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Research paper

Phytochemical analysis, phenolic compounds, condensed tannin content and antioxidant potential in Marwa (*Origanum majorana*) seed extracts

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Abstract

Antioxidant and free radical scavenging potential of seed extracts of *Origanum majorana* was evaluated and correlated with total phenolic content (TPC) and condensed tannin content (CTC). Ethanol, methanol, acetone and chloroform were used to extract bioactive compounds from seeds of *Origanum majorana* at 45 °C for 45 minutes. As compared to other solvents, methanol seems to be an important extraction solvent, as maximum amount of bioactive compounds (1.18 mg GAE/g dwb) with antioxidant potential was observed in methanolic extract. Total phenolic compounds in seeds were evaluated using Folin–Ciocalteu reagent (FC reagent) method. Total phenolic compounds in seeds were in the range of 0.10–1.18 mg gallic acid equivalent/g dry weight basis (mg GAE/g dwb). HPLC study confirmed the presence of catechin, cinnamic acid, gallic acid and ascorbic acid. The antioxidant potential in seed extracts of *Origanum majorana* confirmed the presence of nutraceutical properties in them which will further be helpful in the preparation of various functional food products.

Keywords: Antioxidant potential; TPC; CTC; HPLC

1. Introduction

Whole plants and plant parts rich in bioactive phytochemicals attract the attention of various food industrialists/researchers to prepare functional foods and medicines from them. A huge number of synthetic antioxidant rich foods and food supplements are available in the market but due to the side effects from them, natural antioxidant rich plants/plant parts are gaining lots of popularity. Plants may possess both kinds of phenolic compounds in free form as well as bound phenolics as complex molecules. The phenolic compounds are referred as antioxidative in nature [1–3]. During the natural processes inside the body, various reactive oxygen species are formed and ultimately results in oxidative stress that leads to chronic diseases cancer, heart attack and age related health hazards. Antioxidants neutralize the oxidative stress generated due to free radicals or reactive oxygen species [4].

The presence of bioactive compounds significantly varies according to agro-climatic conditions [5,6]. *Origanum majorana* is locally known as Marwa. Marwa is a rich source of bioactive compounds like phenolic compounds, flavonoids, flavanones, benzoic and cinnamic acid derivatives. In many developing countries, Marwa plant parts are currently being used as a remedy for various chronic diseases. There is a scarcity of information regarding the antioxidant potential in seeds of Marwa plants. This prompted us to design the present study. Thus keeping in view the health benefits from plants, the current study was designed to evaluate the phytochemicals, total phenolic content, condensed tannin content and antioxidant potential of Marwa (*Origanum majorana*) seed extracts.

2. Material and methods

Origanum majorana seeds were purchased from district Sirsa, Haryana, India. Seeds were washed with tap water to remove small stones/debris, dust particles and dried in an oven at 40 °C. The seeds were stored in airtight containers at room temperature until used for further studies.

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2.1. Chemicals and glasswares

All the solvents (Ethanol, Methanol, Acetone and Chloroform) and chemicals *viz.* DPPH (2,2-diphenyl-1-picrylhydrazyl), 2,2′-Azino-bis [3-ethylbenzothiazoline-6-sulfonate (ABTS)], ascorbic acid, gallic acid and catechin used were of analytical grade and obtained from HiMedia, Merck and Sigma-Aldrich respectively. Glasswares used in the experimental work were of Borosilicate. Before using, glasswares were washed with Labolene detergent and rinsed with tap water and sterilized in an oven at 180 °C for 3 hours.

2.2. Preparation of extracts

To obtain fine powdered form, seeds were ground in an electric grinder. The seed sample tested was defatted by blending the ground material with hexane (1:5 w/v, 5 min, thrice) at ambient temperature as reported by Bhanja et al. [7]. Defatted sample was dried in an oven at 45 °C for 24 h–48 h and stored at −20 °C for further analysis. Defatted samples were extracted with ethanol, methanol, acetone and chloroform (1:20 w/v) at 45 °C for 45 minutes separately. After extraction, samples were filtered using Whatman No. 1 filter paper. The filtrate was evaporated using a rotary vacuum evaporator. Extracts were prepared with the same solvent in ratio (1:2). The extracts were frozen at 4 °C for further analysis.

2.3. Phytochemical analysis

Preliminary phytochemical analysis was carried out by following already standardized methods of Purewal [8] and Mir et al. [9].

2.4. Total phenolic content (TPC)

Total phenolic content of the extracts was determined using Folin–Ciocalteu reagent as described by Salar et al. [10]. Absorbance was recorded at 765 nm against a blank. The amount of total phenolic content was calculated as gallic acid equivalent from the standard calibration curve of gallic acid and expressed as mg gallic acid equivalents/g dry weight basis (mg GAE/g dwb).

2.5. Condensed tannin content (CTC)

Condensed tannin content was determined according to the method of Julkunen-Titto [11] with slight modifications as described by Salar and Purewal [12]. The absorbance against blank was read at 500 nm. Catechin was used to prepare the standard curve. The results were expressed as mg Catechin equivalent/g dry weight basis (mg CE/g dwb).

2.6. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The radical scavenging capacity of different samples was measured by the DPPH scavenging method according to Yen and Chen [13] with some modifications. Briefly, 100 µl of extract was taken in spectrophotometric cell and then 3 ml of 100 µM DPPH (4 mg DPPH in 100 ml methanol) was added [10]. The changes in absorbance at 517 nm were recorded after 30 minutes.

Percent (%) DPPH scavenging activity was calculated using the formula:

$$\text{DPPH Scavenging Activity}\,(\%) = (A_C - A_E/A_C) \times 100$$

where A_C and A_E are the absorbance of control and extracts, respectively.

2.7. ABTS radical cation depolarization assay (ABTS)

ABTS, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (HiMedia) radical cation decolorization test is also a spectrophotometric method widely used for the assessment of antioxidant activity of various extracts. Antioxidant activity was measured using the method described by Salar et al. [10].

Percent (%) scavenging activity was calculated using the formula:

$$\text{Decolorization Activity}\,(\%) = (A_C - A_E/A_C) \times 100$$

where A_C and A_E are the absorbance of control and extracts, respectively.

2.8. Total antioxidant capacity (TAC)

Total antioxidant activity of extracts was determined by following the method described by Prieto et al. [14]. The reagent to check the antioxidant activity of extracts was prepared by following Salar and Purewal [12]. Absorbance was recorded at 695 nm. Ascorbic acid was used as the standard to compare antioxidant activity of extracts. Dilution with distilled water was required if the product formed shows dark color.

2.9. Reducing power assay (RPA)

The reducing power was measured using the method described by Oyaizu [15]. Mixture was diluted with distilled water to prepare the final volume of 10 ml. Absorbance was recorded at 700 nm. Quercetin was used as the standard to compare the reducing power potential of extracts [12].

2.10. Hydroxyl free radical scavenging activity (HFRSA)

The antioxidant potential of extracts against hydroxyl radicals was analyzed by following Smirnoff and Cumbes [16]. An aliquot of extracts (100 µl) was mixed with a suitable amount of Smirnoff reagent (3 ml) followed by incubation at 37 °C for 30 minutes. Extraction phase was used as a negative control to check the antioxidant potential of different extracts. The percentage of scavenged OH^- by extracts was calculated using the following formula:

$$\text{Scavenged } OH^- \% = [(Ac - A_E)/Ac \times 100]$$

where Ac is absorbance of control and A_E is absorbance of extract.

2.11. HPLC analysis

2.11.1. Qualitative analysis

All HPLC experiments were carried out on a Shimadzu 10 AVP HPLC system comprising a SCL10 AVP system

controller, two LC-10 AVP pumps CTO-10 AVP column oven with Rheodyne 7120 injection value (20 µl sample loop) and SPD-M10 AVP photodiode-array detector (all from Shimadzu, Tokyo, Japan). Gemini-NX C18 analytical HPLC column (250 × 4.6 mm, 3 µm) with a guard column (40 × 3 mm, 3 µm) both from Phenomenex (Torrance, CA, USA) was used. Analysis was performed at a rate of 0.6 ml/min using 2% v/v acetic acid (solvent A) and methanol: acetonitrile (40:50 v/v) mixture (solvent B) under the following gradient program: 0–8 min 70% acetic acid, 8–19 min 60% acetic acid and 19–30 min 30% acetic acid. Injection volume was 10 µl. The analytes were detected at 280 nm.

2.11.2. Quantitative analysis

For the quantification purpose mobile phase A consisted of methanol, mobile phase B consisted of 0.1% glacial acetic acid (0.1 ml of glacial acetic acid was dissolved in 100 ml of water), mobile phase C consisted of 0.1% ortho-phosphoric acid (0.1 ml of orthophosphoric acid was dissolved in 100 ml of double distilled water) and mobile phase D consisted HPLC grade 100% acetonitrile. Mode of elution: Linear gradient in the ratio A: B: C: D (35:30:30:5). Flow rate was 1.2 ml/min. UV Detector wavelength was set to 280 nm. Column temperature was 30 °C. 1 ml of extract was diluted with 1 ml of diluent and acidified with one drop of Glacial acetic acid and orthophosphoric acid and then filtered through 0.22 µm filter and injected in HPLC instrument. Standard graphs were prepared and R^2 value generated from the graph was used to quantify the said bioactive constituents.

2.12. Statistical analysis

All tests were carried out in triplicate and mean values ± standard deviation are presented. Correlations and means were compared using SPSS 16.0 statistical software. Principal component loading plot for determining the relationship between total phenolic compounds, condensed tannin content and antioxidant potential was drawn using Minitab 16 software.

3. Results and discussion

3.1. Phytochemical composition

The phytochemical analysis of *Origanum majorana* (Seeds) revealed the presence of coumarins, flavonoids, steroids, flavanone, sugars and tannins. All the extracts of *Origanum majorana* (Seeds) showed presence of phytochemicals except chloroform and acetone extracts which showed poor presence of these phytochemicals. The results of preliminary phytochemical analysis represented in Table 1. The results obtained from this work revealed that the *Origanum majorana* seeds contained many bioactive agents.

The above mentioned compounds are known to be biologically active through different mechanisms [17–19]. Steroids, abundant in many plants, have been shown to have hypercholesterolemic effects [19] and are used as emollients, diuretics and as a central nervous system depressant. Singh et al. [20] reported the presence of phytochemicals in leaves and roots of *Ocimum sanctum* methanol extracts whereas Naik et al. [21] studied the effect of methanol, acetone and water on *Ocimum tenuiflorum* leaves and found various phytochemicals in leaf extracts. Moussaid et al. [22] studied aerial parts of *Origanum majorana* and found a variety of phytochemicals in extracts.

Table 1
Results of phytochemical analysis of Marwa (*Origanum majorana*) seeds extracts with different solvents.

Phytochemicals/tests	Ethanol	Methanol	Acetone	Chloroform
Phlabotannin	−	−	−	−
Coumarins	+	+	+	−
Flavonoids	+	+	+	−
Steroids	+	+	+	+
Tannin	−	+	−	−
Protein	−	−	−	−
Anthocyanin	−	−	−	−
Saponin	−	−	−	−
Flavanone	−	−	+	−
Molisch test	+	+	−	−
Fehling test	−	−	+	−
Benedict test	+	+	−	+
Wagner test	−	−	−	−
Mayer test	−	−	−	−
Hager test	−	−	−	−

(+) sign indicates the presence of specific phytochemicals whereas (−) sign indicates the absence.

3.2. TPC (total phenolic content)

Folin–Ciocalteu reagent (FC reagent) is most commonly used by the researchers to detect the presence of phenolic compounds in natural extracts [4,23]. Phenolic compounds are the secondary metabolites that are being extensively studied in plant parts of medicinal value, fruits and vegetables [24,25]. TPC in various extracts of *Origanum majorana* were quantified using Folin–Ciocalteu (FC reagent) method. The amount of extractable phenolic compounds in *Origanum majorana* extracts were expressed as mg gallic acid equivalent/g dry weight basis (mg GAE/g DWB). The results were expressed on dry weight basis (Fig. 1). Roby et al. [26] observed maximum amount of extractable phenolic compounds (5.20 mg GAE/g) in the leaves of *Origanum majorana*. The results of their study also supports that methanol possesses the highest extraction

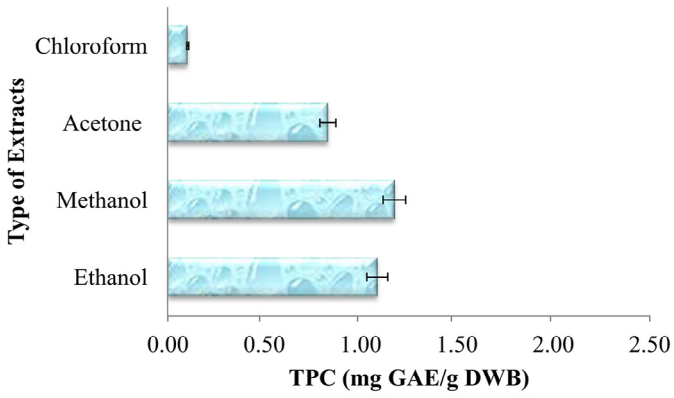

Fig. 1. Total phenolic content in different extracts of *Origanum majorana*.

capability to extract phenolic compounds from the leaves of *Origanum majorana*. Deo et al. [27] reported the presence of bioactive phenolic compounds in different leaf extracts of *Ocimum tenuiflorum*. Like the present study, they used different extraction solvent *viz.* methanol:water (70:30), methanol:water (50:50), ethanol:water (70:30) and ethanol:water (50:50) for extraction of phenolic compounds. They found maximum amount of extractable phenolic compounds in methanol:water (70:30) extract i.e. 4.33 g/100 g. Baatour et al. [28] evaluated the TPC in aerial parts of *Origanum majorana* (8.86 mg GAE/g DWB). Zarin et al. [29] found total phenolic content in the leaves (3.21 mg GAE/g). Amount of phenolic content may differ as different plant and plant parts possess different amount of bioactive compounds. Sometimes, agro-climatic conditions are solely responsible for the occurrence of variable amount of bioactive components in natural resources.

3.3. CTC (condensed tannin content)

Condensed tannin content is another category of bioactive compounds as TPC [12]. CTC provide specific flavor to the natural extracts/food formulations prepared from tannin rich plants. Although, the amount of tannin content is lesser as compared to other bioactive components in plant extracts but, their presence in extracts could not be neglected. The amount of condensed tannin content in various extracts is expressed in the form of Fig. 2. Four solvents viz. ethanol, methanol, acetone and chloroform were used to extract condensed tannin content from *Origanum majorana*. The extracts prepared in acetone show maximal amount of extractable tannin content as compared to other solvents. Baatour et al. [28] described the presence of variable amount of CTC in *Origanum majorana* extracts (20–25 mg/g). Zarin et al. [29] reported the presence of condensed tannin content in leaf extracts (2.06 mg/g). Whereas, in our study the maximum amount of extractable CTC was 6.02 mg CE/g. The results may differ depending on the type of plant, plant part, extraction temperature, extraction time and extraction phase used for recovery of bioactive compounds.

3.4. Antioxidant potential

Various antioxidant assays are currently being used by the researchers to detect the antioxidant potential in natural extracts.

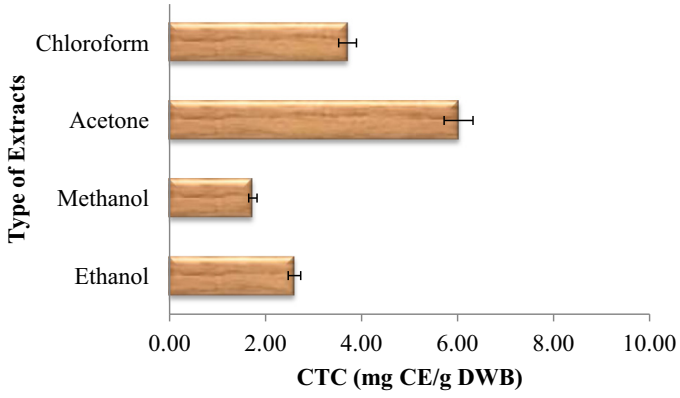

Fig. 2. Condensed tannin content in different extracts of *Origanum majorana*.

Table 2
Antioxidant activity in different extracts of *Origanum majorana*.

Extraction solvent	DPPH	ABTS	TAC	RPA	HFRSA
Ethanol	91.63^{a1}	76.96^{b1}	0.68^{c1}	7.78^{d1}	5.12^{e1}
Methanol	91.89^{a1}	90.79^{a2}	0.70^{b1}	8.69^{c2}	0.89^{b2}
Acetone	91.03^{a1}	71.11^{b3}	0.60^{c1}	6.13^{d3}	0.58^{c2}
Chloroform	84.87^{a2}	0.76^{b4}	0.11^{c2}	1.77^{d4}	0.20^{c2}

Values are the mean of triplicate experiments. Same superscript letters/values in each row/column are not significantly different.

Owing to the complex nature of bioactive compounds in natural extracts, single assay is not enough to quantify the antioxidant potential [25]. However, the most commonly used methods are DPPH, ABTS, TAC, RPA and HFRSA. Phenolic compounds with antioxidant potentials are desirable to prepare medicinal formulation from any natural resource. Various studies support the relationship of phenolic compounds with antioxidant potential [30–33]. Antioxidant potential in various extracts of *Origanum majorana* is represented in Table 2. Each extract shows its own distinct antioxidant potential as compared to other extracts. DPPH is a very simple and useful assay to detect the presence of antioxidant potential in extracts. DPPH is a purple colored reagent which in the presence of antioxidant potential changes to mustard yellow. Although, all the extracts shows antioxidant potential during DPPH assay however, the maximum percent (%) inhibition was shown by the methanolic extracts (91.89%) and the minimum percent (%) inhibition was shown by the extract prepared in chloroform (84.87%) (Table 2). The detection of antioxidant potential in extracts using ABTS assay is a color-based reaction. ABTS reagent comprises a dark blue-green color which turns transparent in the presence of antioxidants. ABTS assay shows similar pattern of antioxidant potential as in DPPH (Table 2). The presence of antioxidant potential in extracts is Methanol > Ethanol > Acetone > Chloroform. The total antioxidant capacity assay is characterized by the reduction of Mo (VI) to Mo (V) and formation of dark bluish-green phosphate Mo (V) complex under acidic conditions. This assay is highly useful for the detection of both water as well as fat soluble antioxidant compounds [34,35]. The results of TAC assay show that the methanolic extract of *Origanum majorana* possesses maximum total antioxidant capacity as compared to other extracts. The order of the TAC in extracts were methanol > ethanol > acetone > chloroform (Table 2). Reducing power assay is an important parameter to detect the presence of bioactive components with antioxidant potential. Reducing power assay shows the specific color (dark blue green) in the presence of antioxidant potential in extracts. In the presence of suitable reductant, Fe^{3+} is reduced into Fe^{2+} form [30]. In this assay performed, a similar pattern was observed as in the case of other antioxidant assays performed. Methanolic extract shows maximum reducing power (8.69 mg AAE/g DWB) and shows the minimum reducing power (1.77 mg AAE/g DWB). HFRSA method is a rapid way to detect the antioxidative nature of natural extracts. This method solely detects the electron donator present in the extracts. As explained in earlier published reports H_2O_2 is a weak oxidizing reagent. Hydrogen peroxide is basically

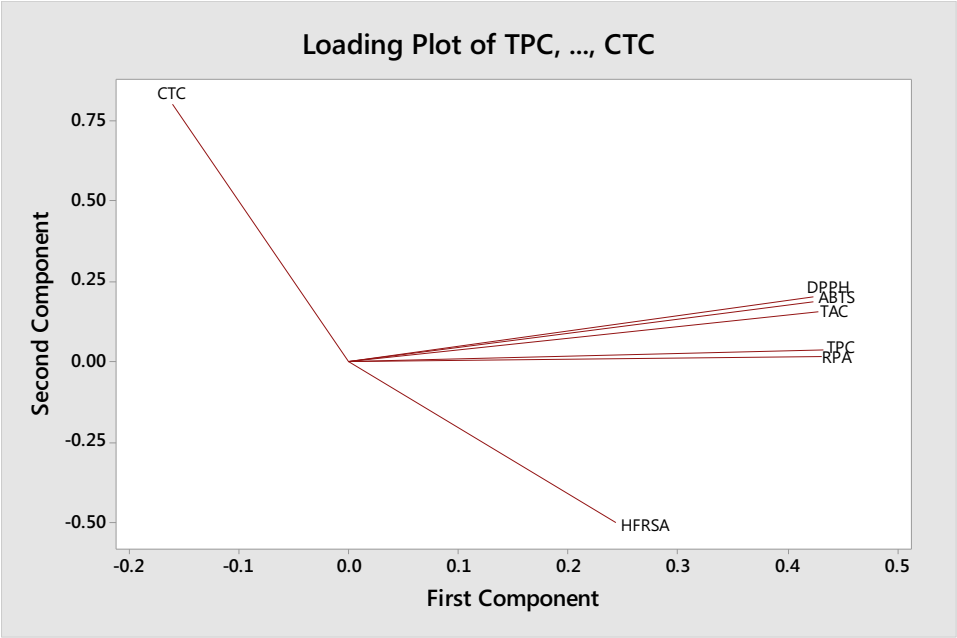

Fig. 3. Principal component loading plot from total phenolic compounds and antioxidant potential of *Origanum majorana* extracts. *TPC: Total phenolic content; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); TAC: Total antioxidant capacity; HFRSA: Hydroxyl free radical scavenging activity; RPA: Reducing power activity; CTC: Condensed tannin content.

involved in the generation of free radicals that creates a major problem in cellular reactions. Antioxidants neutralize the H_2O_2 for the protection of living organisms and to sustain the normal biological phenomenona in human body [36,37]. The results of the HFRSA assay are represented in Table 2. Abdel-Massih et al. [38] reported the maximum percent inhibition (82%) against DPPH radical by *Origanum majorana* extracts. Chun et al. [39] found maximum percent inhibition against DPPH (80%) and ABTS radicals (92%) by *Origanum vulgare* extracts. Further, due to the different methodology adopted, it is more difficult to compare the results of the other assays performed during the present study with earlier published reports.

3.5. PCA (principal control analysis) and correlation

PCA is gaining more popularity as the best statistical tool for the determination of relationship between any two components. It helps to establish a relationship between any variable selected during research analysis. In the present investigation PCA was used to detect the level of relationship between total phenolic compounds, condensed tannin content and antioxidant potential of *Origanum majorana* extracts. The results were generated using Minitab software. The position of each variable in a loading plot describes the relationship with other selected variables. Fig. 3 clearly indicates the excellent relationship between TPC-RPA, TPC-TAC, TPC-ABTS and TPC-DPPH. Other variable CTC and HFRSA showed less correlation with TPC as the distance between them is clearly observable. These relationships were also supported by the data generated from the statistical software SPSS (Table 3). However, further study is required to validate the relationship between TPC-CTC and TPC-HFRSA. Statistical analyses validating the correlation between phenolic compounds and antioxidant potential of the extracts are currently being used by many researchers and food scientists. Earlier published reports suggested the existence of positive correlation between phenolic compounds and antioxidant potential of the extracts [4,12].

3.6. Profile of phenolic compounds

Qualitative analysis for the identification of specific phenolic compounds was carried out via HPLC. Six standards were used for the detection of same compounds in the extracts studied. The main phenolic compound in the best extract was cinnamic acid followed by the ascorbic acid, gallic acid and catechol (Fig. 4) whereas, one unknown peak was also detected in HPLC analysis. The unknown peak might be performing an important role in providing antioxidant potential to the said extract. Further, it is very difficult to compare the results of HPLC generated with earlier published reports as different methods

Table 3
Correlation between total phenolic content, condensed tannin content and antioxidant activity of different extracts of *Origanum majorana*.

	TPC	DPPH	ABTS	TAC	RPA	HFRSA	CTC
TPC	1	.980**	.987**	.988**	.998**	.503	−.369
DPPH	.980**	1	.993**	.999**	.970*	.459	−.176
ABTS	.987**	.993**	1	.994**	.984**	.394	−.242
TAC	.988**	.999**	.994**	1	.980*	.483	−.224
RPA	.998**	.970*	.984**	.980*	1	.477	−.405
HFRSA	.503	.459	.394	.483	.477	1	−.373
CTC	−.369	−.176	−.242	−.224	−.405	−.373	1

** Correlation is significant at the 0.01 level (1-tailed).
* Correlation is significant at the 0.05 level (1-tailed).

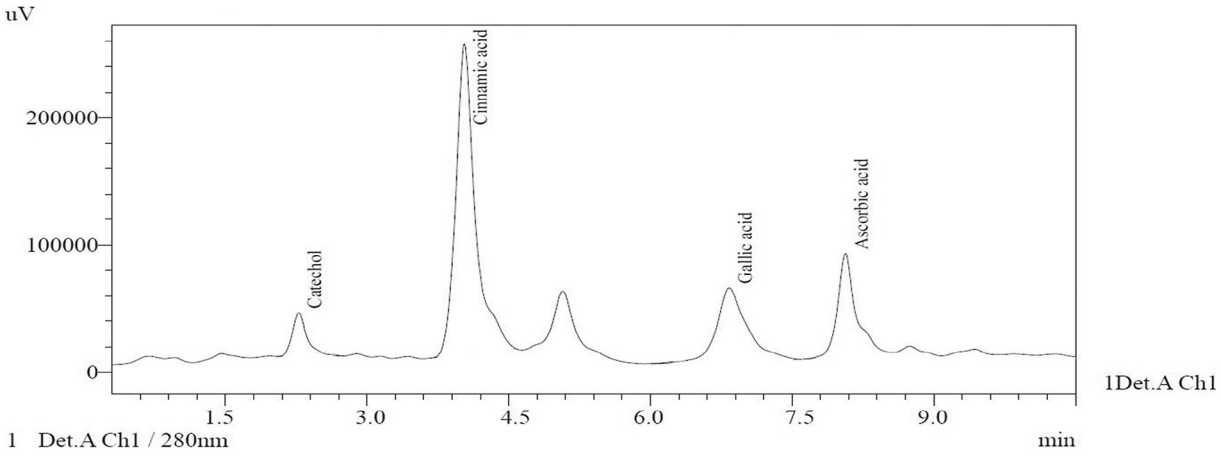

Fig. 4. HPLC chromatogram of *Origanum majorana.*

Table 4
Quantification profile of bioactive compounds in *Origanum majorana* extracts.

Constituents found	Area	Concentration (x) µg/ml
Catechol	192,861	1.76
Cinnamic acid	22,881	63.02
Gallic acid	53,278	0.89
Ascorbic acid	74,288	9.11

have been used for extraction purpose. Moreover, phenolic compounds are solely responsible for the antioxidant potential in natural extracts [12]. Quantitative analysis of bioactive compounds present in *Origanum majorana* revealed that cinnamic acid (63.02 µg/ml) is the major bioactive compound present in the extract followed by ascorbic acid (9.11 µg/ml), catechol (1.76 µg/ml) and gallic acid (0.89 µg/ml) (Table 4). An extract may possess a mixture of other important bioactive compounds that might be responsible for the antioxidant potential. As per our study, out of the selected six major bioactive compounds, seed extract possesses only four major bioactive components.

4. Conclusion

The extraction phase significantly affects the leaching out phenomenon within natural extracts. The amount of phenolic content, condensed tannin content and antioxidant potential varied significantly among different extracts, possibly indicating the role of extraction solvent. The extraction of *Origanum majorana* seeds with different solvents revealed methanol to be the best to recover the bioactive components from the seeds of the plant studied, followed by ethanol, acetone and chloroform. *Origanum majorana* is an important plant. Extracts of *Origanum majorana* is used as a liver tonic, as an effective stimulant, in bleeding control, and in controlling digestive problems such as nausea, intestinal spasm and diarrhea. Medicines and extracts prepared from *Origanum majorana* help in flushing toxins from the body. The oil extracted from the plant is used in toothache, soothes joints and muscular pain. Various parts are currently being used in the treatment of asthma, skin diseases and common cold. The presence of bioactive compounds in *Origanum majorana* makes it a potential source for the production of functional food products/food supplements and various nutraceuticals.

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Conflict of interest

The authors declare no conflict of interest.

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