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Original Article

Phytochemicals and antioxidant activity of alcoholic/hydroalcoholic extract of *Trifolium pratense*

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ABSTRACT

Objective: *Trifolium pratense* has many healing properties, including fewer complications of menopause, cancer cell suppression, reducing blood glucose and lipids, as well as cardiovascular beneficial effects. The purpose of this study was to identify the phytochemical and mineral composition of *T. pratense*.
Methods: Plant aerial parts were harvested and dried, and then hydroalcoholic and alcoholic extracts were prepared. Gas chromatography–mass spectrometry (GC–MS) analytical method was used to identify volatile compounds then liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) was used to identify polyphenols and the mineral elements were identify by inductively coupled plasma atomic emission spectrometer/ICP-AES and scanning electron microscope-energy-dispersive X-ray spectroscopy (SEM-EDS) methods. Total phenolic content (TPC) was determined based on colorimetric method, and total flavonoid content (TFC) was established based on the folin-chiocalteau reagent. Furthermore, two assays (DPPH and FRAP) were used to measure the antioxidant capacity of *T. pratense* ethanolic extract.
Results: A total of 37 polyphenols and 107 peaks were identified by LC-ESI-MS analysis, and the GC/MS method also detected 21 volatile compounds, the most important of which were methylcyclopentane, dimethylpentanal and hexadecanol. A total of 18 mineral elements, including K, Mg, Al, Si, Zn, Ni, Cu, Se, Co, Fe, Mn, and Ca in the plant, were identified ICP-AES and SEM-EDS analysis.
Conclusion: *T. pratense* has many therapeutic compounds such as polyphenol (isoflavone and flavonoids), volatile compounds, and essential mineral elements, which can be formulated purely and used in the pharmaceutical and traditional medicine industries.

1. Introduction

Red clover (*Trifolium pratense* L.) is one of the most important leguminous forage plants in temperate and humid areas, which is very valuable in livestock feeds with a height of 10–15 cm and oval, three-leafed leaves (Fig. 1). There are about 300 species of clover in the world, of which 25 species are important for agriculture, and only nine species are commercially grown (Kroyer, 2004). *T. pratense* is considered as an herb with a variety of therapeutic properties in human studies and animal models. This plant can be used to treat common diseases such as diabetes, high blood pressure and blood lipids (Khazaei & Pazhouhi, 2018). Nowadays, researches have another dimension due to the identification of various compounds such as polyphenol and isflavonoids with antitumor properties and supporting the immune system and treating diseases such as Alzheimer's (Occhiuto, Zangla, Samperi, Palumbo, Pino, & De Pasquale, 2008).

Natural polyphenols (e.g., phenolic acids, flavonoids, and tannins etc.) are produced by plants and are involved in their defense mechanisms against biotic and abiotic stresses. They have the scavenger capacity for oxidative free radicals, such as those derived from lipids and nucleic acids that underlie their utility in reducing the risk of certain age-related degeneration's diseases (Tyagi, Singh, Sharma, & Aggarwal, 2010). Phytoestrogens, a large group of compounds containing a number of phenolic hydroxyl groups, are the major components of soybean, and have been shown to be protective of several diseases, particularly cancers (Shahrokhi, Ghanimatdan, Bazm, & Karimi, 2014).

Isoflavone compounds such as formononetin, biochanin A, genistein, and daidzein presented in *T. pratense* have estrogenic properties in the form of glucoside and malonate, which are hydrolyzed by flora and intestinal mucosal cells (Setchell, Brown, Zimmer-Nechemias, Brashear, Wolfe, & Kirschner, 2002). LC-MS analysis identified about 10 malonates in *T. pratense* flowers and

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Fig. 1. *T. pratense* cultivated in research field of Kermanshah University of Medical Sciences.

other LC-ESI-MS study revealed the existence of > 30 flavonoids, including about 20 malonates (Lin, He, Lindenmaier, Yang, Cleary, & Qiu, 2000). LC-ESI-MS study of ethanolic extract of *T. pratense* revealed the existence of daidzin, glycetin, calycosin 7-*O*-α-*D*-glucoside, luteolin 7-*O*-α-*D*-glucoside, genistin, hyperoside, isoquercitrin, ononin, apigenin, 7-*O*-α-*D*-glucoside, and biochanin A (Klejdus, Vitamvásová-Štěrbová, & Kubáň, 2001).

Numerous analytical procedures (TLC, LC-DAD, LC-FLD, LC-MS, GC and CE) have been developed for the quantification of phenolic and isoflavone compounds in herbs. Liquid chromatography-mass spectrometry (LC-MS) is extensively used for the analysis of phenolic compounds and mineral elements of the herbs or medicinal plants and tea leaves from all over the world (Akbari, Khazaei, Khazaei, & Naseri, 2019). Different techniques, such as flame- or electrothermal- atomic absorption spectrometry (FAAS, ETAAS), inductively coupled plasma-optical emission spectrometry (ICP-OES) or mass spectrometry (ICP-MS), and scanning electron microscope (SEM) are used to identify the mineral elements in solvent extracts (Rezić, 2011).

The combination ofSEM and energy dispersive spectroscopy (EDS) allows for an elemental analysis performed on different microscopic sections of the various samples such as stone, herbal extracts, and synthetic nanoparticles with uncertain elemental composition. EDS can detect major (higher than 10% wt) and minor (between 1 and 10% wt) elements concentrations (Kabata-Pendias, 2000; Ramamurthy & Kannan, 2009). EDS technique is capable of producing elemental distribution maps. SEM-EDS method is used to identify the elements in solid samples, while ICP-MS used for solvent samples. ICP-MS is used to identify the elements in industrial solutions, drinking water, herbal extracts, drugs and radioactive specimens. In ICP-AES the extract is transformed into aerosols by nebulizer and sprayed. The hot argon gas in the machine after the drying of the extract causes its particles to atomize (Maher et al., 2001). The present study describes the development of an LC-ESI-MS/MS method for analyzing the phytochemical content, GC/MS to identify volatile compounds with low molecular weight, and SEM-EDS/ICP-AES method for analyzing the mineral elements in the different parts of *T. pratense*.

2. Materials and methods

2.1. Plant sample preparation

Plant seeds were obtained from Karaj Seed and Plant Improvement Institute and cultivated at a research farm. In spring, the leaf and stem of the plant were collected and immediately frozen in liquid nitrogen (samples can be stored at − 70 °C until used). All samples were surface sterilized with 0.1% sodium hypochlorite (20 min) and rinsed with double-deionized water (four times). Lyophilize frozen samples for 2 to 3 d (dried samples can be stored at − 20 °C until use). After drying, cut samples into small pieces and grind them into fine powder.

For LC-ESI/MS analyzing, 2 g of the powder was mixed with 2 mL of 0.1 mol/L HCl and 10 mL of acetonitrile (ACN) in a 125 mL screw-top flask, stirred (2 h at room temperature) and filtered (Whatman No. 41 UK). The filtrate was dried in a vacuum evaporator and then dissolved in 10 mL of 80% methanol. The sample was filtered (0.45 μm filter unit, Cameo syringe-filter, nylon), transferred to 1 mL vials. Analytical standards of coumaroylquinic acid (≥98%), cinnamic acid (≥97%), quercetin-3,7-diglucoside (≥98%), myricetin-3-*O*-rhamnoside (≥97%), coumaric acid-*O*-pentoside (≥97%), myricetin-3-*O*-acetyl rhamnoside (≥97%), genistein (≥98%), daidzein 7-*O*-β-*D*-glucoside (≥98%), formononetin (≥98%), apigenin 6,8-diglucoside (≥98%), apigenin-7-*O*-glucoside (≥98%), ellagic acetyl rhaminoside (≥98%) and biochanin A (≥98%) were purchased from Sigma-Aldrich. Standards were dissolved in methanol–water (1:1, volume percentage). Due to low solubility of standard compounds, solutions were filtered (0.20 μm filter) (Naseri, Khazaei, Ghanbari, & Bazm, 2019).

For GC–MS analyzing, 10 g of the powdered leaves was soaked in 99.6% ethyl alcohol (72 h) then incubated overnight and filtered along with sodium sulfate to remove the sediments and traces of water in the filter paper (Whatman No. 41 UK). The paper was wet with 95% ethanol and sodium sulfate. The filtrate is then concentrated to 1 mL by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phytocomponents of the plant material. Then, 2 μL of the extract was used for injection in the device GC–MS for analysis. To condense the solution, a vacuum distillation unit (Heidolph Collegiate, LABOROTA 4000) at 55 °C was used. (Khazaei & Pazhouhi, 2019).

2.2. LC-ESI/MS analysis

MS analysis was carried out on an ESI (negative mode) using the Agilent Zorbax Eclipse Atlantis T3 C_{18} Mass Spectrometer (3 μm, 2. 1 × 150 mm) column coupled to a HCT ultra ion trap MS detector fitted with an ESI ion source (Shimadzu LC/MS device 2010 A). For chromatographic separation, the same column and conditions as described above were used. The standard analysis of the machine was carried out according to the following conditions: injection volumes: 10 μL; dwell time: 500 ms; flow rate: 0.2 mL/min; detection gain: 1.8 kV; fragmentor:135; operation mode: negative. Mobile phase: A, acetonitrile with 0.1% formic acid; B, H_2O with 0.1% formic acid; column temperature:25 °C; capillary voltage: 3.5 kV; dry gas flow (N_2-grade 5): 1.2 L/min; nebulizer pressure: 15 psi; and capillary temperature: 285 °C.

Mass spectra were recorded between mass ranges 130–1100 amu. Gradient condition due to the percentage of solvent flow in the column is according to the time and flow velocity, respectively: Time (min) 0, 15, 20, 25, and 30; phase B (%) 95, 10, 5, 5, and stop; phase A (%) 5, 10, 95, 95 and stop; flow rate: 0.3 mL/min in all times (source conditions on a set of polyphenolic compounds standards include flavonoid aglycones and glycosylated flavonoids). Quality control (QC) samples were then prepared by diluting separate analyte stock solutions with diluent with the same amount of known internal standard (Akbari, Khazaei, Khazaei, & Naseri, 2019; Namera, Kawamura, Nakamoto, Saito, & Nagao, 2015).

2.3. GC–MS analysis

GC–MS analysis of the ethanol extract of *T. pratense* was performed using a GC Clarius 500 Perkin Elmer comprising an AOC-

20i auto-sampler and a GC–MS equipped with an Elite-1 (100% dimethyl poly siloxane) column (30 × 0.25 mm 1 μm df) fused a capillary column (30 × 0.25 μm ID × 0.25 μm df). Helium (99.999%) (5 nines) was used as the carrier gas at a flow rate of 1 mL/min in the split mode (10:1). For GC–MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV.

An aliquot of 2 μL of ethanol solution of the sample was injected into the column with the injector temperature at 200 °C. The ion-source temperature was 200 °C, the GC oven temperature started at 110 °C and holding for 2 min, raised to 200 °C with an increase of 10 °C/min, then 5 °C/min to 280 °C, ending with a 9 min at 280 °C. Ion source temperature was maintained at 200 °C. Mass spectra were taken at 70 eV. The solvent delay was 0 to 2 min, and the total GC/MS running time was 48 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. A scan interval of 0.5 s and fragments from 45 to 450 Da was maintained. The total running time was 36 min. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2 (Ezhilan & Neelamegam, 2012; Akbari Bazm, Goodarzi, Shahrokhi, & Khazaei, 2020).

2.4. SEM-EDS analysis

One gram of *T. pratense* alcoholic extract (99.6% ethyl alcohol) was prepared and coated with gold, placed in a special stage and placed in the microscope's (Seron Technology's high competitive normal SEM, AIS2300C, Korea) sample chamber with an energy dispersive x-ray spectrometer (EDS). The analysis conditions of the device for identifying the elements in the extract were as follows: Accelerating Voltage: 12 kV, Beam Shift & Rotation: 250 μm (X, Y), Image Rotation (360°), Vacuum: ~10^{-4} Torr (Starlin, Ragavendran, Raj, Perumal, & Gopalakrishnan, 2012).

2.5. ICP analysis

Fifteen gram of *T. pratense* powdered was soaked in 99.6% ethyl alcohol for 8 h by soxhlet extraction, then incubated on water bath at 60 °C and filtered (Whatman No. 41 UK). The dark greenish brown thick solution was stored in a glass vial at 4 °C. One g of the extract was placed in a 55% nitric acid solution in a warm water bath at 85 °C for 10 min. After cooling, 50 mL of distilled water was poured on it. Finally, the solution was analyzed under standard conditions by inductively coupled plasma atomic emission spectrometer.

The analysis was performed using a standard instrument, and the software interface version 5 was evaluated. The analysis was performed using an ARCOS model instrument from M/s Spectro, Germany and the software interface Smart Analyzer Vision 5.01.0921 was evaluated. The analysis conditions of the device for identifying the elements in the extract were as follows: Nebulizer flow: 0.801 L/min, Auxiliary flow: 1.009 L/min, Coolant flow: 11.04 L/min Pump speed, 34 rpm, and Plasma power: 1500 W, R.F generator: 1.6 KW (Bazm et al., 2018; Senila, Drolc, Pintar, Senila, & Levei, 2014; Goodarzi, & Akbari, 2016).

2.6. Total phenolic compounds

Thephenolic content of the extracts was measured using the modified Folin-Ciocalteu assay (Slinkard and Singleton method) by placing gallic acid as standard. First, to determine the calibration curve, 50 μL of 0.024, 0.075, 0.105, 0.3, and 0.4 mg/mL gallic acid with methanol was mixed. Samples were introduced into 0.8 mL of sodium carbonate (Na_2CO_3) (7.5%) and 1.0 mL Folin-Ciocalteu's reagent (diluted 10-fold) and were mixed. 50 μL of the ethanolic extract was added to above described solution. All samples were read after 2 h of incubation at 30 °C with a wavelength of 765 nm (Shimadzu UV–Vis spectrophotometer). Results were expressed as milligrams of gallic acid equivalent per gram of fresh plant (mg GAE/g) (Slinkard & Singleton, 1977).

2.7. Total flavonoid content

Total flavonoid content was determined using a modified method (Miliauskas et al., 2004). A total of 10 mg of ethanolic extract was dissolved in 5 mL of methanol, and then 1 mL of the resulting solution was added to 1 mL of methanol dissolved in aluminum trichloride ($AlCl_3$), 0.1 mL of potassium acetate (1 mol/L), 2.8 mL of water and the volume was increased to 25 mL with methanol. Then the absorbance of the resulting solution was read after incubation at 25 °C for 30 min at 415 nm. Total flavonols were expressed as milligrams of rutin equivalent (RE) per 1 g of extract (mg RUE/g).

2.8. DPPH radical scavenging activity

DPPH was determined using a modified method (Brand-Williams, Cuvelier, & Berset, 1995). Aliquots (25–170 μL) of the *T. pratense* extracts were placed in a cuvette, and 3 mL of 0.06 mmol/L methanol DPPH$^{\bullet}$ radical solution was added. Absorbance measurements commenced immediately. The DPPH radical scavenging effect, after 3 h for all samples measured in absorbance at 516 nm during in the dark condition. Then, according to the Trolox calibration curve, The DPPH radical scavenging effect of the extracts was expressed as μmol Trolox equivalent per 100 g of dried *T. pratense* (μmol eq. Trolox/10 g plant).

2.9. Ferric reducing antioxidant power (FRAP) assay

FRAP method based on the pH-dependent (pH 3.6) color change where the yellow Fe^{3+}-TPTZ complex is reduced to the Fe^{2+}-TPTZ complex. FRAP method was adjusted according to Benzie and Strain modified method (Benzie & Strain, 1996). In this method, the extract was dissolved in methanol solution (1 mg/mL). Then added 20 μL of the resulting solution to 180 μL of distilled water and 2 μL of FRAP solution. Finally, the absorbance of the solution and methanol as blank was read at 593 nm and expressed as mmol Fe^{2+} sulfate heptahydrate per mg of plant (mmol Fe^{2+}/mg plant).

3. Results

3.1. LC-ESI/MS analysis

Liquid chromatographic gradient conditions were optimized. The optimized gradient allowed good peak resolution with total runtime lower than 20 min (Fig. 2).

To obtain an acceptable separation of major isoflavonoids and other interference compounds (flavonoids, saponins, etc.) a 20-min chromatographic run was used. LC-ESI/MS analysis was then applied for the full characterization of the different compounds in the extracts of *T. pratense*. After chromatograms were prepared in the negative ion mode, 46 compounds and 104 peak (intensity > 5 × 10^4) were identified. The chromatograms obtained are displayed in Fig. 2 and they demonstrated the hydroalcoholic extract of *T. pratense*. A full MS scan, in the form of a total ion current chromatogram (TIC), was initially acquired, following which reconstructed ion chromatograms (RICs) were generated for each of the expected *m*/*z* values based on the molecular weights of the possible constituents. The extracts are separated from each other

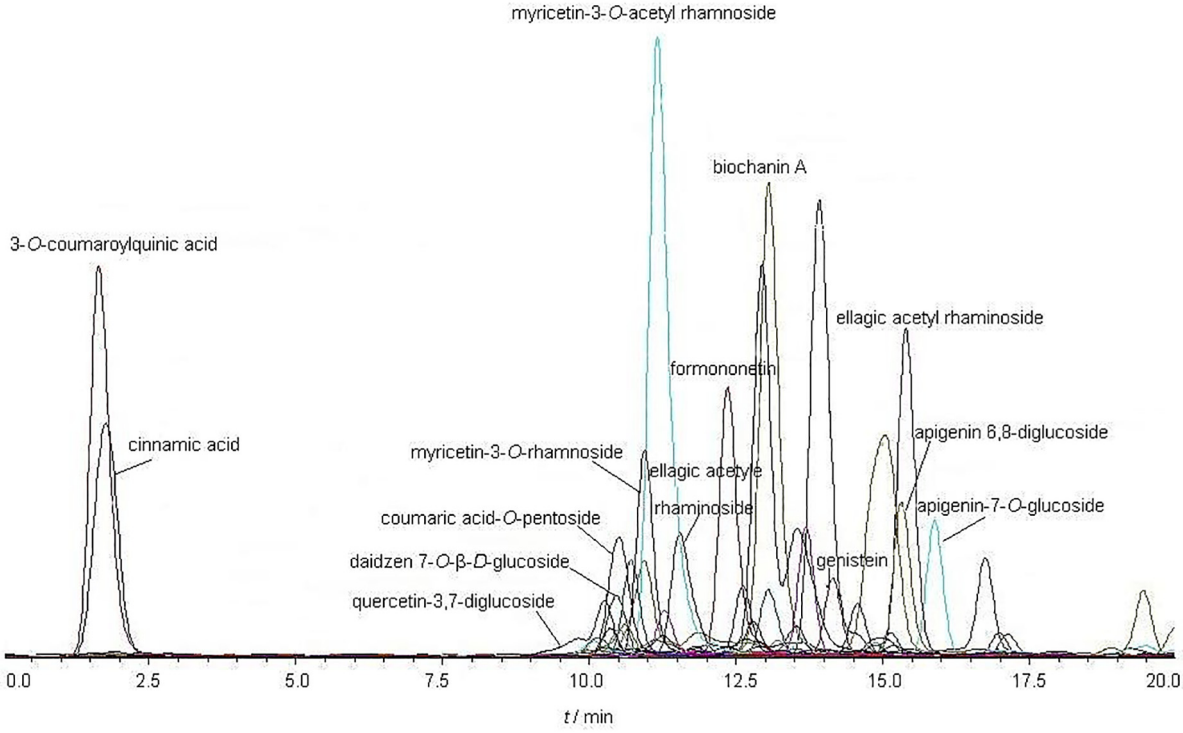

Fig. 2. Representative chromatogram of standard solution containing 3-*O*-coumaroylquinic acid, cinnamic acid, quercetin-3,7-diglucoside, myricetin-3-*O*-rhamnoside, coumaric acid-*O*-pentoside, myricetin-3-*O*-acetyl rhamnoside, daidzein 7-*O*-β-*D*-glucoside, formononetin, apigenin 6,8-diglucoside, apigenin-7-*O*-glucoside, genistein, ellagic acetyl rhaminoside and biochanin A.

according to the molecular weight $[M-H]^-$ of the expected compounds and acquisition time, Counts (Mass-to-charge) $\times 10^4$ and counts listed in Table 1.

Compounds characterized for the first time by LC-ESI-MS (intensity > 5×10^4). The identification was confirmed by direct comparison with standard compound, respectively.

Various isoflavone were identified by LC-ESI/MS. The retention time, *m/z*, $[M-H]^-$ and typical fragment ions for individual peaks confirmed the presence of isoflavones in *T. pratense* leaves and stem. By comparing the retention time and mass spectrum with corresponding standards, the followings were present in the plant, respectively: the cinnamic acid (*m/z* 149 $[M-H]^-$), 3-*O*-(Z)-p-coumaroylquinic acid (*m/z* 337 $[M-H]^-$), epigallocatechin(*m/z* 306 $[M-H]^-$), caffeic acid (*m/z* 179 $[M-H]^-$), coumaric acid-*O*-pentoside (*m/z* 295 $[M-H]^-$), kaempferol galloylglucoside (*m/z* 599 $[M-H]^-$), quercetin-3,7-diglucoside (*m/z* 625 $[M-H]^-$), kaempferol-3,7-di-*O*-glucoside (*m/z* 609 $[M-H]^-$), myricetin-3-*O*-rhamnoside (*m/z* 463 $[M-H]^-$), quercetin-3-*O*-galactoside (*m/z* 464 $[M-H]^-$), myricetin-3-O-acetyl rhamnoside (*m/z* 505 $[M-H]^-$), fraxidin (*m/z* 223 $[M-H]^-$), ellagic acetyl rhaminoside (*m/z* 489 $[M-H]^-$), vitexin-*O*-(maloyl)-rhamnoside (*m/z* 693 $[M-H]^-$), myricetin-3-O-acetyl rhamnoside (*m/z* 505 $[M-H]^-$), rhamnetin-3-*O*-(6-*O*''-acetylglucoside) (*m/z* 519 $[M-H]^-$), formononetin (*m/z* 267 $[M-H]^-$), genistein (*m/z* 268 $[M-H]^-$), biochanin A-7-glucoside (*m/z* 445 $[M-H]^-$), daidzein 7-O-β-*D*-glucoside (*m/z* 417 $[M-H]^-$), α-tocopherol (*m/z* 501 $[M-H]^-$), ferulic acid-O-hexoside derivative (*m/z* 689 $[M-H]^-$), biochanin A (*m/z* 283 $[M-H]^-$), coumaric acid-O-rhamnoside (*m/z* 309 $[M-H]^-$), ophiopogonanone A (*m/z* 327 $[M-H]^-$), epimedoside A (*m/z* 663 $[M-H]^-$), apigenin-7-O-glucoside (*m/z* 431 $[M-H]^-$), hesperetin (*m/z* 301 $[M-H]^-$), 9,12,13-trihydroxy-10,15-octadecadienoic acid (*m/z* 327 $[M-H]^-$), nosylicariside II (*m/z* 661 $[M-H]^-$), malvidin (*m/z* 331 $[M-H]^-$), ethyl gallate (*m/z* 197 $[M-H]^-$), gallocatechin (*m/z* 307 $[M-H]^-$), apigenin 6,8-diglucoside (*m/z* 593 $[M-H]^-$), galloyl-HHDP-hexoside (*m/z* 633 $[M-H]^-$), daidzin 4′-*O*-glucuronide (*m/z* 592 $[M-H]^-$), kaempferol-3-*O*-sophoroside-7-*O*-glucoside (*m/z* 777 $[M-H]^-$), ganoderic acid AM_1 (*m/z* 513 $[M-H]^-$), 6-C-pentosyl-8-C-hexosyl apigenin (*m/z* 564 $[M-H]^-$), paeoniflorin (*m/z* 480 $[M-H]^-$), genistein 8-C-glucoside-xyloside (*m/z* 311 $[M-H]^-$), quercetin-3-*O*-pentoside (*m/z* 433 $[M-H]^-$), kaempferol galloylglucoside (*m/z* 599 $[M-H]^-$599), 8-prenylnaringenin (*m/z* 339 $[M-H]^-$339),chlorogenic acid derivative (*m/z* 451 $[M-H]^-$) and oxypaeoniflorin isomer/ mono galloyl hexoside (*m/z* 494 $[M-H]^-$) (Table 1). Further LC-ESI/MS analysis were carried out on-line using the dependent full scanning mode in which the MS software selected ions of certain intensity for further fragmentation experiments on the basis of a set of parameters predetermined by the operator.

3.2. GC–MS analysis

GC–MS chromatogram analysis of the ethanolic extract of *T. pratense*was shown in Figs. 3 and 4. Comparison of the mass spectra of the constituents with the NIST library, showed 21 peaks which indicating the presence of 21 phytochemical constituents (Table 2).

This analysis showed that according to the amount of compounds, respectively 3,7,11,15-tetramethyl-2-hexadecen-1-ol (25.03%), methylcyclopentane (7.18%), 2,3-dimethylpentanal (7.07%), 1-hexadecanol (6.22%), cyclohexane (5.991%), 4-hydroxy-4-methyl-2-pentanone (5.8%), 3,7,11,15-tetramethyl-2-hexade cen-1-ol (5.05%), 1-nonadecene (4.63%), *n*-tetracosanol-1 (4.14%), 3-eicosyne (4.05%), 7-tetradecene (3.65%), phytol (3.36%), hexane (2.96%), *n*-tridecanol (2.9%), *n*-hexadecanoic acid (2.29%), 2-methyl-1-hexadecanol (2.04%), 9-eicosyne (1.96%), hexylcyclohexane (1.68%), octylcyclohexane (1.54%), 10-heneicosene (1.5%), and 3-trifluoroacetoxypentadecane (1.05%) is present in this plant.

Table 1
Qualitative analyses by LC-ESI/MS and LC-ESI/MS of *T. pratense*.

Compounds	*m/z* $[M-H]^-$	MS/MS fragments	Acquisition time	Counts (Mass-to-charge) × 10^4
Cinnamic acid (149)	149	149	1.72	4.8
3-*O*- (Z)-*p*-Coumaroylquinic acid (337)		337		
Epigallocatechin (306)		306		
Caffeic acid (179)		179		
Coumaric acid-*O*-pentoside (295)	295	295	10.3	23.4
Kaempferol galloylglucoside (599)		599		
Quercetin-3,7-diglucoside (625)		625		
Kaempferol-3,7-di-*O*-glucoside (609)	609	609	10.7	49.2
Myricetin-3-*O*-rhamnoside (463)	463	463	10.92	234.6
Quercetin-3-*O*-galactoside (464)		464		
Myricetin-3-*O*-acetyl rhamnoside (505)	505	505	11.3	441.9
Fraxidin (223)		223		
Ellagic acetyl rhaminoside (489)	489	489	11.5	121.6
Myricetin-3-*O*-acetyl rhamnoside (505)		505		
Rhamnetin-3-*O*-(6-*O*''-acetylglucoside) (519)		519		
Formononetin (267)	267	267	12.3	8.6
Genistein (268)		268		
Biochanin A-7-glucoside (445)	445	445	12.6	6.8
Formononetin (267)		267		
Genistein (268)		268		
Daidzein 7-*O*-β-*D*-glucoside (417)		417		
α-Tocopherol (501)	501	501	12.7	7.8
Formononetin (267)		267		
Daidzein 7-*O*-β-*D*-glucoside (417)		417		
Biochanin A-7-glucoside (445)		445		
Biochanin A (283)	283	283	12.91	23.4
Ophiopogonanone A (327)		327		
Daidzein 7-*O*-β-*D*-glucoside (417)		417		
Epimedoside A (663)		663		
Apigenin-7-*O*-glucoside (431)	431	431	13.5	11.22
Hesperetin (301)		265		
Biochanin A (283)		283		
9,12,13-trihydroxy-10,15-octadecadienoic acid (327)		301		
		327		
Nosylicariside II (661)	661	661	13.7	6.91
Biochanin A (283)		283		
Hesperetin (301)		301		
9,12,13-Trihydroxy-10,15-octadecadienoic acid (327)		327		
Vitexin-*O*-(maloyl)rhamnoside (693)		693		
Formononetin (267)	267	267	14.1	37.2
Malvidin (331)	331	331	14.6	4.06
Ethyl gallate (197)		197		
Gallocatechin (307)		307		
Apigenin 6,8-diglucoside (593)		593		
Apigenin 6,8-diglucoside (593)	593	593	14.8	3.58
Galloyl-HHDP-hexoside (633)		633		
Daidzin 4′-*O*-glucuronide (592)	592	592	15	5.36
Galloyl-HHDP-hexoside (633)		309		
Kaempferol-3-*O*-sophoroside-7-*O*-glucoside (777)		633		
Coumaric acid-*O*-rhamnoside (309)		777		
Epigallocatechin (305)	305	305	15.1	3.31
Apigenin 6,8-diglucoside (593)		593		
Galloyl-HHDP-hexoside (633)		633		
Hydroxy-octadecatrienoic acid (293)		293		
Biochanin A (283)	283	283	15.3	648.6
Apigenin 7-*O*-glucoside (435)	435	435	15.8	6.21
Ethyl gallate (197)		197		
Biochanin A (283)		283		
Ganoderic acid AM_1 (513)	513	513	16.75	3.52
Ethyl gallate (197)		197		
Hydroxy-octadecatrienoic acid (293)		293		
6-C-Pentosyl-8-C-hexosyl apigenin (564)		564		
Ferulic acid-*O*-hexoside derivative (689)		689		
Paeoniflorin (480)	480	480	17	6.52
Genistein 8-C-glucoside-xyloside (311)		311		
Quercetin-3-*O*-pentoside (433)		433		
Kaempferol galloylglucoside (599)		599		
Chlorogenic acid derivative (451)	451	451	19.44	2.38
Ethyl gallate (197)		197		
8-Prenylnaringenin (339)		339		
Apigenin 7-*O*-glucoside (435)		435		
Oxypaeoniflorin isomer		494		
/Mono galloyl hexoside (494)				

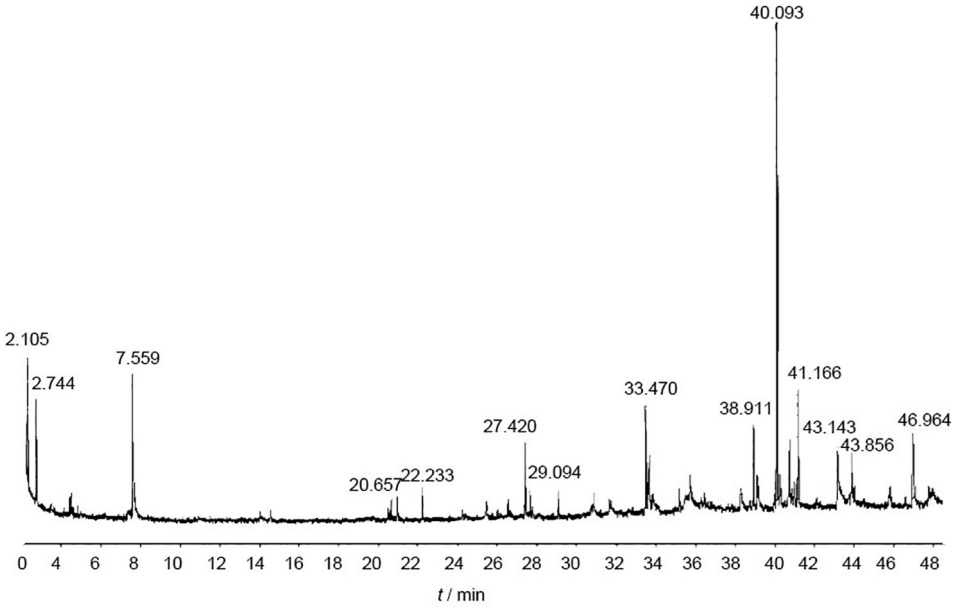

Fig. 3. GC–MS Chromatogram of ethanolic extract of *T. pratense.*

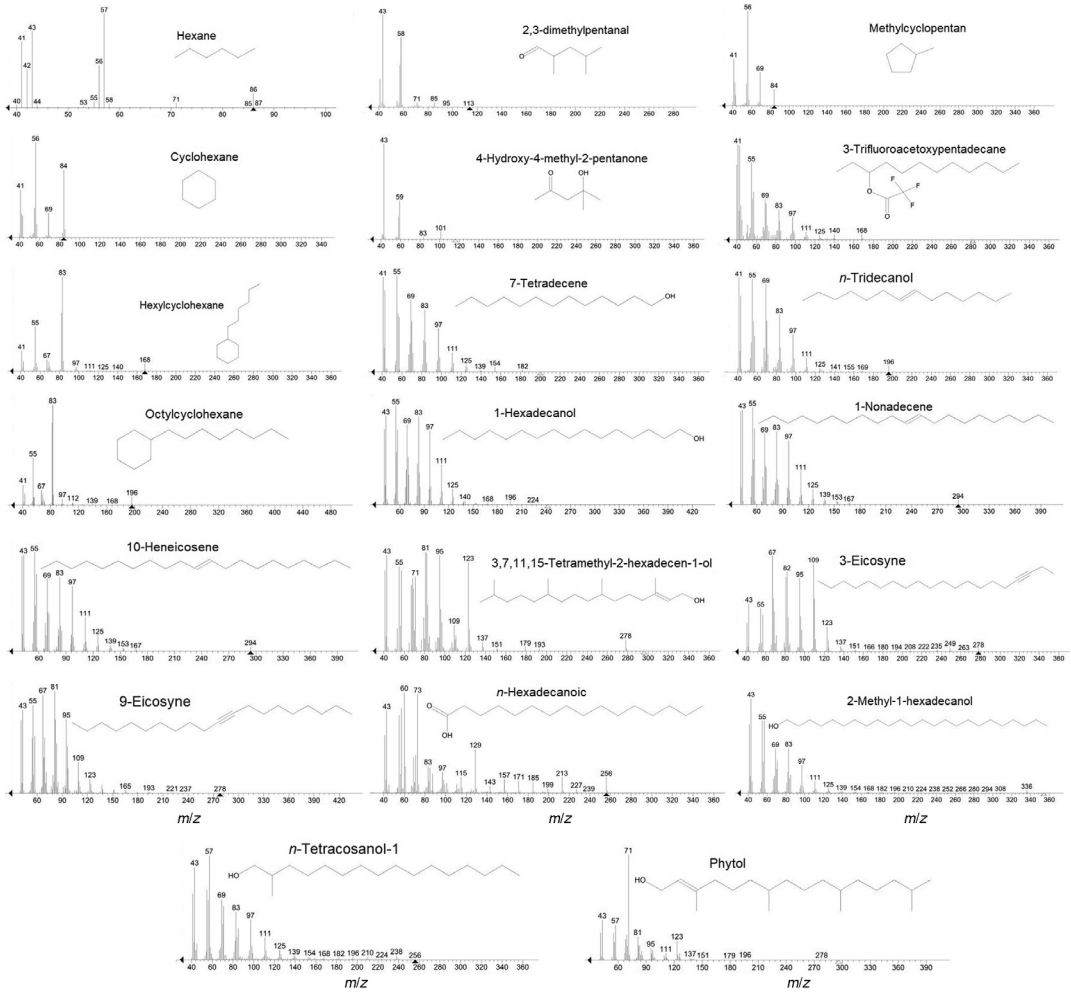

Fig. 4. Mass spectrum and structure of phytocomponents identified by GC–MS in ethanolic extracts of *T. pratense.*

Table 2
Phytocomponents identified in methanolic leaf extract of *T. pratense* by GC–MS.

Peak	RT/min	Compounds	Molecular formula	MW/(g·mol^{-1})	Area sum/%
1	2.066	Hexane	C_6H_{14}	86.18	2.96
2	2.105	2,3-dimethylpentanal	$C_7H_{14}O$	114.19	7.07
3	2.299	Methylcyclopentane	C_6H_{12}	84.162	7.18
4	2.744	Cyclohexane	C_6H_{12}	84.16	5.91
5	7.559	4-Hydroxy-4-methyl-2-pentanone	$C_6H_{12}O_2$	116.16	5.8
6	20.657	3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324.428	1.05
7	22.233	Hexylcyclohexane	$C_{12}H_{24}$		1.68
8	27.42	7-Tetradecene	$C_{14}H_{28}$	168.324	3.65
9	27.42	*n*-Tridecanol	$C_{13}H_{28}O$	200.366	2.9
10	29.094	Octylcyclohexane	$C_{14}H_{28}$	196.378	1.54
11	33.47	1-Hexadecanol	$C_{16}H_{34}O$	242.44	6.22
12	38.911	1-Nonadecene	$C_{19}H_{38}$	266.513	4.63
13	38.911	10-Heneicosene	$C_{21}H_{42}$	294.567	1.5
14	40.098	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.539	25.03
15	40.098	3-Eicosyne	$C_{20}H_{38}$	278.524	4.05
16	41.166	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.539	5.05
17	41.166	9-Eicosyne	$C_{20}H_{38}$	278.524	1.96
18	43.148	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	2.29
19	43.856	2-Methyl-1-hexadecanol	$C_{17}H_{36}O$	256.474	2.04
20	43.856	*n*-Tetracosanol-1	$C_{24}H_{50}O$	354.65	4.14
21	46.964	Phytol	$C_{20}H_{40}O$	128.1705	3.36

3.3. SEM analysis

Elemental analysis using a SEM-EDS spectrum has revealed the presence in order of C (534.40) > O (56.47) > K (53.17) > Cl (30.18) > Ge (7.89) > Mg (6.08) > Al (4.27) > Si (2.45) > Zn (1.47) > Ni (1.40) > Cu (1.26) > Co (1.08) > Fe (0.91) > Pb (0.87) > Mn (0.63) > Ca (0.52). The results show higher concentration of carbon (Fig. 5, Table 3).

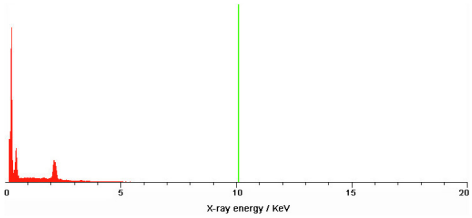

Fig. 5. Elements concentration (wt %) and intensity (c/s) in *T. pratense* plant using a SEM-EDS spectrum that include the characteristic X-ray peaks of the element (0–20 keV X-ray energy/2K X-ray intensity).

Table 3
Elements concentration (wt %) and intensity (c/s) in *T. pratense* plant using a SEM-EDS (n = 3).

Element	Intensity (C/S)	Concentrations (Wt. %, Mean ± SD)*
C	534.4	70.127 ± 5.21
O	56.47	22.197 ± 2.20
Mg	6.08	0.321 ± 0.011
Al	4.27	0.186 ± 0.0062
Si	2.45	0.093 ± 0.0051
Cl	30.18	1.131 ± 0.094
K	53.17	2.17 ± 0.26
Ca	0.52	0.022 ± 0.0016
Mn	0.63	0.045 ± 0.0069
Fe	0.91	0.072 ± 0.0091
Co	1.08	0.099 ± 0.011
Ni	1.40	0.144 ± 0.029
Cu	1.26	0.160 ± 0.042
Zn	1.47	0.222 ± 0.063
Ge	7.89	2.091 ± 0.92
Pb	0.87	0.091 ± 0.0081

3.4. ICP-AES analysis

Elemental analysis has revealed the presence in order of C > O > K > Cl > Ge > Mg > Al > Si > Zn > Ni > Cu > Se > Co > Fe > Pb > Hg > Mn > I > Ca. The results showed higher concentration of carbon (Table 4).

Elements such as Sc, N, Ba, Li, S, P, Cd, and Na were not detected. Elements such as iron, copper, zinc, manganese, and selenium etc. have immunomodulatory functions and thus the extract of this plant can be used to treat various diseases, including cancer, because it has multimineral properties. Of course, the amount of elements in the plant extracts depends on the climatic conditions

Table 4
Concentration of elements found in alcholic soxhlet leaf extracts of *T. pratense* plants using ICP-AES technique (mean ± SD, n = 3).

Elements	Wavelength / nm	Concentrations / (μg/mL)
C	127.72	780.124 ± 12.12
O	130.22	214.215 ± 9.812
Mg	279.079	44.120 ± 0.912
Al	176.640	31.089 ± 0.814
N	131.05	ND
Si	251.612	29.099 ± 0.614
Cl	134.724	50.140 ± 1.124
K	766.490	110.161 ± 4.112
Ba	455.404	ND
Se	196.10	1.123 ± 0.061
Ca	422.673	0.371 ± 0.044
Li	670.780	ND
Mn	257.611	0.410 ± 0.012
Fe	259.940	0.981 ± 0.023
I	178.262	0.382 ± 0.00
P	178.276	ND
S	180.731	ND
Co	230.786	1.061 ± 0.018
Ni	231.604	3.910 ± 0.16
Cd	214.438	ND
Cu	324.754	2.044 ± 0.23
Zn	213.856	6.090 ± 0.17
Na	589.592	ND
As	189.042	ND
Ge	164.919	42.092 ± 2.06
Pb	220.353	0.713 ± 0.94
Sc	361.384	ND
Hg	184.95	0.642 ± 0.073

ND = Not detected means less than 0.01 μg/mL.

(including rainfall, soil, type of crop and type of fertilizers used for plant growth) and its cultivation and harvesting season.

Feed supplements with selenium content (0.2–0.3 μg/mL) are added to diets to prevent deficiency and resultant diseases such as white muscle disease in cattle and sheep and acute myopathy in horses (Edmonson, Norman, & Suther, 1993). Selenium supplementation (10 μg/mL for 30 d) might modulate the lipid profile, mainly reducing the level of TC, non-HDL-C, and atherogenic index in animal models (Sartori, Pinton, da Rocha, Gai, & Nogueira, 2016). Furthermore, 1 μg/mL selenium supplementation could restore the reduced T3 and T4 hormones in the serum of high-fat diet-fed rabbits and same dose in drinking water could reduce aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in diabetic rats (Kang, Bansal, & Mehta, 2000).

The results of this study show that due to the high levels of selenium in this plant (1.123 ppm), it can be used as a plant source to compensate for this deficiency. However, the Selenium in the diet at > 5 μg/mL may produce mild clinical effects after prolonged exposure of ≥ 30 d (Edmonson, Norman, & Suther, 1993). The target supplementary dietary levels of copper and zinc are 5–15 and 20–80 μg/mL, respectively, which more or less over time can cause symptoms of deficiency of these minerals (Olukosi, Van Kuijk, & Han, 2018). Due to the amount of copper (2.044 μg/mL) and zinc (6.09 μg/mL) present in this plant, it could be used as a suitable source of therapeutic dose in people with a deficiency of these minerals.

Iron deficiency is the most common cause of anemia, because it is essential for hemoglobin formation. Of about 15 mg/d of dietary iron, adults absorb only 1 mg, which is the approximate amount lost daily by cell destruction. An adult needs 60 μg/mL of iron per day in food, which is higher in women and growing children (0.5 mg/day than the other people) (Saljoughian, 2007). Although it is not high in iron (0.981 μg/mL), it could help iron deficiency in postmenopausal women (due to the presence of isoflavonoids). A safe reference dose (RfD) for mercury (5.5×10^{-3} mg/d to 1.0×10^{-3} mg/d for a person weighing between 120 and 220 lb) was determined based on methyl mercury poisonings and deaths that occurred in Japan (1953–1960, contaminated fish) from, and Iraq (1971–1972, contaminated grain).

Furthermore, the U.S. Food and Drug Administration (FDA) in 2002 set a limit of 1 μg/mL of mercury in foods and 0.3 ppm in fish for human consumption (Echols, Meadows, & Orazio, 2009). FDA at 2006 established an RfD for lead (Pb) at 0.5 μg/mL. But in some food products, at least this has been changed, for example, in dairy products, especially products containing a maximum detectable maximum chocolate content, which should not exceed 0.1 μg/mL (US Food and Drug Administration, 2006). However, the amount of leads and mercury in red clover are 0.713 ppm and 0.642 μg/mL respectively, which should be taken into account when consuming.

3.5. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacities (DPPH and FRAP assay)

The TPC of *T. pratense* (58.12 ± 6.21 mg GAE/g plant) and TFC (39.21 ± 4.26 mg RUE/g plant) was determined. Antioxidant compounds can reduce free radicals primarily by two mechanisms: hydrogen atom transfer (HAT) and single electron transfer (ET). The antioxidant capacity or radical scavenging capacity (RSC) of the ethanol extract of the flowers from leaves and stem of *T .pratense* was measured by DPPH assays. In the DPPH assay the ethanol extract gave a value at (149 ± 11.61) μmol eq. Trolox/10 g plant. In the FRAP method under acidic conditions, the blue ferrous complex from the yellow ferric-tripyridyltriazine complex is formed by electron donors. *T. pratense* is already confirmed as being a good antioxidant capacity, and it was also a fine source of compounds with reducing capacity as it can be concluded by the FRAP value at 6620.15 ± 43.26 mmol Fe^{2+}/mg that reducing free radicals (Table 5).

Table 5
TPC, DPPH,TFC and FRAP assays results for ethanolic extracts from leaves and stems of *T. pratense* (mean ± SD, *n* = 3).

Methods	Parameter value/Unit
Total phenolic content	58.12 ± 6.21 (mg GAE/ g dried plant)
Total flavonoid content	39.21 ± 4.26 (mg RUE/ g dried plant)
DPPH	149 ± 11.61 (μmol eq. Trolox/10 g dried plant)
FRAP	6620.15 ± 43.26 (mmol Fe(II)/mg dried plant)

4. Discussion

In this study, for the first time, the introduction of SED-EDS for the identification of minerals in plant extracts as well as the analysis of volatile and polyphenolic compounds (flavonoids and isoflavones) of red clover species in Iran for therapeutic applications was investigated. The flavonoids are a large family of polyphenols including the flavanones, flavones, isoflavones, flavonols, and flavanonols which are derived from various phenylproponoid biosynthetic pathways. After LC-ESI/MS analysis, it was determined that the red clover contains formononetin, daidzein 7-*O*-β-*D*-glucoside, daidzin 4′-*O*-glucuronide, genistein, Genistein 8-C-glucoside-xyloside and biochanin A, biochanin A-7-glucoside isoflavones. In the LC-APCI-MS analysis of de Rijke et al., it was found that the plant contains daidzin, daidzein, genistin, genistein, ononin, sissotrin and biochanin A isoflavones compounds de (de Rijke, Zafra-Gómez, Ariese, Udo, and Gooijer, 2001).

In another LC-ESI/MS analysis in the aqueous methanolic extract of the flowers of *T. pratense*, the isoflavones and flavonoids, including genistin, genistin 6′′-*O*-malonate, genistein, formononetin, formononetin 7-*O*-α-*D*-glucoside 6′′-*O*-malonate, prunetin, biochanin a, ononin, irilone 4′-*O*-α-*D*-glucoside 6′′-*O*-malonate, *trans*-clovamide, caffeic acid derivative, pratensein 7-*O*-α-*D*-glucoside, isoquercitrin, isoquercitrin 6′′-*O*-malonate and afrormosin 7-*O*-α-*D*-glucoside were identified (Lin, He, Lindenmaier, Yang, Cleary, & Qiu, 2000).

Isoflavone compounds such as formononetin, biochanin A, genistein and daidzein, presents in this plant have estrogenic properties. These compounds also affect cognitive and mood performance by binding to β-estrogen receptor and changing dopaminergic, serotonergic, and cholinergic systems and reduce bone loss and aging of the skin due to menopause. Due to the presence of isoflavones in this plant, the extract of this plant or its compounds could be used purely for the treatment of various diseases, especially the reduction of menopausal symptoms (Han, Soares, Haidar, De Lima, & Baracat, 2002).

This is while the polyphenol compounds identified in our study included cinnamic acid, 3-*O*-(Z)-p-coumaroylquinic acid, epigallocatechin, caffeic acid, coumaric acid-*O*-pentoside, kaempferol galloylglucoside, quercetin-3,7-diglucoside, kaempferol-3,7-di-*O*-glucoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, myricetin-3-*O*-acetyl rhamnoside, myricetin-3-*O*-acetyl rhamnoside, ferulic acid-*O*-hexoside derivative, apigenin-7-*O*-glucoside, apigenin 6,8-diglucoside, gallocatechin, quercetin-3-*O*-pentoside and kaempferol galloylglucoside. Saviranta et al. after HPLC-MS analyzing in red clover leaves, flavonoids, including quercetin-galactoside, quercetin-glucoside, quercetin, kaempferol, pseudobaptigenin and maackiainhave been identified (Saviranta, Julkunen-Tiitto, Oksanen, & Karjalainen, 2010).

Accumulation of mineral elements in plants depends on soil properties, cultivation, total and plant-available amount of ele-

ments, and fertilization system, climate, as well as plant properties (Bengtsson, Öborn, Jonsson, Nilsson, & Andersson, 2003). In our study, all the elements in this plant were identified so that by using two methods (SEM and ICP), the amount of each elements were measured. However, few of the elements were identified in similar studies on mineral elements in this plant. Our SEM and ICP analysis showed that the red clover contains important minerals such as K, Mg, Al, Si, Zn, Ni, Cu, Se, Co, Fe, Mn, and Ca. Also, the presence of very low toxic compounds, including Lead (Pb) and mercury (Hg) in this plant was proven.

Meanwhile, Whittaker et al. identified the Al, Mg, Co, Ni, Ca, Ba, S, and K elements in the plant using path analysis (Whittaker, Vazzana, Vecchio, & Benedettelli, 2009). Another study showed that the plant contains elements such as Ca, P, K, Mg, Na, Cu, Zn, Mn, Fe and C. Reay et al. showed that the plant contains Fe, Zn, Mn, Cu, Ca, Mg, K, Na, P, and N elements and N (3670 μmol/g DW) and K (790 μmol/g DW) is the most common elements in this plant (Reay & Marsh, 1976; Shokri & Maadi, 2009). In our study, the amounts of C, O, K, Cl, Ge and Mg in this plant were higher than the rest of the elements in this plant. Some of the important elements in this plant, including selenium and phosphorus are in the other clover family (*Trifolium alexandrium* L. and *T. repens* L.) (Singh & Malhotra, 1976).

In the study of volatile compounds of this plant, it was found that it contains fugitive compounds such as 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, methylcyclopentane, 2, 3-dimethylpentanal, 1-hexadecanol, cyclohexane, 4-hydroxy-4-methyl-2-pentanone and 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol (Reay & Marsh, 1976). Using a GLC-MS analysis, showed that the plant contains volatile compounds, including 2-methylbutanol, hexanol, 3-hexenol, 2, 3-dihydroxybutane, β-ocimene, 3-hexenyl acetate, acetic acid, methyl salicylate and 1-phenylethano.Another study, using GC/MS techniques, also showed other volatile compounds, including 3-methyl-1-butanol, ethyl butanoate, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, hexadecanoic acid, 3-methylbutanoic acid, dimethylpyridine, methyl hexanoate, benzaldehyde and 1-heptanol (Rodrigues & do Céu Costa, 2007).

An analysis of GC/MS has shown in the studies of the presence of volatile compounds, including hexane (Higgins & Smith, 1972), methylcyclopentane (Fukui & Doskey, 2000), cyclohexane (Yoshihara, Yoshikawa, Kunimatsu, Sakamura, & Sakuma, 1977), 4-hydroxy-4-methyl-2-pentanone (Coote, Wayne, Regtop, & Biffin, 2004), 7-tetradecene, 1-nonadecen (Parekh & Felice, 2014), 10-heneicosene (Kami, 1978) and *n*-tetracosanol-1 (Houx, Garrett, & McGraw, 2008) which were identified in our analysis of these compounds in this plant.In addition, at the end of our study, new volatile compounds were identified that were not reported in previous studies of the plant, which are 3-trifluoroacetoxypentadecane, n-tridecanol, octylcyclohexane, 1-hexadecanol, 3-eicosyne, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 9-eicosyne and 2-methyl-1-hexadecanol.

Some of these compounds have therapeutic properties, including 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (R/T 40.98) can be an antioxidant, antimicrobial and anti-inflammatory (Jananie, Priya, & Vijayalakshmi, 2011), *n*-hexadecanoic acid (R/T 43.148) can be an antifungal, antioxidant, hypocholesterolemic, nematicide, anti-androgenic flavor, hemolytic, 5α-reductase inhibitor, potent antimicrobial agent, antimalarial and antifungal (Akpuaka, Ekwenchi, Dashak, & Dildar, 2013), 4-hydroxy-4-methyl-2-pentanone (R/T 7.559)9-eicosane (R/T 41.166), 1-hexadecanol (R/T 33.47) and 10-heneicosene (R/T 38.911) can be an antioxidant and potent antimicrobial agent (Neelamma, Rao, & Anuradha, 2011). Due to flavonoids and isoflavones and essential minerals identified for this study, the clover extracts or its purified compounds can be used for various treatments.

A study showed that the value of total phenolics of *T. pratense* leaves was 62.65 and flowers 58.53 mg GAE/gdried plant. It was in line with our result of the value of total phenolics of Iranian red clover (58.12 mg GAE/g dried plant) (Tava, Pecio, Lo Scalzo, Stochmal, & Pecetti, 2019). However, in another study on the total phenolics and total flavonoid of the *T. pratense* (aerial parts) methanolic extract, the amount of total phenolics 31.94 mg GAE/g was reported. In the same study, the amount of total flavonoid was reported at 13.03 mg RUE/g (Khorasani, Mat, Mohajer, & Banisalam, 2015), while our study showed that the leaf and stem extracts contained 39.21 mg RUE/g. Also, in the present study, the antioxidant capacity was 149 ± 11.61 (μmol eq. Trolox/10 g dried plant) using DPPH solution and 6620.15 ± 43.26 (mmol Fe (II)/mg dried plant) in FRAP solution.

5. Conclusion

Isoflavone compounds such as formononetin, biochanin A, genistein and daidzein, as well as flavonoid compounds including apigenin, kaempferol, ferulic acid, quercetin, caffeic acid, coumaric acid and myricetin, which have many medicinal properties that can be purified and used in the pharmaceutical industry. Volatile compounds such as 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, methylcyclopentane, 2, 3-dimethylpentanal,1-hexadecanol, cyclohexane and 4-hydroxy-4-methyl-2-pentanone have the highest concentrations (5%<) in this plant. Using two different methods (ICP and SEM), 18 mineral elements were identified in this plant that some essential mineral elements, including Se, Cu, Fe, Co, and Zn, along with therapeutic and medicinal properties play a role in the metabolism of plant cells and the production of their compounds. In addition to mineral elements with therapeutic properties, two toxic elements (Pb and Hg) were detected in this plant, which had lower concentrations than other elements. In future studies, a comparison between the effect of season, plant genus and geographical area on the amount of minerals and polyphenolic compounds in clover will be examined.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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