

Phytochemical Analysis, HPLC Profiling and Anti-oxidant Potential of *Euphorbia nivulia* Buch.-Ham.

Abstract

Euphorbia nivulia (EN) one of the members of *Euphorbiaceae* family, is a medicinal plant of Cholistan Desert (Punjab, Pakistan) that is traditionally used for a number of diseases. The plant is enriched with many phyto-constituents including flavonoids, triterpenes and polyphenols. In present study, crude extract as well as various fractions were assessed for phytochemical analysis, HPLC profiling, radical scavenging property and total phenolic and flavonoid contents. Hydro-alcoholic (70%) crude extract of EN was subjected to fractionation using different solvents including *n*-hexane, chloroform, butanol and aqueous. After performing the phytochemical screening and HPLC profiling, antioxidant activity was estimated by using DPPH and FRAP assays. Total flavonoid and phenolic contents were also estimated. HPLC analysis confirmed the presence of flavonoids and polyphenols like quercetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, chlorogenic acid, syringic acid and ferulic acid in crude extract as well as butanol and aqueous fractions. Results revealed that butanol fraction showed maximum phenolic (143.26 ± 2.65 mg/g GA/g) and crude extract showed maximum flavonoid (69.80 ± 1.212

mg/g Q) contents. Maximum antioxidant potential was displaced by butanol fraction which was $IC_{50}=0.04\pm 0.02$ by DPPH and 1193.77 ± 12.4 $\mu\text{mol TE/ml}$ by FRAP respectively. Current study is the first information about the HPLC profiling of phenolic compounds in *EN* and its antioxidant potential that could be a step forward towards ethno-pharmacological based phyto-medicine.

Keywords: *Euphorbia nivulia*, antioxidant, polyphenols, flavonoids, HPLC.

INTRODUCTION

Free radicals and/or reactive oxygen species (ROS) are produced by numerous physiological and biochemical metabolic processes in the body and are involved in some beneficial functions such as cell signaling, redox regulation and defense against pathogenic microorganisms. But their overproduction can damage the macromolecules of the body such as lipids, proteins and nucleic acid. This leads to the pathogenesis of degenerative diseases like aging, diabetes mellitus, Parkinson disease, Alzheimer disease, cataracts, rheumatoid arthritis, and cardiovascular as well as respiratory disorders (Phaniendra, Jestadi et al. 2015). Antioxidants are molecules that neutralize these harmful ROS by inhibiting oxidative chain reaction, preventing lipid peroxidation, reducing free radical concentration and chelating metal ions (Zhou and Yu 2004). Even in low concentrations antioxidants prevent or slow down the oxidation of the substrate significantly (Marques, Tóth et al. 2016); and provide shelter against many pathological and physiological processes caused by free radical reactions (Zhou, Yuping et al. 2015). Complex antioxidant system which constitute non-enzymatic antioxidants such as albumin, β -carotene, α -tocopherol, bilirubin, vitamin C, uric acid, flavonoids and glutathione and, enzymatic antioxidants (catalase, superoxide dismutase) present in the living organisms interact with reactive species which are deleterious to human life (Ramakrishna, Murthy et al. 2012). Some synthetic commercial antioxidants i.e. propyl gallate, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which have been widely used in pharmaceuticals and food industry for the preservation of food products, are found to be toxic and carcinogenic (Sasaki, Kawaguchi et al. 2002, Chen, Tan et al. 2015, Ye, Liang et al. 2015, Wattanasiritham, Theerakulkait et al. 2016). Impact of oxidative stress on human health and increasing safety concerns about synthetic antioxidants, shift the focus of the scientific community to search for new safe and feasible natural antioxidants. Vegetables, cereals, fruits, and mushrooms have been screened worldwide; however, medicinal plants are more potent source of natural antioxidants

(Cai, Luo et al. 2004, Li, Wong et al. 2008, Albouchi, Hassen et al. 2013, Baba, Malik et al. 2015). Thus, there is a growing interest in the antioxidant prospective of phytochemicals (Noda, Anzai et al. 1997). In this capacity, besides nutritional importance, medicinal plants can provide health benefits which are associated with their secondary metabolites such as polyphenolic and flavonoid compounds (Ksouri, Megdiche et al. 2007, Falleh, Msilini et al. 2013). These flavonoid and phenolic compounds are established universally as antioxidants or free radical scavengers and act as reducing agents, metal chelators and singlet oxygen quenchers (Chew, Goh et al. 2009). Owing to these properties they show antioxidant, anti-carcinogenic, anti-diabetic, anti-viral, antimicrobial, anti-inflammatory and anti-mutagenic activity (Ye, Liang et al. 2015). Plants/herbs belonging to Euphorbiaceae family are reported to have antioxidant bioactive molecules like triterpenes and flavonoids, both of which are reported to possess hepato-protective and antioxidant activity (Lanhers, Fleurentin et al. 1991, Evans, Evans et al. 2009). Out of approximately 195 of species, *Euphorbia nivulia* (EN) has been recorded from India. It is enrich in various phyto-constituents including flavonoids and polyphenolics. Both of which are reported to possess antioxidant activity (Sunitha, Nagaraj et al. 2001).

The present work was aimed to investigate the phyto-constituents and evaluate the antioxidant potency of the aerial parts of *EN*.

MATERIALS AND METHODS:

All chemicals /solvents used were of analytical grade and purchased from Sigma Aldrich , B.D. H. and Merck. Solid DPPH, ammonium molybdate, sodium phosphate, sulphuric acid, potassium ferricyanide, trichloroacetic acid, ferric chloride, methanol, ascorbic acid, quercitin, gallic acid, pyrogallol, brilliant green, ferrous sulphate, hydrogen peroxide, Folin-Ciocalteu reagent, sodium bicarbonate ,microplate reader, DMSO, spectrophotometer (DAD 8453 Agilent), laboratory centrifuge model (YJ03-043-4000).

Experimental design

Preliminary Phytochemical Screening

Preliminary qualitative phytochemical screening of EN crude extract (ENCr) as well as its four fractions i.e. Hexane (ENHF), Chloroform (ENCF), Butanol (ENBF) and Aqueous (ENAF) to

identify the phyto-constituents like alkaloids, glycosides, flavonoids, tannins, saponins and phenols, etc., was carried out by using standard conventional procedures (Baba and Onanuga 2011).

Antioxidant activity

Antioxidant activity of the crude extract and its various fractions was measured by DPPH reagent method. A mixture of 90 μ l of 0.3mM DPPH solution and 10 μ l of sample solution was mixed and incubated for 30 min at room temperature in dark in 96-well plate. Absorbance was measured at 517 nm by using LT-4500 96-well microplate reader, Labtech, UK. Absorbance of blank and standard were also measured. Both positive and negative controls were included in the triplicated assays. The percentage of total inhibition of DPPH radicals was measured by using the equation given below:

$$\text{Inhibition (\%)} = \left(\frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \right) \times 100$$

Antioxidant activities of the samples after suitable dilutions were determined and IC₅₀ values were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, (USA) (Shimada, Fujikawa et al. 1992).

Total Phenolic Content (TPC)

Total phenolic content was determined using Folin–Ciocalteu’s methods. Briefly colorimetric method was used with some modifications. An aliquot of 0.3 mL (various concentrations) extract was mixed with 2.25 ml Folin–Ciocalteu’s phenol reagent. After 5 min, 20 to 25 ml of Na₂CO₃ (6%) was added. The solution was allowed to stand for 90 min. After wards absorbance was measured at 725 nm. TPC was calculated using the standard calibration curve (ranging from 0-200 μ g/mL) and data was expressed as milligram gallic acid equivalent per gram of dry extract (mg of GAE/g of DE). Assays were carried out in triplicates (Chlopicka, Pasko et al. 2012).

Total flavonoid content

Total flavonoid content was measured by modified colorimetric method. A calibration curve was established using Quercetin as standard, 1mg/ml in methanol, ranging from 0 to 100 μ l (0-100 μ g). All solutions were made in methanol. 100 μ l of sample solution was mixed with 25 μ l of

1% sodium nitrite solution and allowed to stand for 5 min, followed by the addition of 10 μ l of 10% aluminum chloride solution and again allowed to react for 5 min. Finally, 35 μ l of 4% sodium hydroxide solution was added and mixture was diluted with 30 μ l of methanol. Absorbance was measured at 510 nm. TFC was calculated using the calibration curve equation and expressed as milligram quercetin equivalent per gram of dry extract (mg of QE/g of DE) (Wolfe, Wu et al. 2003).

HPLC Analysis of Phenolic Compounds

Hydrolysis of *Euphorbia nivulia* extracts was performed as described previously (Pak-Dek, Osman et al. 2011). The test samples (50 mg) of each extract were dissolved in 24 ml methanol and were homogenized. 16 mL distilled water was added followed by 10 mL of 6M HCl. Mixture was then thermostated for 2 hr at 95°C. The final solution was filtered using 0.45 μ m nylon membrane filter (Biotech, Germany) prior to high performance liquid chromatography (HPLC) analysis.

Separation of plant samples on gradient HPLC (LC-10A, SHIMADZU, JAPAN) was performed using shim-pack CLC-ODS (C118), 25cm X 4.6 mm, 5 μ m column. Chromatographic separation was carried out using as mobile phase gradient: A (H₂O: Acetic acid-94:6, pH = 2.27), B (acetonitrile 100%). The gradient used was 15% solvent B (0-15 min), 45% solvent B (15-30 min) and 100% solvent B (35-45 min) with 1 mL/min flow rate. UV- visible detector (λ max 280 nm) was used for separation of phenolic compounds. Identification of phenolic compounds was established by comparing the retention time and UV-Visible spectra of the peaks with those previously obtained by injection of standards. Quantification was performed by external calibration.

Assay of total antioxidant capacity (TAC)/ reducing power assay

Total antioxidant capacity was measured by ferric reducing ability of tissue (FRAP) method. This method is based on the ability of tissue in reducing Fe³⁺ to Fe²⁺ in the presence of TPTZ (2,4,6-tri[2-pyridyl]-s-triasine). The reaction between Fe²⁺ and TPTZ gives a blue complex with the maximum absorbance at 593 nm (Benzie and Strain 1996). Fe-reducing power of plant extract was determined by the method of Nile and Park with slight modifications (Nile and Park 2015). The reaction flask consists of 100 mL extract, 0.5% v/v dimethyl sulfoxide, and 5 mL of potassium ferric cyanide (1 mM) solution which was incubated for 30 min in water bath. Finally, the reaction was terminated using 3 mL of trichloro acetic acid (TCA) (10%) solution. The upper

portion of the reaction mixture (5 mL) was mixed with 5 mL distilled water, and 1 mL FeCl₃ solution (0.01%). The absorbance of the sample was measured at 593 nm spectrophotometrically after cooling for 10 min at room temperature, using an appropriate blank solution. The calibration curve was constructed using Trolox (100–2000 IM) and the results were expressed in μmol Trolox/g extract. All values were taken in triplicates, and mean ± SD values were calculated.

Results and Discussion

In last four decades, researchers have expressed a great zeal in phyto-constituents as potential source of medicinal agents because plants have been used as medicines from ancient times. Polyphenols or phenolic compounds constitute the largest group of secondary plant metabolites in plants possessing key properties for their adaptation to the environment (Oh, Trick et al. 2009, Kaurinovic and Vastag 2019). Thousands of phenolic compounds occur in the medicinal herbs. Typical phenolic compounds in the plant were of several major types, including phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids. For example, more than 4,000 kinds of flavonoids and hundreds of coumarins and lignans have been reported as naturally occurring constituents (Iwashina 2000, Xiao, Yang et al. 2000). In present study, preliminary phytochemical screening showed the presence of different compounds including phenols, flavonoids and tannins. Results for presence of various phytochemicals like alkaloids, glycosides, flavonoids, phenols, saponins, tannins and carbohydrates in crude as well as various fractions are presented in **Table 1**. Our findings showed that crude extract and polar fractions contain phenols, flavonoids and tannins. These results are again confirmed by the TPC, TFC and HPLC analysis and correspond with the available research data of *EN* and other *Euphorbia* species (Thorat and Bolli 2017, Younus, Hasan et al. 2019). Our findings showed that polar fractions, in current study, exhibited highest phenolic and flavonoid content. These results are in accordance with previous data that showed that phenolic compounds being polar in nature are found in polar fractions (Mahayasih, Elya et al. 2018). In current study, ENBF exhibited highest phenolic content (143.26±2.65 mg/g GA) followed by ENCr (125.6±1.32 mg/g GA), ENAF (96.53±2.01 mg/g GA), ENCF (38.27±3.21 mg/g GA) and ENHF (15.49±1.92 mg/g GA) as shown in **Figure 2 (A)**. Unlike

phenolic content, highest flavonoid content was exhibited by ENCr as shown in **Figure 2 (B)**. It was 69.80 ± 1.212 mg/g Q followed by 41.26 ± 1.234 , 31.65 ± 1.224 , 26.85 ± 0.934 and 19.63 ± 1.136 mg/g Q possessed by ENCF, ENAF, ENBF and ENHF respectively. In the same way, *EN* has very good antioxidant activity as depicted by DPPH and FRAP assays. Results of DPPH are shown in **Figure 1 (A)**. Butanol fraction was found to have highest radical scavenging activity followed by ENCr, ENCF, ENAF and ENHF with IC_{50} values of 0.04 ± 0.02 , 0.14 ± 0.019 , 0.29 ± 0.007 , 0.338 ± 0.021 and 0.58 ± 0.008 $\mu\text{g/ml}$ respectively when compared with rutin as standard (0.014 ± 0.009 $\mu\text{g/ml}$). Ferric reducing ability power assay was employed to investigate antioxidant potential of *EN* extract and its various fractions. The FRAP values expressed as $\mu\text{mol Trolox equivalents (TE/g)}$ of dry weight of extract are shown in **Figure 1 (B)**. Among the fractions and crude extract of *EN*, butanol fraction showed maximum FRAP value (1193.77 ± 12.4 $\mu\text{mol TE/ml}$) while minimum was showed by hexane fraction (365 ± 9.28 $\mu\text{mol TE/ml}$). While, ENCr, ENAF and ENCF showed FRAP values 708.810 ± 10.30 , 708.320 ± 13.0 and 366.75 ± 5.76 $\mu\text{mol TE/ml}$ respectively. These are widely used methods to evaluate the free radical scavenging ability and reducing power of various plant species in short period of time (Ebrahimzadeh, Ehsanifar et al. 2009). According to results of both assays the polar fraction and butanol fraction (ENBF) showed maximum antioxidant potential. Chromatographic fingerprinting and quantitative sketching of *EN* crude extract and its various fractions was done. HPLC confirmed the presence of a number of polyphenols in crude as well as butanol and aqueous fractions of *EN*. These include quercetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, chlorogenic acid, cinnamic acid, coumaric acid, syringic acid and ferulic acid. HPLC Chromatograms of *EN* crude extract, butanol and aqueous fractions are depicted in **Figure 3, 4 and 5** while quantity of each polyphenolic compound is mentioned in **Table 2, 3 and 4** respectively. Moreover, our results showed a direct relationship between antioxidant activity and total phenolic content which proved that antioxidant activity of plants may be attributed to the presence of phenolic compounds in them (Velioglu, Mazza et al. 1998, Sun, Chu et al. 2002). Polyphenols have got high importance due to their antioxidant ability to prevent oxidation (Khasawneh, Elwy et al. 2011, Jaafar, Ibrahim et al. 2012). Pharmacological activities of many plants, fruits and vegetables are due to the presence of natural antioxidants especially of these phenolic acids and flavonoids. Antioxidants significantly

decrease the adverse effect of reactive species and at the same time antioxidant therapy has great impact in the treatment of many other diseases including microbial infections, cancer, AIDS and heart diseases (Di Carlo, Mascolo et al. 1999, Yao, Jiang et al. 2004, Mradu, Saumyakanti et al. 2012).

Conclusion

The results of this study are first information about the HPLC profiling of phenolic compounds in *EN*. Present study also revealed that *EN* is very rich in polyphenolic compounds including phenolic acids and flavonoids with excellent antioxidant properties which may be a potential therapeutic agent for various degenerative diseases. Moreover, the study gives basis for the standardization, development and isolation of these useful phenolic compounds.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Legends to Tables

Table 1 Preliminary phytochemical evaluation of various fractions of *EN* extract

Table 2 Qualitative and quantitative analysis of phenolic compounds in ENCr fraction using HPLC

Table 3 Qualitative and quantitative analysis of phenolic compounds in ENBF fraction using HPLC

Table 4 Qualitative and quantitative analysis of phenolic compounds in ENAF fraction using HPLC

Legends to Figures

Figure 1 Antioxidant activity using DPPH and FRAP assays of EN crude extract and its various fractions

Figure 2 Total phenolic and flavonoid content of EN crude extract and its various fractions

Figure 3 HPLC chromatogram of ENCr fraction

Figure 4 HPLC chromatogram of ENBF fraction

Figure 5 HPLC chromatogram of ENAF fraction

List of Tables

Table 1

Test	ENCr	ENHF	ENCF	ENBF	ENAF
Carbohydrates					
<i>Fehling's Test</i>	+	-	+	+	+
Alkaloids					
<i>Hager's Test</i>	+	-	-	-	-
<i>Mayer's Test</i>	+	-	-	-	-
<i>Wagner's Test</i>	+	-	-	-	-
Glycosides					
<i>Keller Kiliani Test</i>	+	-	-	-	-

Phenolic Compounds					
<i>FeCl₃ Test</i>	+	-	+	+	+
Flavonoids					
<i>Alkali Test</i>	+	-	+	+	+
Tannins					
<i>FeCl₃ Test</i>	+	-	+	+	+
Saponins					
<i>Froth Test</i>	-	-	-	-	-

Table 2

Compounds	RT (min)	Area (mV.s)	Quantity (ppm/mg)
Quercetin	3.36	27.85	1.47
Gallic acid	4.58	27.59	0.99
Caffeic acid	12.85	9.55	0.43
Syringic acid	16.26	9.78	0.24
m-coumaric acid	20.14	16.67	0.19
Ferulic acid	22.15	17.43	1.25
Cinnamic acid	25.38	2.97	0.11

Table 3

Compounds	RT (min)	Area (mV.s)	Quantity (ppm/mg)
Quercetin	3.26	37.86	2.13
Gallic acid	4.6	61.19	2.19
Caffeic acid	12.49	33.71	1.55
Benzoic acid	14.34	24.51	2.59
Chlorogenic acid	15.56	23.83	1.85
Syringic acid	16.85	18.9	0.47
p-coumaric acid	17.93	52.81	0.68
m-coumaric acid	20.38	83.84	1.58
Ferulic acid	22.43	17.18	1.22
Cinnamic acid	25.44	46.69	1.63

Table 4

Compounds	RT (min)	Area (mV.s)	Quantity (ppm/mg)
Quercetin	3.23	35.47	1.87
Gallic acid	4.48	34.6	1.24
Caffeic acid	12.2	17.74	0.81

Vanillic acid	13.58	19.62	1.22
Benzoic acid	14.7	14.97	1.57
Chlorogenic acid	15.32	11.82	0.92
Syringic acid	16.14	13.04	0.32
Ferulic acid	22.02	34.57	2.48

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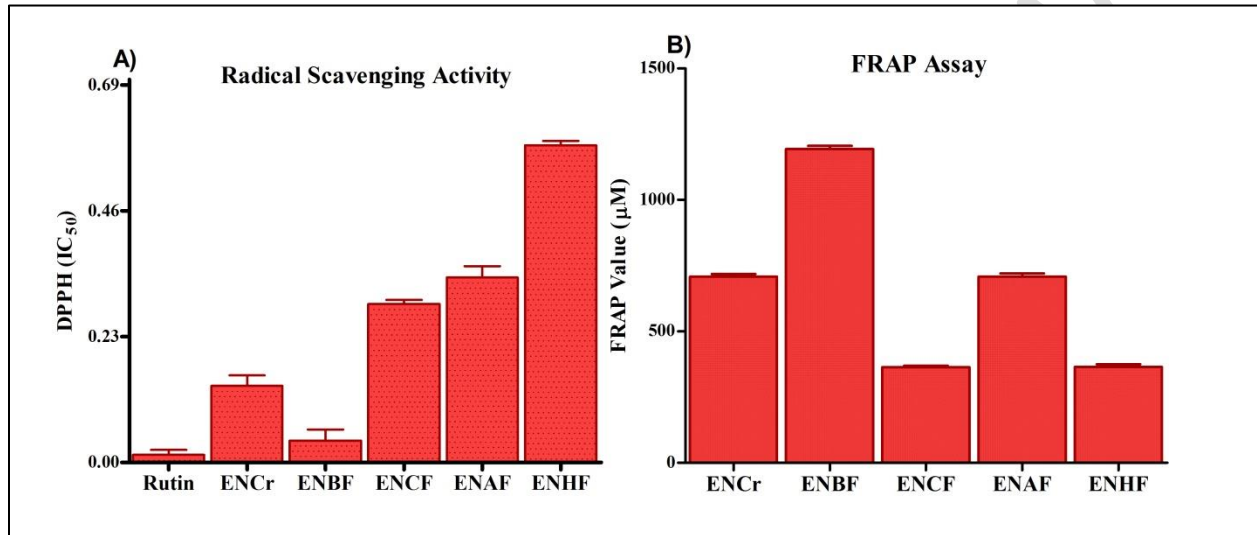


Figure 1

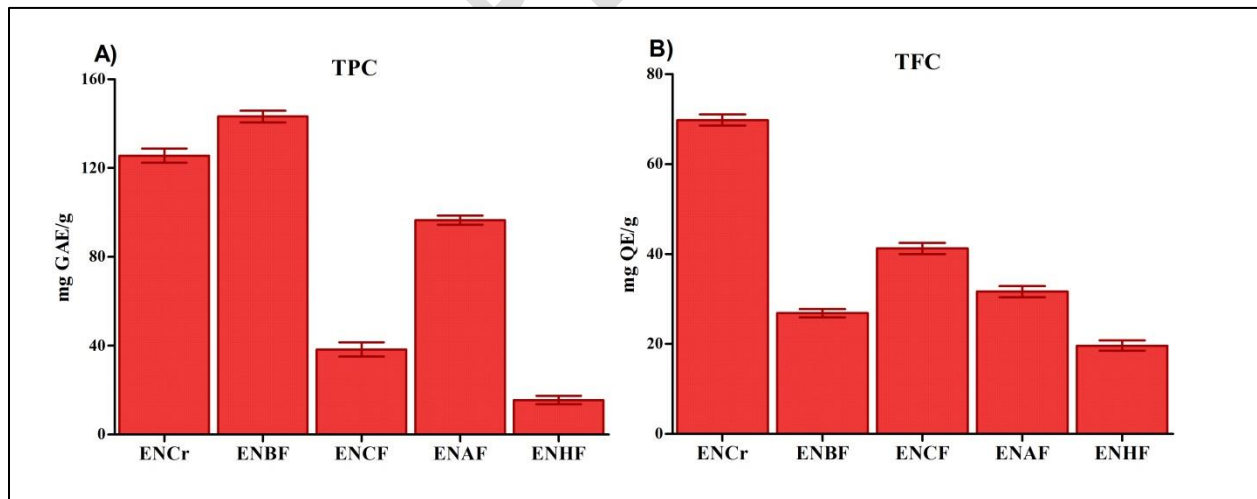


Figure 2

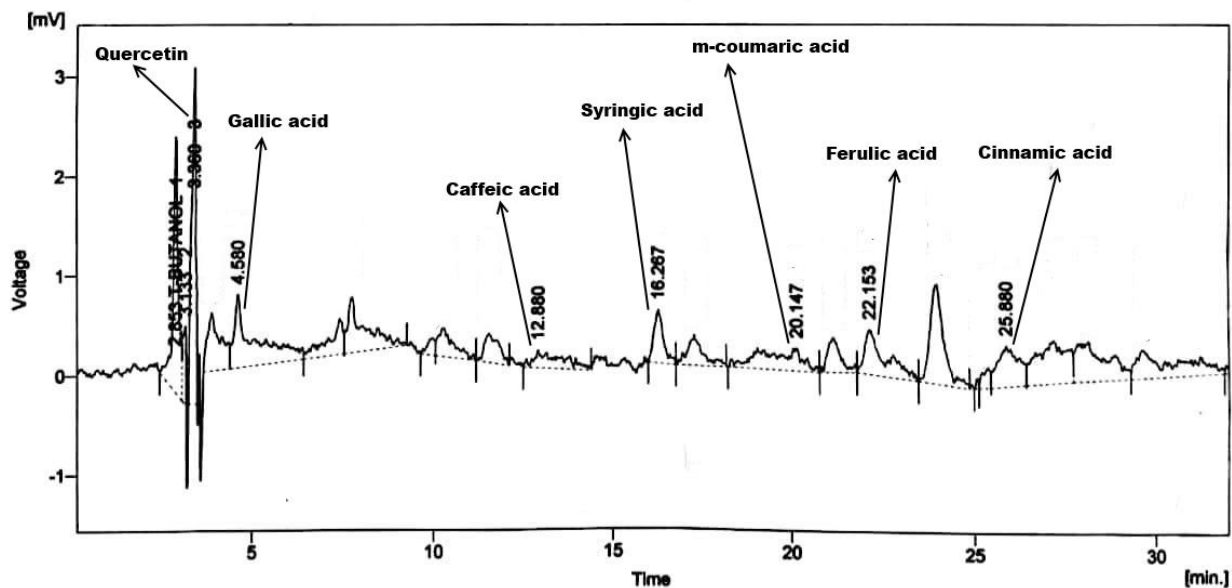


Figure 3

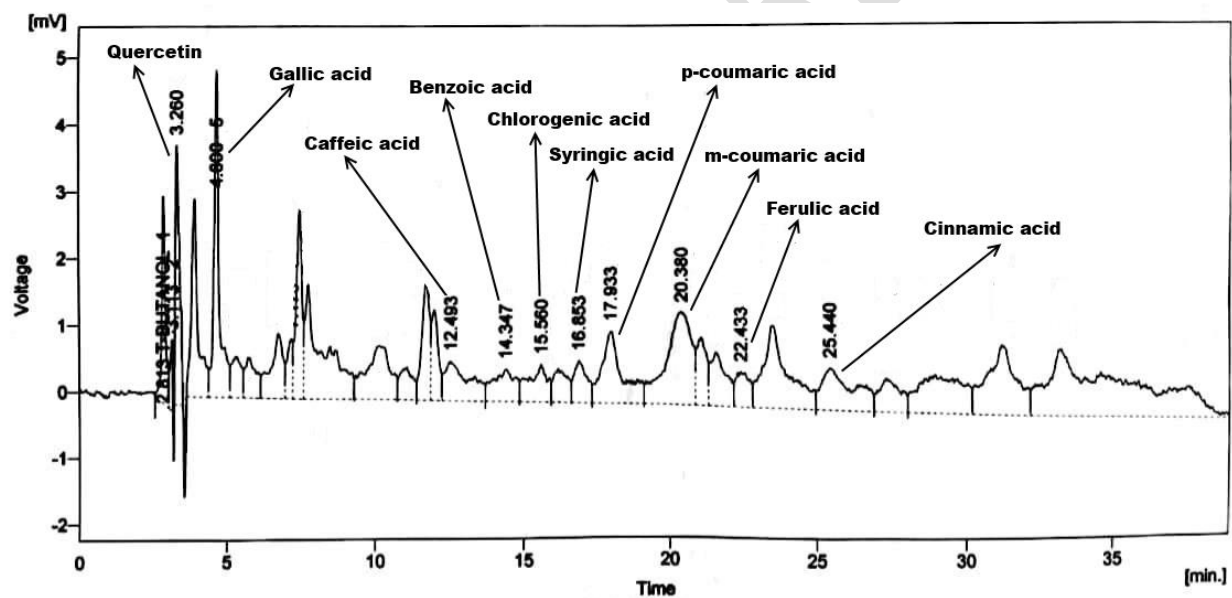


Figure 4

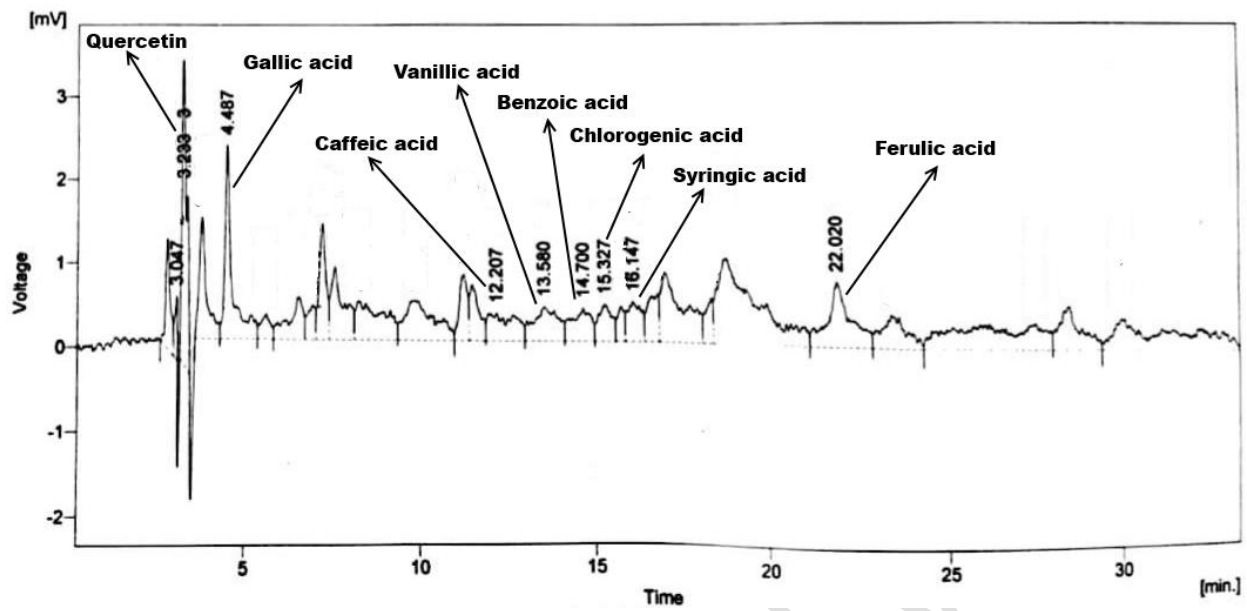


Figure 5

UNDER PEER REVIEW