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HPLC Profiling and Evaluation of *In-vitro* Antioxidant Activity of *Cirsium arvense* L. (Family: Asteraceae)

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Abstract

This study evaluated HPLC analysis and *in-vitro* antioxidant activity of *Cirsium arvense* commonly known as Canada thistle that has been used to treat various diseases as traditional medicine. The different antioxidant assays such as DPPH free radical scavenging, nitric oxide scavenging, hydrogen peroxide scavenging, superoxide radical scavenging, reducing power, ferrous ions chelating ability, total phenolic, flavonoids and tannin contents were studied. Moreover, high performance liquid chromatography (HPLC) coupled with diode-array detection was used to identify and quantify the phenolic compounds present in *C. arvense*. The ethanol extract of *Cirsium arvense* showed dose-dependent antioxidant activity compared to the standard in different antioxidant activity tests. *p*-coumaric acid, rutin hydrate, quercetin and kaempferol were identified in the extract. Among the phenolic compounds, quercetin was abundantly present in the extract. Our exploration suggests that *C. arvense* contains high amount of phenolic compounds which may be responsible for its biological activities in folkloric medicine.

Keywords: *Cirsium arvense*; Asteraceae; HPLC; Antioxidant.

Introduction

The plants that possess therapeutic properties or exert beneficial pharmacological effect on the living body are generally known as “Medicinal Plant”. Medicinal Plants are a large group of plants used in medicine or veterinary practice for therapeutic or prophylactic purposes. Secondary metabolites of plants exert therapeutic actions in human. Free radical induced oxidative stress causes damage to our body that can be protected various antioxidants [1]. Now a days, natural antioxidants e.g. polyphenols are in prime consideration to prevent oxidative damage [2]. Thus, it is vital to characterize different types of medicinal plants for their antioxidant potentiality [3, 4, 5]. Large number of the world population depends on drugs obtained from plants [6]. The use of plant drugs is increasing in the developing countries because of the cost of modern medicines. In future the use of plant drugs will be more and more than present to reduce the financial burden on the developing countries [6].

Cirsium arvense belongs to the family of Asteraceae that has been used to treat many diseases traditionally. North American Indians used infusions and extracts for mouth diseases and infections and considered to be useful as a health promoting tonic, diuretic and astringent. Its leaves can also be chewed to relieve the pain of toothaches, cancer sores and sore throats as they have anti-inflammatory properties and can be a source of fiber, vitamins and minerals much like other herbs. If swallowed, the juice of the roots is purportedly effective at killing intestinal parasites and reducing the symptoms of poison ivy and gastritis. The pharmacological actions of *C. arvense* such as antibacterial, antifungal and hepatoprotective activity have been described previously [7, 8]. However, very few reports have been found in literature which showed the analysis of phenolic compounds. As a part of our ongoing study about natural antioxidant from local medicinal plants of Bangladesh [9, 10, 11, 12], here we evaluated the antioxidant activity of *C. arvense* extract and analyzed the phenolic compounds present using HPLC-DAD system.

2. Materials and Methods

2.1. Chemicals

Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), myricetin (MC), kaempferol (KF), and quercetin (QU) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was

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obtained from Merck (Darmstadt, Germany). Ascorbic acid, butylated hydroxyl toluene and potassium ferricyanide were purchased from Merck, Germany. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu's reagent and all types of analytical grade solvents and reagents were obtained from Sigma Chemical Co. Ltd., (St. Louis, MO, USA).

2.2. Collection and Identification of Plant Material

The plant was collected from Khulna city, Bangladesh on February 12, 2013 at the daytime. During collection any type of adulteration was strictly prohibited. The plants were mounted on paper and the sample was identified by the experts of Forestry and Wood Technology (FWT) Discipline, Khulna University, Khulna-9208, Bangladesh.

2.3. Preparation of Crude Extract

The collected aerial parts were separated from undesirable materials or parts and were shade-dried for 10-12 days to ensure that the active constituents were free from decomposition and also to avoid any photochemical degradation. The collected aerial part of *Cirsium arvense* was powdered with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China) and extracted by 96% ethanol using soxhlet apparatus. The weight of the crude extract was 25 gm and yield was 12.5 %. Then crude extract was stored in a refrigerator at 4°C.

2.4. Phytochemical Test

To identify the chemical constituents such as carbohydrates, alkaloids, glycosides, phenolic compounds, flavonoids, tannins, steroids, gum, saponins and acidic compounds of plant extract standard procedures were followed.

2.5. In-vitro Antioxidant Assays

2.5.1. Total Phenolic Content

Total phenolic content of the extract was determined by Folin-Ciocalteu method [13] in which the absorbance was measured at 765 nm. Total phenolic content was determined as mg of Gallic acid equivalent (GAE) per gm of dry extract using the equation obtained from a standard Gallic acid calibration curve.

2.5.2. Total Flavonoids Content

AlCl₃ colorimetric method was used to determine the total flavonoids content in the extract [14]. After reading the quercetin equivalent from the calibration line, total content of flavonoids was expressed as mg quercetin equivalent (QE) per gram of dry extract.

2.5.3. Total Tannin Content

The Folin-Ciocalteu phenol reagent was used to determine the total tannins in the extracts as reported by Amorim *et al.*, (2008) [15]. Total tannin content was estimated and expressed as mg gallic acid equivalent per gm of dry extract.

2.5.4. DPPH Free Radical Scavenging Assay

The *in-vitro* DPPH free radical scavenging activity was quantitatively estimated according to the method of Sadhu SK *et al.*, 2003 [16]. % inhibition was calculated from the equation:

$$\% \text{ Inhibition} = \frac{(\text{Blank absorbance} - \text{Sample absorbance})}{\text{Blank absorbance}} \times 100.$$

50% inhibitory concentration (IC₅₀) of extract and ascorbic acid were determined from % inhibition versus concentration graph.

2.5.5 Hydrogen Peroxide Assay

Antioxidant activity by hydrogen peroxide scavenging assay was determined according to the method of Nabavi *et al.*, 2008 [17].

2.5.6. Superoxide Radical Scavenging Activity

Measurement of superoxide radical scavenging activity was done using standard method [1] with slight modification. The superoxide radical scavenging activity was calculated according to the following equation:

$$\% \text{ Superoxide Radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A₀ was the absorbance of the blank and A₁ was the absorbance in the presence of the sample of extract and standard.

2.5.7. Ferrous ion (Fe²⁺) Chelating Ability Assay

The ferrous ion chelating ability of the extract was monitored by measuring the formation of the ferrous ion ferrozine complex according to the method of Dinis [2]. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percent inhibition of ferrozine-Fe²⁺ complex formation was given in the below formula:

$$\text{Ferrous ions chelating ability (\%)} = \frac{(A_0 - A)}{A_0} \times 100$$

Where, A₀ is the absorbance of the control solution (containing all reagents except plant extracts); A is the absorbance in the presence of the sample. The control contains FeCl₂ and ferrozine, complex formation molecules. The IC₅₀ value was compared with EDTA.

2.5.8. Nitric Oxide (NO) Scavenging Assay

Nitric oxide scavenging activity was measured spectrophotometrically according to Govindarajan R *et al.*, 2003 [13]. The nitric oxide (NO) radical scavenging activity was expressed as the percent inhibition (I %) and calculated as per the equation:

$$I \% = \frac{(A_{\text{Blank}} - A_{\text{Sample}})}{A_{\text{Blank}}} \times 100$$

Where A_{Blank} is the absorbance of the control reaction and A_{Sample} is the absorbance of the sample. IC₅₀ value is the concentration of sample required to scavenge 50% nitric oxide free radical and was calculated from the plot of percent inhibition (%) versus the sample concentration. Ascorbic acid was used as standard for this study.

2.5.9. Reducing Power Assay

The reducing power of the sample was determined by the method of Oyaizu (1986) [14] with modifications. The absorbance was measured at 700 nm and ascorbic acid was used as standard.

2.6. High Performance Liquid Chromatography (HPLC) System

Chromatographic analyses were carried out on a Thermo Scientific Dionex Ulti Mate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS auto sampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated on an Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionex, USA) which was controlled at 30 °C using a temperature controlled column compartment (TCC-

3000). Data acquisition, peak integration and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

2.6.1. Chromatographic Conditions

The phenolic composition of the ethanol extract of *Cirsium arvense* was determined by HPLC-DAD, as described previously with some modifications. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30 min, 100%A. There was a 5 min post run at initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 ml/min and the injection volume was 20 µl. For UV detection, the wavelength program was optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was set at an acquisition range from 200 nm to 700 nm. The detection and quantification of GA, CH, VA, CA, and EC was done at 280 nm, of PCA, RH, and EA at 320 nm, and of MC, QU, and KF at 380 nm, respectively.

2.6.2. Standard and Sample Preparation

A stock standard solution (100 µg/ml) of each phenolic compound was prepared in methanol by weighing out approximately 5 mg of the analyte into 50 ml volumetric flask. The mixed standard solution was prepared by dilution the mixed stock standard solutions in methanol to give a concentration of 5 µg/ml for each polyphenols except (+)-catechinhydrate, caffeic acid, rutin hydrate (4 µg/ml) and quercetin (3 µg/ml). All standard solutions were stored in the dark at 5°C. The calibration curves of the standards were made by a dilution of the stock standards (five set of standard dilutions) with methanol to yield 1.0 - 5.0 µg/ml for GA, CH, VA, EC, PCA, EA, MC, KF; 0.5 - 4.0 µg/ml for CH, CA, RH, and 0.25 - 3.0 µg/ml for QU. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard. A solution of the extract at a concentration of 10 mg/ml was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were stored in the dark at low temperature (5°C). Spiking the sample solution with phenolic standards was done for additional identification of individual polyphenols. Prior to HPLC analysis, all solutions (mixed standards, sample, and spiked solutions) were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 minutes.

3. Results and Discussion

Medicinal plants play an essential role in primary healthcare as they are used to treat a wide range of diseases. Polyphenolic compounds that are commonly found in different plants are responsible for multiple biological activities like antioxidant, antimicrobial, anthelmintic and antidiarrhoeal activities [18, 19, 20, 21 22]. There has been growing interest in the involvement of reactive oxygen species (ROS) in different pathological conditions in the past few years. ROS produced *in-vivo* includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hypochlorous (HOCl).

Due to presence of hydroxyl groups [23], conjugated ring structures and carboxylic group [23, 24] phenolic compounds are capable to act as free radical scavenger [25], hydrogen donors, singlet oxygen quenchers [24, 26], metal chelator, lipid peroxidator [27] and other physiological properties.

Preliminary phytochemical screening of the present study revealed the presence of alkaloid, phenolic compounds, flavonoids, tannins, reducing sugar, glycoside and proteins (Table 1).

Table 1: Result of Phytochemical screening.

Constituents	Result
Reducing sugar	+
Combined reducing sugar	+
Tannins	+
Flavonoids	+
Saponin	-
Phenolic compounds	+
Gums	-
Steroids	-
Alkaloids	+
Glycoside	+
Proteins	+
Acidic compounds	-

Here, + = Presence; - = Absence

The most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds because of their possible usage in processed foods as a natural antioxidant [28] that have reached a new high in recent years [29]. *C. arvense* contains phenolic contents of ~25 mg GAE/gm of dry extract and it may be considered moderate amount of phenolic compounds. Within the antioxidant compounds, flavonoids with a large distribution in nature have been studied more comprehensively [30, 31, 32]. The positions of the substituents also affect the physiological properties of different flavonoids. The flavonols having ortho or para hydroxyl group in the 2-phenyl ring are known to have strong antioxidant properties, while free hydroxyl at the 5, 7- positions proved to have a pro-oxidant effect. A positive correlation between the content of flavonoids and the antioxidant capacity in plant extract has been found [33]. The flavonoids were found to have a content of ~ 22 mg QE/gm of the dry extract. The tannins are widely distributed in almost all plant foods, species [34]. Tannins possess anticarcinogenic and antimutagenic potentials as well as antimicrobial properties. Present study suggests that *C. arvense* contains very little amount (~ 5 mg GAE/gm of dry extract) of tannins.

Table 2: Antioxidant components of *C. arvense* (n = 5).

Phenolic	Total polyphenolic ^a	Total flavonoids ^b	Total tannin ^c
Result	25 ± 0.273	22 ± 0.424	5 ± 0.052

^{a, c} Expressed as mg gallic acid equivalent (GAE) /gm of dry extract,

^b Expressed as mg quercetin equivalent (QE) /gm of dry extract

Free radicals have been claimed to play an important role in affecting human health by causing several diseases including cancer, hypertension, heart attack and diabetes [35], atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [36]. Flavonoids and other phenolic compounds of plant origin have been reported as scavengers of free radicals [37]. The most appropriate, easy, fast and sensitive way to evaluate the antioxidant activity of the natural source is the use

of DPPH based free radical scavenging method [38, 39]. Smaller the antioxidant molecules, greater the antioxidant activity due to greater accessibility to the reaction site of the DPPH free radical [40]. In DPPH assay, the obtained IC_{50} value of $\sim 118 \mu\text{g/ml}$ (Fig. 1) may be due to the presence of lower amount of polyphenolic compounds and/or antioxidant compounds may be larger in size and shape. The reducing power of a compound may therefore serve as a significant indicator of its potential antioxidant activity [41]. Reductants react directly with peroxides and also with certain precursors of peroxide [42]. The number and position of hydroxyl group of phenolic compounds rule reducing power activity [43]. The data of reducing power assay showed that extract contains very less amount of reductant and consequently exhibits low reducing activity. The sample exhibited little H_2O_2 scavenging assay (Fig. 3). In super oxide radical scavenging assay, the extract exhibited moderate scavenging activity that was comparable to that of standard due to the presence of flavonoids and tannins (Fig. 4). Ferrous ion, commonly found in food systems, is well known as an effective pro-oxidant. Chelation property may afford protection against oxidative damage and iron-overload. Chelating ability of the extract was dose-dependent manner. It provides a strategy to avoid free-radical generation and iron-overload by chelation of metal ion. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions. NO works as an atypical neural modulator that is involved in neurotransmitter release, neuronal excitability and learning and memory. Additionally, evidence shows that NO modulates neurotoxin induced cell damage and is involved in neuronal cell death in Parkinson's disease and other neurodegenerative disorders such as Alzheimer disease. The extract showed scavenging activity of NO (IC_{50} , $100 \mu\text{g/ml}$) compared to that of standard (IC_{50} , $17 \mu\text{g/ml}$). In the reducing power assay the extract exhibited maximum absorption of 0.391 at a concentration of 1 mg/ml and the minimum absorbance of 0.034 at 0.1 mg/ml whereas the standard shows maximum absorption of 1.118 at a concentration of 1 mg/ml and the minimum absorbance of 0.340 at 0.1 mg/ml (Fig. 2). The reducing power of *C. arvense* was comparable to standard Butylated Hydroxy Toluene.

Table 3: Antioxidant activity of sample and standard (n = 5).

Test	IC_{50} of sample	IC_{50} of standard
DPPH assay	$118 \pm 0.17 \mu\text{g/ml}$	$12 \pm 0.011 \mu\text{g/ml}$
Hydrogen peroxide	$142 \pm 0.157 \mu\text{g/ml}$	$48 \pm 0.034 \mu\text{g/ml}$
Superoxide scavenging	$110 \pm 0.134 \mu\text{g/ml}$	$25 \pm 0.076 \mu\text{g/ml}$
Ferrous ion chelating	$92 \pm 0.124 \mu\text{g/ml}$	$17 \pm 0.110 \mu\text{g/ml}$
Nitric oxide scavenging	$100 \pm 0.181 \mu\text{g/ml}$	$14 \pm 0.134 \mu\text{g/ml}$

HPLC chromatogram of the extract showed the presence of *p*-coumaric acid, rutin hydrate, quercetin and kaempferol. The chromatographic separations of polyphenols in standard and ethanol extract are shown in Fig. 5 and 6, respectively. The content of each phenolic compound was calculated from the corresponding calibration curve and presented as the mean of five determinations as shown in Table 4.

Table 4: Contents of polyphenolic compounds in the ethanol extract of *C. arvense* (n = 5).

Polyphenolic Compound	Ethanol extract of <i>Cirsium arvense</i>	
	Content (mg/100 gm of dry extract)	% RSD
PCA	12.67	0.09
RH	30.41	1.26
QU	39.99	1.43
KF	14.72	1.01

The experimental results indicated that ethanol extract of *C. arvense* contained a moderate concentration of rutin hydrate, and quercetin (30.41 and 39.99 mg/100 gm of dry extract, respectively). Kaempferol and *p*-coumaric acid were also detected in low concentration in the extract (14.72 and 12.67 mg/100 gm of dry extract). The other polyphenolic compounds were not detected in the ethanol extract. Since the phenolic compounds have been of interest of health benefits, the present study could be a potential application to identify and quantify the polyphenolic compounds present in the plant.

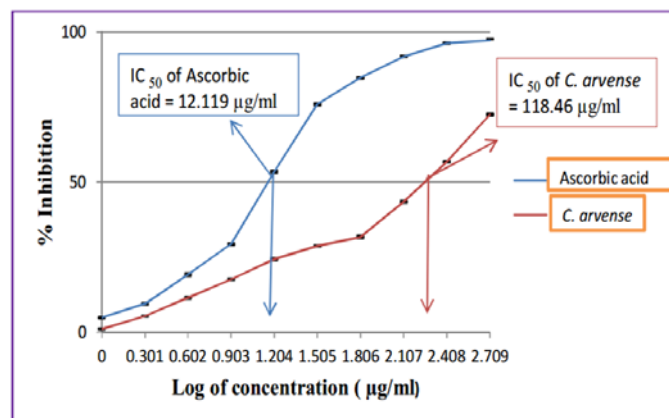


Fig 1: Comparison of IC_{50} value of Ascorbic acid and *C. arvense* in DPPH (SD = ± 0.17 , n = 3).

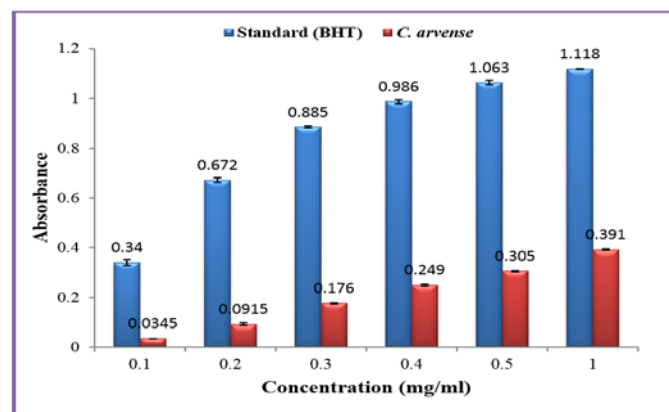


Fig 2: Comparison of reducing power of *C. arvense* with standard BHT scavenging assay (\pm DE, n = 3).

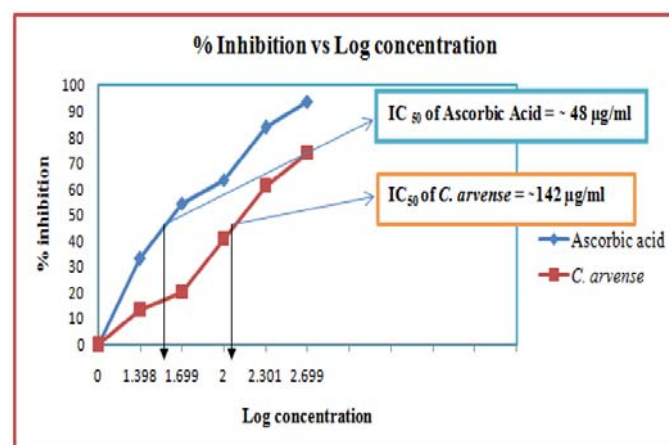


Fig 3: Comparison of Hydrogen peroxide Scavenging Assay of Ascorbic acid and *C. arvense* (SD = ± 0.157 , n = 3).

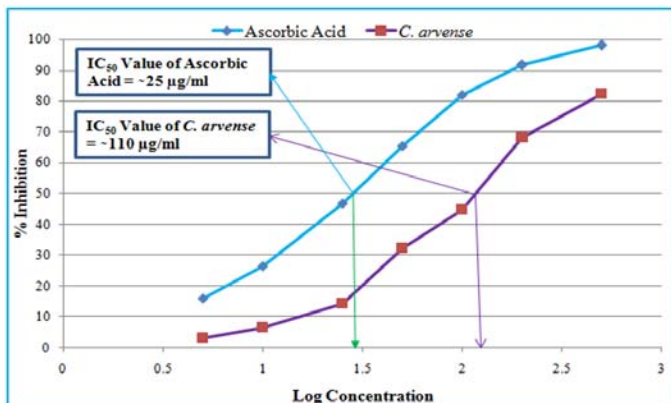


Fig 4: Comparison of superoxide scavenging activity of ascorbic acid and sample (SD = \pm 0.134, n = 3).

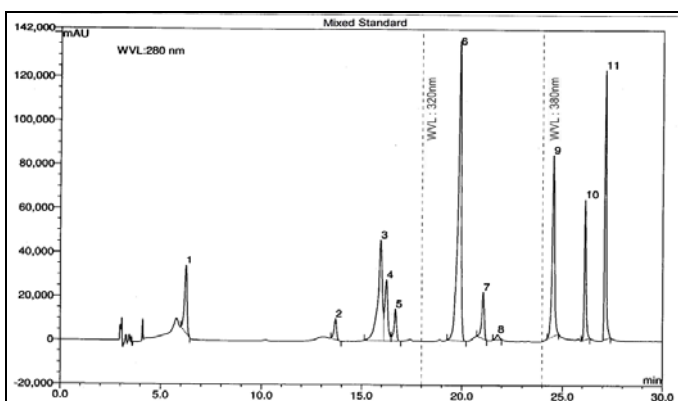


Fig 5: HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (-)-epicatechin; 6, p-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

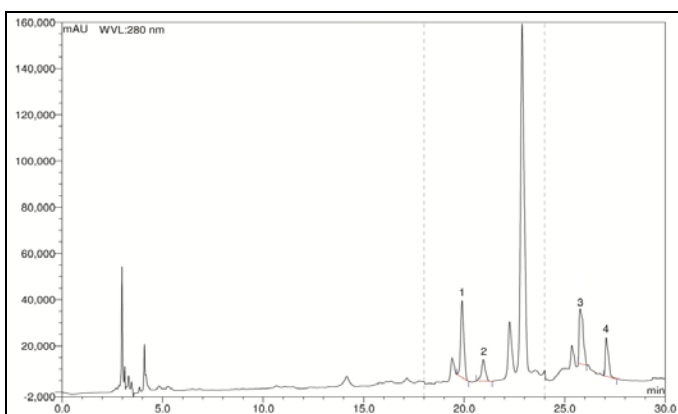


Fig 6: HPLC chromatogram of ethanol extract of *C. arvense*. Peaks: 1, p-coumaric acid; 2, rutin hydrate; 3, quercetin; 4, kaempferol.

4. Conclusion

On the basis of the results obtained in the present study, it can be concluded that phytochemical, pharmacological evaluation and HPLC analysis of the extract provides the strong evidence of the antioxidant property of the plant.

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