to 950µL of DPPH radical.

The solution was vortexed and allowed to stand at room temperature in darkness. The absorbance of the samples was assayed at 517nm after 15min. The percentage of DPPH radical scavenging capacity was calculated according to the following equation:

DPPH scavenging effect (%) = ((A0 –A1)/A0)) × 100.

Where; A0 is the absorbance of control DPPH solution at 0min and A1 is the absorbance in the presence of test sample at 15min.

*Trans- resveratrol content (TRC)*

The resveratrol analysis was carried out by a reversed phase high-performance liquid chromatography (HPLC) system (Waters, 1525, Milford, USA) equipped with a UV-Visible detector (Waters Dual λ Absorbance 2487). Detection was monitored at 306 nm. The column was a Symmetry C18 (4.6×150mm, 5µm; Waters, Dublin, Ireland).

The mobile phase was HPLC grade acidified water containing 3% acetic acid (A) and acetonitrile (B). The gradient program was: B: 0.00-5.00min, 0–8.5%; 5.00-16.50min, 8.5–2.0%;16.50–35.00 min, 2.0–18%; 35.00–50.00min, 18-20%; 50.00-65.00min, 20-30%; 65.00-70.00min, 30-0%. The column was held at 25°C and flushed at a flow rate of 1mL/min. Detection, quantification and calculation of trans-resveratrol were accomplished using the external standard. The standard resveratrol was prepared by the standard calibration curve as Fig 3.

Standard procedure was established by injection of 0.25, 0.125, 0.062 and 0.031mg/l. Chromatographic identification and confirmation of trans- resveratrol were based on comparing retention times with the authentic standard and on-line UV absorption spectrum data (Zhang *et al.*, 2011). 10mL 1N HCl/methanol/water, 1/80/19, v/v/v was added to 1gr sample and was shaken on a shaker at 100rpm overnight at room temperature. The aqueous phase was poured in a 250mL flask and concentrated in a rotary evaporator (R 2119, Heidolph) at 35°C to a volume of 2mL. The concentrated sample was centrifuged at 10000rpm for 10min.

The supernatant fluids were filtered through disposable 0.45µm syringe filter and stored at – 20°C until analysis (Zhang *et al.*, 2011).

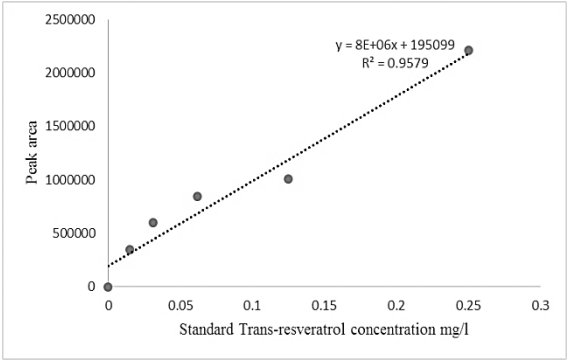


**Fig. 3.** Standard calibration curve of standard resveratrol.

*Resveratrol purification*

One of the conventional methods of the biomass extraction for obtaining stilbenoids is Soxhlet extraction (Pietarinen *et al.* 2006, Välimaa *et al.* 2007). A weighed sample (10g) of powdered material was extracted at 70-80°C for 6h under reflux with 50mL dichloromethane solvent. Then, the solvent was evaporated due to its low boiling point. Although the extract sample was fat -free, it was fattened three times with 10ml of hexane. Subsequently, some proteins were initially removed with ammonium sulfate and the remainder was injected into the Sephadex column (G-25). Resveratrol was isolated according to the comparison of the retention volume with the standard sample and control of its UV spectrum. The GPC (gel permeation chromatography) process was repeated three times recurrently to match the obtained melting point with the resveratrol point. Subsequently, the FT-IR and MASS spectra were recorded and monitored to ensure the identity of the purified material (Fig. 4).

The Agilent 5977A Series MSD mass spectrometer was equipped with an EI ionization detector and the Single Quadrupole analyzer was used to provide resveratrol spectra. The DIP system connects to the MSD detector directly and allows the sample to be detected without the need for GC use in the shortest time.

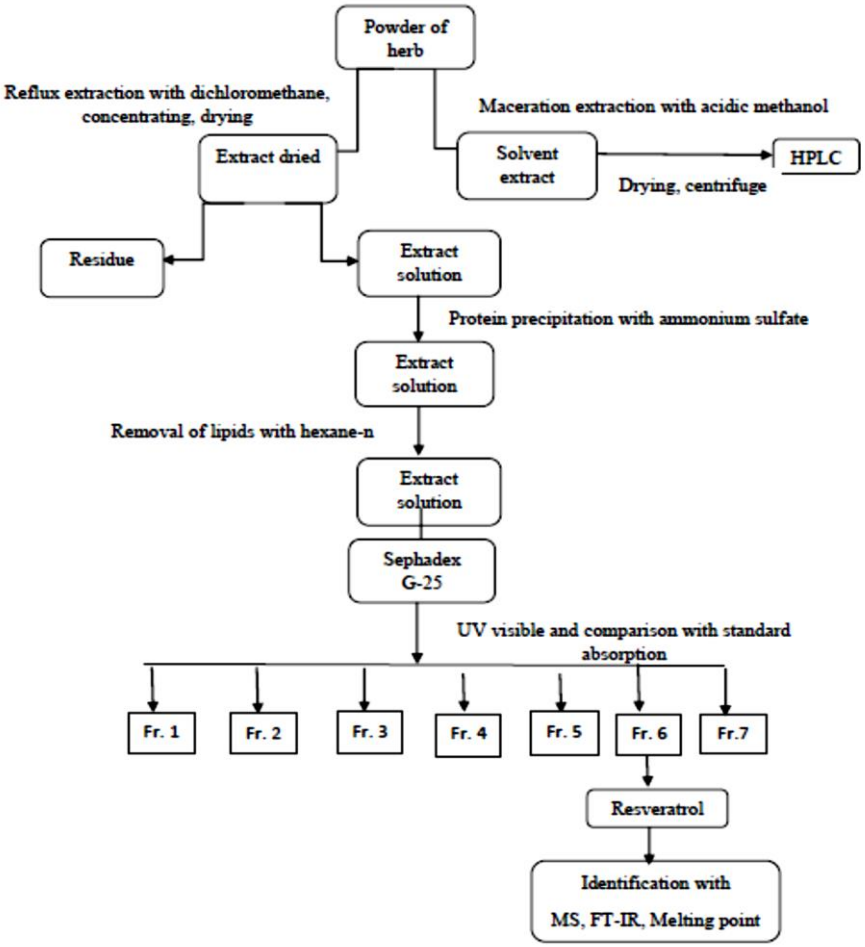


**Fig. 4.** Flow chart of the extraction and purification processes of resveratrol.

**Result and discussion**

The results showed that wild grapes are a rich source of phenolic compounds, especially resveratrol (Table 1.) Guder *et al* (2014) reported that wild grape in turkey had the high content of phenolics (252µg/mg DW) and flavonoids (68µg/mg DW). Pudel *et al* (2008) reported skins of wild grapes contained the highest amount of total phenolics (13.8mg/g gallic acid equivalent) and antiradical activities (61.7mmol/g trolox equivalent of fresh weight) which is again dissimilar from the deployed method in this study. In another research in Spain, total phenol of *Vitis vinifera* ssp. *sylvestris* was reported 180mg/l (Ocete *et al.*, 2011). Duan *et al* (2015) emphasized on the high stilbene content of wild grape. They collected 86 different genotypes of wild grapes from various regions of Germany and France to evaluate and compare the content of stilbenes. As a result, two rich clusters for the production of stilbene compounds were introduced. Resveratrol production potential of these genotypes was reported to be around 10 to 80mg/g DW which were supported by the results of the current research (Duan *et al.*, 2015).

**Table1.** Total phenol content, total flavonoid content, antioxidant capacity and resveratrol content of wild grape.

| Plant material | TPC (mg GAE/g dw) | TFC (mg CTE/g dw) | AOX (%) | TRC (mg/100g dw) |
|---|---|---|---|---|
| *Vitis sylvestris* | 8.8±0.23 | 2.9±0.05 | 50.5±0.08 | 14.0±0.90 |

Resveratrol levels in wild grapes have been studied recently in Korea, Japan and China (S.-J. Kim *et al.*, 2010; Shiozaki *et al.*, 2012; Zheng *et al.*, 2017). It has been announced that stilbene content mainly depends on the genetic background (Vincenzi *et al.*, 2013). In American wild grapes (*Vitis labrusca*), fruit's exocarp was also introduced as a high-capacity for resveratrol production (Hall and De Luca, 2007). In another study on 86 genotypes of wild grape, two clusters with

high potential of stilbenes production were highlighted (Zhang *et al.*, 2011). Scholars in a further research showed that wild grape had a higher amount of resveratrol than the ordinary one.

They reported the amount of resveratrol in wild grapes 340-440µg/mg FW at different stages of harvesting. (Jeandet *et al.*, 1991).

The HPLC results demonstrated that wild grape (*Vitis silvestris*) had a high trans-resveratrol (14.0±0.90mg/100g D.W). The retention times of Trans resveratrol was 48 min in our analysis conditions (Fig 5). The yield of 95% ethanol extract acquired from the wild grape by maceration extraction was 11.3%, and the content of resveratrol in the extract was 0.014%.

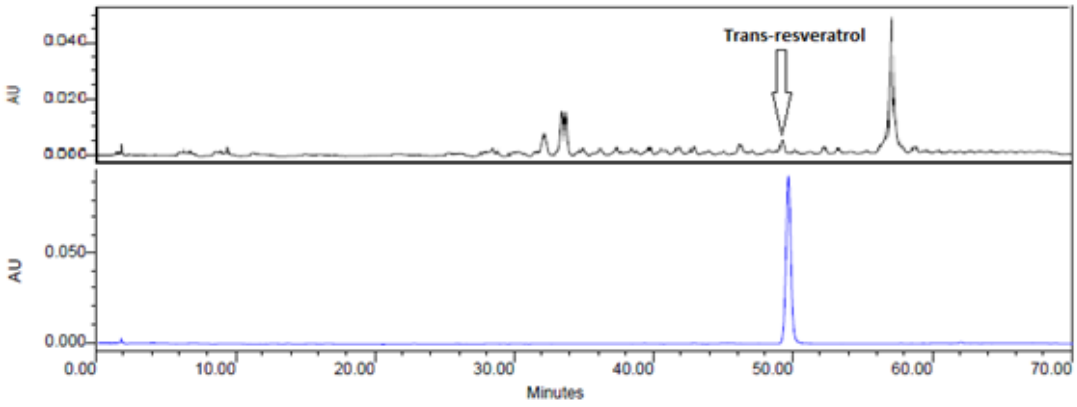


**Fig. 5.** HPLC chromatogram obtained from wild grape extracts (top) and Trans resveratrol standard (bottom) captured at 306nm.

Ammonium sulfate was used to precipitate the proteins after extraction. This method was used for protein precipitation of peanut and grape extract in the resveratrol purification process (Derckel *et al.*, 1998; Schöppner and Kindl, 1984). In addition, this material can play an effective role in removing pigments from the extract (Nollet and Toldrá, 2012).

Removal of lipids with normal hexane or dichloromethane is also recommended in most sources for fattening of samples containing resveratrol (Counet *et al.*, 2006; Fan *et al.*, 2009; Jerkovic *et al.*, 2010).

For separation and purification of resveratrol, sephdex G-25 column was used which showed a satisfactory result. In the past, sephadex G-25 was used for separation and purification of resveratrol and viniferin from various genotypes of grapes in Georgia (Bezhuashvili *et al.*, 2011). In Turkey, Sephadex and silica gel column were employed in chromatography to purify resveratrol from wild grapevine (Güder *et al.*, 2014).

The combination of sephadex LH-20 and TLC were used for purification of resveratrol and its derivatives from wild vine root (*Vitis thunbergii*) by Chinese researchers (Huang *et al.*, 2005). Utilization of Sephadex has been reported to purify resveratrol in several studies and in different plant sources (Hall and De Luca, 2007; H. J. Kim *et al.*, 2002; S.-J. Kim *et al.*, 2010; Pryce and Langcake, 1977). However, in most of them, hybrid techniques involving two or more chromatography column with other chromatographic methods were used; while the method employed in this literature is simple and preparative and it is also applicable on industrial scale. After purification process, the content of resveratrol in the dried product reached 0.1%.

IR spectra of the resveratrol in infra-red region is shown in the Fig. 6 and Table 2. Infra-red spectrum of resveratrol showed narrow band of O-H stretching at 3294 cm-1. FT-IR resveratrol spectrum extracted from wild grape in comparison with spectra pure resveratrol contained more information, however, a significant similarity was traced between them.

The great benefit of FT-IR spectroscopy is the detection possibility of aromatic structures in the analyzed extracts. Similar to the present study, researchers obtained the FT-IR spectrum of purified resveratrol of wild grapevine with three strong stretching. They reported the OH bond in the range of 3,200 to 3,450.

The bending vibration of the C-C group and the C-H group is very close to the results (Al-Jumaily *et al.*, 2013; Güder *et al.*, 2014). Mass spectra of trans-resveratrol presented similar fragmentation pattern with main peaks being at 76, 115, 181 and 228m/z (Fig. 7), which is in line with the reported MS data of Trans resveratrol (Camont *et al.*, 2009; Zhao *et al.*, 2015).

**Table 2.** The FT- IR frequencies region for the functional groups of the standard Trans- resveratrol and the extracted pure resveratrol of wild grape.

| Functional group | IR Frequencies of resveratrol standard, $cm^{-1}$ | IR Frequencies of extracted resveratrol, $cm^{-1}$ |
|---|---|---|
| Phenolic–OH group | 3291.98 | 3294.19 |
| Aromatic C–H group | 3006.13 | 3016.46 |
| Aliphatic C=C | 1593.21 | 1589.23 |
| Aromatic C=C | 1151.10 | 1149.50 |

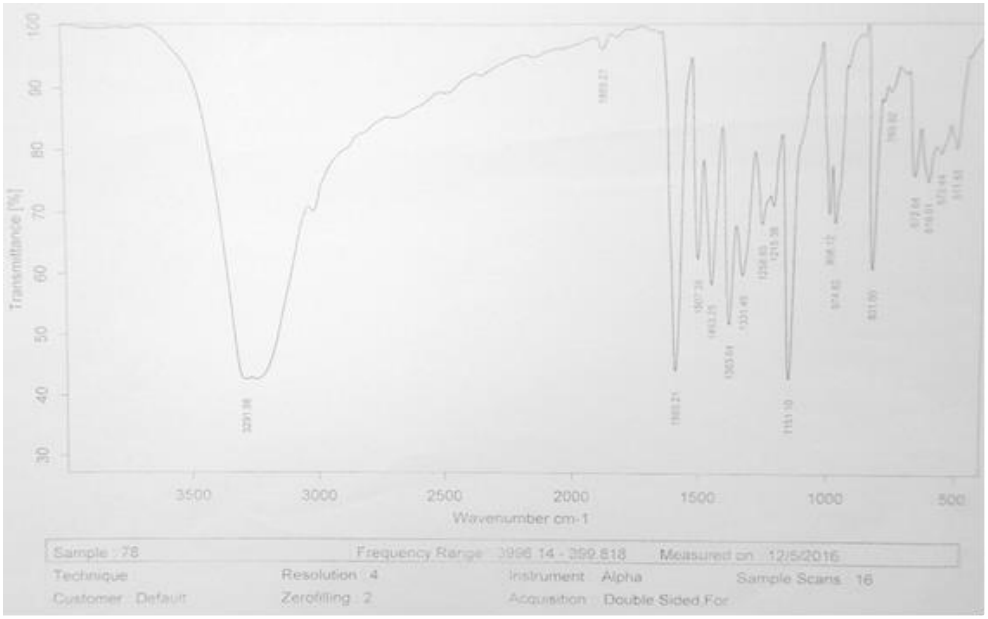


**Fig. 6.** FTIR spectra in infra-red region 4000-400 cm-1 of extracted pure resveratrol.

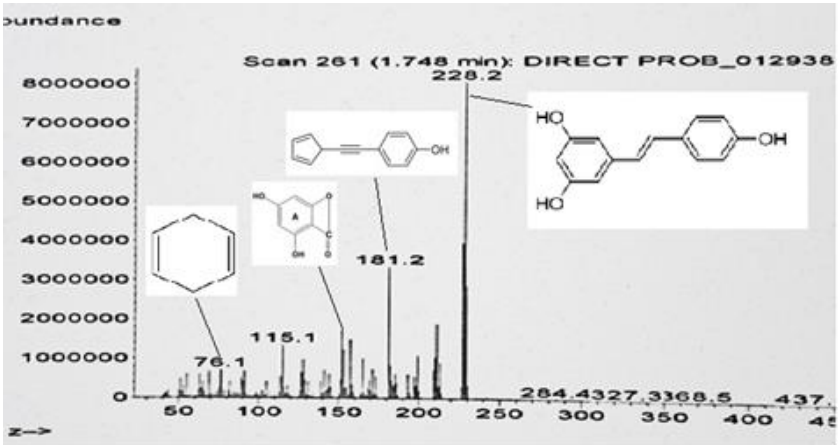


**Fig. 7.** Mass spectra of extracted pure trans-resveratrol.

## Conclusion

Wild grapes are a rich source of phenolic compounds, especially resveratrol. By evaluating wild grapes, we may be able to assess the value of these indigenous natural resources and promote their cultivation once the pharmalogical and nutraceutical benefits have been identified. The use of four-step resveratrol-separation methods as reflux extraction, protein precipitation, remove of lipids and gel permeation chromatography on sepahdex column can be considered for extracting this valuable compound.

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