

HPLC profile of bioactive compounds present in *Oleauropeae*, *Myrtuscommunis* and *Monotheabuxifolia* and their antibacterial activities

Abstract

Aims: The aqueous extract of *Oleauropeae*, *Myrtuscommunis* and *Monotheabuxifolia* were screened for antibacterial activity, total phenolic content, polyphenol, chlorophyll a, chlorophyll b, β -carotene and lycopene.

Design of the study: The study is composed of HPLC profile of bioactive compounds in selected plants.

Place and duration of the study: Biochemistry Lab Department of Chemistry University of Malakand Pakistan. The study duration was from September 2015 to August 2016.

Methodology: The antibacterial activities of the extracts were determined using agar well diffusion, minimum inhibitory concentration and minimum bactericidal concentration methods. The total phenolic contents and polyphenol were determined by FollinCiocalteu reagent method. The extract was subjected to HPLC analysis for antioxidants concentration.

Results: The zone of inhibition against *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis* and *Bacillus cereus* were 1.82, 1.52, 1.72 and 2.46 cm for *Oleauropeae* extract, 1.51, 2.06, 1.98, 1.79 cm for *Myrtuscommunis* extract, 1.98, 2.28, 2.36, 1.62 cm for stem extract and 1.78, 2.05, 1.80, 1.77 cm for root extract of *Monotheabuxifolia* respectively. Morin, Quercetin and Chlorogenic acid were detected at retention times; 12.518, 10.022 and 6.305 minutes respectively in *Oleauropeae* extract. Morein and Hydroxy benzoic acid were detected at 12.858 and 6.079 minutes retention time respectively in *Myrtuscommunis* extract. Chlorogenic acid, Quercetin, Morin, Pyrogallol and Phloroglucinol were detected in root extract of *Monotheabuxifolia* while in its stem, Chlorogenic acid, Quercetin, Morin, Rutin, Mandalic acid, Phloroglucinol and Hydroxy benzoic acid were detected.

Conclusion: A number of bioactive compounds were detected in the selected plants and on the basis of findings it was concluded that selected plants could be used medicinally.

Keywords

Olea europaea, *Myrtus communis*, *Monothea buxifolia*, total phenolic contents, polyphenols, antioxidants, pigments, inhibition.

1. Introduction

Prehistoric men have used medicinal plants for the treatment of various diseases [1]. Medicinal plants may be defined as those plants which are sources of some drugs or precursors used to treat, mitigate or to prevent a disease or to bring about a change in physiological and pathological processes. Medicinal plants have an important role in developing countries as they are sources of money on one hand and improving their quality of life and health on the other hand. According to reports 80% of human population prefer herbal medicines for the treatment of ailments [2].

Different antioxidants are obtained from different plants which provides a powerful defense to free radicals in human body. Damage to cell caused by free radicals is believed to play a central role in the aging process and in diseases progression. Antioxidants are our first line of defense to the damage caused by these free radicals and are critical for maintaining optimum health and well-being [3]. Chlorophyll content is one of the important component of photosynthetic activity. It is of particular importance in agriculture and is an indicator of photosynthetic activity [4].

Olive (*Olea europaea*, family *Oleaceae*) is an ever green tree grown for its edible fruits, which yield oil and are also marketed as table or pickled olives [5]. The olive tree (*Olea europaea*) is one of the most important fruit trees in the world. Almost 90% of all olive trees are planted in Mediterranean countries [6]. *Myrtus communis* Linn (Family *Myrtaceae*) is an aromatic evergreen perennial shrub or small tree [7]. 8 to 2.4m in height with small foliage and deep fissured bark. It is native to Southern Europe, North Africa and West Asia [8]. *Monothea buxifolia* is a broad-leaved evergreen small tree belonging to the family Sapotaceae. This species is found in the hilly regions of Afghanistan and in Northern Pakistan. [9].

In the present study, the antioxidant activity, antibacterial activity, total phenolic content, polyphenol, pigments that include chlorophyll a, chlorophyll b, β -carotene and lycopene were determined for the selected medicinal plants. Certain phenolic compound were determined

MBC High performance liquid chromatography (HPLC) in *Olea europaea*,
Myrtus communis and *Monothea buxifolia*.

2. Material and method

2.1. Collection of plant materials

Olea europaea plant was collected from Village Ouch lower Dir KPK Pakistan,
Myrtus communis plant stem was collected from the local regions of lower Dir KPK, Pakistan
and *Monothea buxifolia* plant was collected from Village Kityari (Safara Mountain), Lower
Dir KPK Pakistan. The plant samples were washed with clean water and kept in a shady
place for drying at room temperature for 3 weeks. The dried plants then were powdered by
grinding with the help of mechanical grinder (locally made). The powdered plants were
dissolved in 90% methanol and kept it for one week with constant shaking (at room
temperature and 300 rpm) at different intervals. After one week the mixtures were filtered
through filter paper. The extract was obtained in semisolid form using rotary evaporator. It
was then kept in open atmosphere to evaporate the remaining solvent. The crude extracts
were kept in a refrigerator at a temperature at 4°C.

2.2. Antibacterial activity

Amoxicillin (Oxide UK) was used as a standard drug for the antimicrobial activity. Two
Gram positive [*Bacillus cereus* (ATCC8035), *Enterococcus faecalis* (ATCC29212)] and two
Gram negative [*Klebsiella pneumonia* (ATCC13833), *Escherichia coli* (ATCC25922)]
bacterial strains were used in this study. Powders of the antibiotic amoxicillin (purity 100%)
were accurately weighed and dissolved in sterile distilled water that give appropriate dilutions
yield (0.03g/15ml) the required concentration. The standard solutions were stored at -20°C.
The growth media nutrient agar and the petri dishes were sterilized at 121°C for 3 hours in
autoclave. Then four petri dishes were taken and the agar nutrient solution was transferred to
each petri dish. Holes were made by cork borer in each agar plate at a proper distance from
one another and standard antibiotic and crude extracts of watercress were introduced in the
holes by using the micropipette. Now inoculate each agar plate with different bacterial strains
with the help of cotton swab. After 24 hours of incubation period at 37°C in incubator the
antimicrobial activities were determined from the diameter of the inhibition zone formed by
the extracts of the selected plants around the holes which was then compared with the
inhibition zone created by standard antibiotic (amoxicillin). The Minimum Inhibitory
Concentration (MIC) was found by preparing five different solution of all three plants 0.02,

0.04, 0.06, 0.08 and 0.1 (making 10 ml of such solution). Now nutrient broth solution was prepared and 9 ml of broth solution was taken in five test-tube. After that add 1, 1 ml from each of the five extract solution to the test-tube were added and one type of bacterial strain to these five test tube were also added. The test tube were placed in incubator for 24 hours and after this minimum inhibitory concentration against selected bacteria were found. The same tubes were used for the determination of minimum bactericidal concentration (MBC) and after 3 days MBC was estimated from changes in turbidity in the test tubes.

2.3.Determination of free radical scavenging activity

First of all 0.039gm of DPPH was accurately weighed with the help of digital balance and dissolve in the distilled methanol to give an appropriate solution of 100ml (0.039gm/100ml) of the required concentration.

1 gm of each extract was accurately weighed and dissolved separately in the distilled methanol to give the required solutions of 20 ml. Then this solution was stored as plant extract stock solutions. One solution of 5ml pure distilled methanol + 0ml plant sample solution was also taken which was termed as control solution. Then 1 ml of stock solution of DPPH was added to the control solution and diluted solution of extract solution. Then all these solutions were kept in dark place for 30 minutes. After this their absorbance reading was taken at 517 nm with the help of spectrophotometer (PG instruments, T60) by the given formula;

$$\%RSA = \frac{(\text{Control solution absorbance} - \text{plant sample solution absorbance}) \times 100}{(\text{Control solution absorbance})} \quad (1)$$

2.4.Total Polyphenol Assay

Total polyphenol was measured using Follin-ciocalteu method [10]. The 1 g of each extract was mixed with 2 ml of Follin-ciocalteu reagent and 20 ml of H₂O and incubated at room temperature for 3 minute. Then added 10 ml of 20 % of Na₂CO₃ to the mixture. Then total polyphenol was determined after 1 hour of incubation at room temperature .The absorbance of the resulting mixture was measured at 765 nm .Quantification was done with respect to the standard curve of Gallic acid. The result was expressed as GAE, mg /g of dry weight.

2.5.Determination of Pigments Contents

Pigments such as Chlorophyll α , Chlorophyll β , β -carotene and Lycopene were determined by Spectrophotometer. 1 g of each sample was mixed with acetone-hexane (4:6) and then placed on shaker for 1 hour. After shaking, the absorbance were noted at 453, 505, 645 and 663 nm respectively and the results were expressed as mg/100 ml.

2.6.Determination of phenolic contents through HPLC

For the preparation of extracts of each plant, 10 mL of methanol and water were mixed with 1 gram plant powdered sample. These mixtures were then heated on hot plate at 70°C for 1 hour. After cooling, the samples were filtered with Whattman filter paper then these filtered samples were centrifuged at 6000 rpm for 15 minutes, after centrifugation it was again filtered through syringe filters and put into HPLC vials. The vials containing the samples were labelled with proper code. The determination of phenolic compounds were carried out by means of Agilent 1260 (HPLC) system. The separation was achieved via Agilent Zorbax Eclipse C18 column. The identification was performed by using retention times, available standards and UV spectra. Quantification of the identified compounds was on the basis of percent peaks values. HPLC chromatogram of each plant is shown in the figure 3. Only polyphenolic compound was identified. The detailed identification of compound with its peaks position in chromatogram and retention time (RT) of the standard phenolic compounds is given in figure 2. The injection volume was 10 micro liters and lambda max was 320 nm.

3. Result

3.1.Antimicrobial activities of the extracts

The extract of *Olea europaea*, *Myrtus communis* and *Monothea buxifolia* were tested for its antimicrobial activity and their values are shown in the table 1.

The MIC and MBC of *Olea europaea*, *Myrtus communis* and *Monothea buxifolia* against *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis* and *Bacillus cereus* are given below in table 2.

3.2.Antioxidant activity

The free radical scavenging activities of the extract of *Olea europaea*, *Myrtus communis* and *Monothea buxifolia* are shown in table 3.

3.3.Total Polyphenols

Total polyphenol was measured using Follin-ciocalteu method. Quantification was done with respect to the standard curve of Gallic acid. The result was expressed as GAE, mg /g of dry weight is found by formula 2. The results have been shown in table 4.

$$TPC = \frac{C_{cal} * D * V(ml)}{wt} \quad (2)$$

3.4.Determination of Pigments Contents

The pigment contents were determined by the simple method devised by Nagata and Yamashita (Nagata *et al.*,2009). The results obtained have been shown in table 5. The equation devised by them is given as follow:

$$\text{Chlorophyll a (mg/100ml)} = 0.999A_{663} - 0.0989A_{645} \quad (3)$$

$$\text{Chlorophyll b (mg/100ml)} = -0.328A_{663} + 1.77A_{645} \quad (4)$$

$$\text{Lycopene (mg/100ml)} = -0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453} \quad (5)$$

$$\beta\text{- Carotene (mg/100ml)} = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453} \quad (6)$$

3.5.Determination of phenolic contents through HPLC

The extracts were subjected to HPLC analysis for antioxidants concentration. Morin, Quercetin and Chlorogenic acid were detected at retention times were 12.518, 10.022 and 6.305 respectively for *Oleauropeae*, Morein and Hydroxy benzoic acid were detected at 12.858 and 6.079 retention time respectively for *Myrtuscommunis*, Chlorogenic acid, Quercetin, Morin, Pyrogallol and Phloroglucinol at 6.212, 10.506, 12.997, 28.245 and 35.598 retention time were detected in root extract and while in stem, Chlorogenic acid, Quercetin, Morin, Rutin, Mandalic acid, Phloroglucinol and Hydroxy benzoic acid at 6.611, 10.480, 22.495, 30.827, 35.236 and 36.221 retention times respectively were detected in *Monothea buxifolia*, these are found by formula as

$$Cx = \frac{Ax * Cs \left(\frac{\mu g}{ml} \right) * V(ml)}{As * Sample \text{ (wt in g)}} \quad (7)$$

Where

C_X = Concentration of unknown

176 A_s = Peak area of standard

177 A_x = Peak area of unknown

178 C_s = Concentration of standard

179 **4. Discussion**

180 As new drug-resistant bacterial strains emerges, herbal drugs are being looked as very
181 important source for discovery of new agents for treating various ailments related to bacterial
182 infections. We conducted a prospective observational study of antibacterial activity of
183 aqueous extract. This standard showed significant zone of inhibition against all bacterial
184 strains used as compared to the extracts.

185 It was found that the free radical-scavenging activities of the extracts increased with
186 increasing concentration which is clear from the figure. The present study correlates with the
187 study of Ozenet *al.*[11] in which he investigate the antioxidant activity of the plant extracts
188 both in vivo and in vitro.

189 The total polyphenol were measured using Follin-ciocalteu method [10], the absorbance of
190 the resulting mixture was measured at 765 nm .Quantification was done with respect to the
191 standard curve of Gallic acid. The result was expressed as GAE, mg /g of dry weight. This
192 study correlates with Silveriraet *al.*[6], and Mazandarani *et al.*, [12]in which the total
193 polyphenol contents were determined for the extract obtained after homogenizing 1 g of
194 frozen watercress leaves with 3 mL of methanol/water solution.

195

196 Pigments such as Chlorophyll α , Chlorophyll b, β -carotene and Lycopene with the help of
197 spectrophotometer. These pigments showed absorbance at 453,505,645 and 663. This study
198 correlates with Gonclaveset *al.*[13], in which he determined different pigments at three
199 different temperatures.

200 The HPLC chromatogram of all plants extractsshowed different Polyphenolic compounds
201 that were identified through comparing with standard chromatogram containing eight
202 standard antioxidant. These compounds were shown with their respective peak position,
203 retention time, and wavelength and identification references. The different chromatograms
204 are shown in figure 1-5 along with standard. This study correlates with Boligonet *al.* [14], in
205 which he study the phenolic contents through HPLC.

5. Conclusion

In this study, antibacterial activity, total phenolic content, polyphenol, chlorophyll a, chlorophyll b, β -carotene and lycopene and the phenolic compound through HPLC were determined in aqueous extract of *Oleauropeae*, *Myrtus communis* and *Monothea buxifolia*. The antioxidant activity of the extract were also determined. *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis* and *Bacillus cereus*, were screened by three methods, an agar well diffusion method, minimum inhibitory concentration and minimum bactericidal concentration. The extracts were subjected to HPLC analysis for antioxidants concentration. Morin, Quercetin and Chlorogenic acid were detected at retention times were 12.518, 10.022 and 6.305 respectively for *Oleauropeae*, Morein and Hydroxy benzoic acid were detected at 12.858 and 6.079 retention time respectively for *Myrtus communis*, Chlorogenic acid, Quercetin, Morin, Pyrogallol and Phloroglucinol at 6.212, 10.506, 12.997, 28.245 and 35.598 retention time were detected in root extract and while in stem, Chlorogenic acid, Quercetin, Morin, Rutin, Mandalic acid, Phloroglucinol and Hydroxy benzoic acid at 6.611, 10.480, 22.495, 30.827, 35.236 and 36.221 retention times respectively were detected in *Monothea buxifolia*.

The *Oleauropeae*, *Myrtus communis* and *Monothea buxifolia* extracts in the form of aqueous extracts exhibit antibacterial, antioxidants and other phytochemicals and as a potent candidate is used as medicinal plant. The present study will open a new research area to be investigated in future.

References

- [1]. Ahmad R, Srivastava SP, Maurya R, Rajendran SM, Arya KR, Srivastava AK.. Mild anti-hyperglycaemic activity in *Eclipta alba*, *Berberis aristata*, *Betula utilis*, *Cedrus deodora*, *Myristica* Fragrance and *Terminalia chebula*. Indian J Sci Technol. 2008;1(5): 1-6.
- [2]. Tahira JJ, Khan SN, Suliman R, Anwar W. Weed flora of curcuma longa fields of district Kasur, Pakistan. Pak J Weed Sci Res. 2010;16:241-46.
- [3]. Percival M. Antioxidants. Clin Nutr Insights. 1998;31:1-4.
- [4]. Bojovic B, Stojanovic J. Chlorophyll and carotenoid content in wheat cultivars as a function of mineral nutrition. Arch Biol Sci. 2005;57:283-90.

- [5].Mahesh B, Satish S.Antimicrobial activity of some important medicinal plant against plant and human pathogens.World J Agr Sci.2008;4:839–43
- [6].Srivastava J, Lambert J, Vietmeyer N. Medicinal plants: An expanding role in development. World Bank Technical Report No: 320. 1996.
- [7].Ahmad I, Ibrar M, Barkatullah AN. Ethnobotanical study of Tehsil Kabal, Swat District, KPK, Pakistan. Pak J Bot.2011;20:1-9
- [8].Kirtikar KR, Basu BD. Indian Medicinal Plants.International Book Distributors, Dehra Dun, India. 1988:1040-42
- [9].Silverira AC, Araneda C, Hinojosa A, Escalona VH. Effect of non-conventional modified atmosphere packaging on fresh cut watercress (*Nasturtium officinale* R.Br.).Postharvest Biol Technol.2014;92:114–20
- [10].AndressaB, Lopes GC, De Mello JCP. Application and Analysis of the Follin-ciocalteu Method forthe Determination of the Total Phenolic Content from*Limoniumbrasiliense*L. Molecules.2013;18:6852-65.
- [11].Ozen T (2009).Investigation of antioxidant properties of *Nasturtium Officinale*(Watercress) Leaf extracts. *ActaPoloniae Pharm. Drug Res.* 66:187-93.
- [12].Mazandarani M, Momeji A, Moghaddam PZ.Evaluation of phytochemical and antioxidant activities from different parts of *Nasturtium officinale* R.Br. in Mazandaran.Iran J. Plant Physiol.2013;3:659-64
- [13].Gonclaves EM, Cruz RMS, Abreu M, Brandao TRS,Silva CLM. Biochemical and colour changes of watercress (*Nasturtiumofficinale* R.Br) during freezing and frozen storage.J Food Eng.2009;93:32–9
- [14].Boligon AA, Janovik V, Pivetta CR, Pereira, Da Rocha JBT,Athayde ML. HPLC Analysis of Polyphenolic Compounds and Antioxidant Activity in *Nasturtium officinale*. Int J Food Prop.2013;16: 61-9

263 **Table 1:**Antibacterial activity of aqueous extracts of the selected plants

Plant Extract	Bacterial Strain	Mean of zone diameter of inhibition (cm)	Zone diameter of inhibition (cm) of standard
<i>Olea europeae</i>,	<i>Escherichia coli</i>	1.82	3.92
	<i>Klebsiella pneumonia</i>	1.52	2.3
	<i>Bacillus cereus</i>	2.46	3.58
	<i>Enterococcus faecalis</i>	1.72	3.15
<i>Myrtus communis</i>	<i>Escherichia coli</i>	1.51	3.66
	<i>Klebsiella pneumoniae</i>	2.06	3.78
	<i>Enterococcus Faecalis</i>	1.98	2.48
	<i>Bacillus cereus</i>	1.79	4.06
<i>Monothea buxifolia</i>(Stem)	<i>Escherichia coli</i>	1.98	2.56
	<i>Klebsiella pneumonia</i>	2.28	1.68
	<i>Bacillus cereus</i>	1.62	2.4
	<i>Enterococcus faecalis</i>	2.36	2.42
<i>Monothea buxifolia</i>(root)	<i>Escherichia coli</i>	1.78	2.8
	<i>Klebsiella pneumonia</i>	2.05	3.7
	<i>Bacillus cereus</i>	1.77	3.54
	<i>Enterococcus faecalis</i>	1.80	2.8

264

265 **Table 2:** MIC and MBC of aqueous extracts of selected plants

Plant Extract	Parameter	<i>Escherichia coli</i>	<i>Klebsiella pneumonia</i>	<i>Enterococcus faecalis</i>	<i>Bacillus cereus</i>
<i>Olea europeae</i>	MIC	0.06	0.04	0.08	0.06
	MBC	0.10	0.08	0.10	0.08
<i>Myrtuscommunis</i>	MIC	0.1	0.06	0.04	0.08
	MBC	0.06	0.08	0.08	0.1
<i>Monotheabuxifolia</i>(Stem)	MIC	0.02	0.06	0.04	0.08

)	MBC	0.10	0.10	0.02	0.06
<i>Monothea buxifolia</i>(root)	MIC	0.06	0.02	0.04	0.08
	MBC	0.10	0.08	0.02	0.06

Table 3: Free radical scavenging activity of aqueous extracts of selected plant.

Plant Extract	20 ppm	40 ppm	60 ppm	80 ppm
<i>Olea europeae</i>	53.6	52.0	48.5	57.7
<i>Myrtus communis</i>	45.0	49.0	78.0	89.0
<i>Monothea buxifolia</i> (Stem)	47.6	46.32	68.0	79.0
<i>Monothea buxifolia</i> (root)	49.04	53.0	65.0	76.0

Table 4: Total polyphenol and phenolic acid contents of the selected plants

Plants	Total phenolic contents	Total polyphenols
<i>Olea europeae</i>	5.5	5.89
<i>Myrtus communis</i>	5.25	5.21
<i>Monothea buxifolia</i> (root)	5.0	3.52
<i>Monothea buxifolia</i> (stem)	7.5	6.72

Table 5: Pigment contents in the selected plants extracts (mg/100ml)

Plant	Chlorophyll a	Chlorophyll b	β-carotene	Lycopene
<i>Olea europeae</i>	0.15873	0.4399	0.63712	0.087
<i>Myrtus communis</i>	0.899	0.274	0.5004	0.0889
<i>Monothea buxifolia</i> (stem)	0.262	0.0732	0.1910	0.1731
<i>Monothea buxifolia</i> (root)	0.254	0.0543	0.132	0.1543

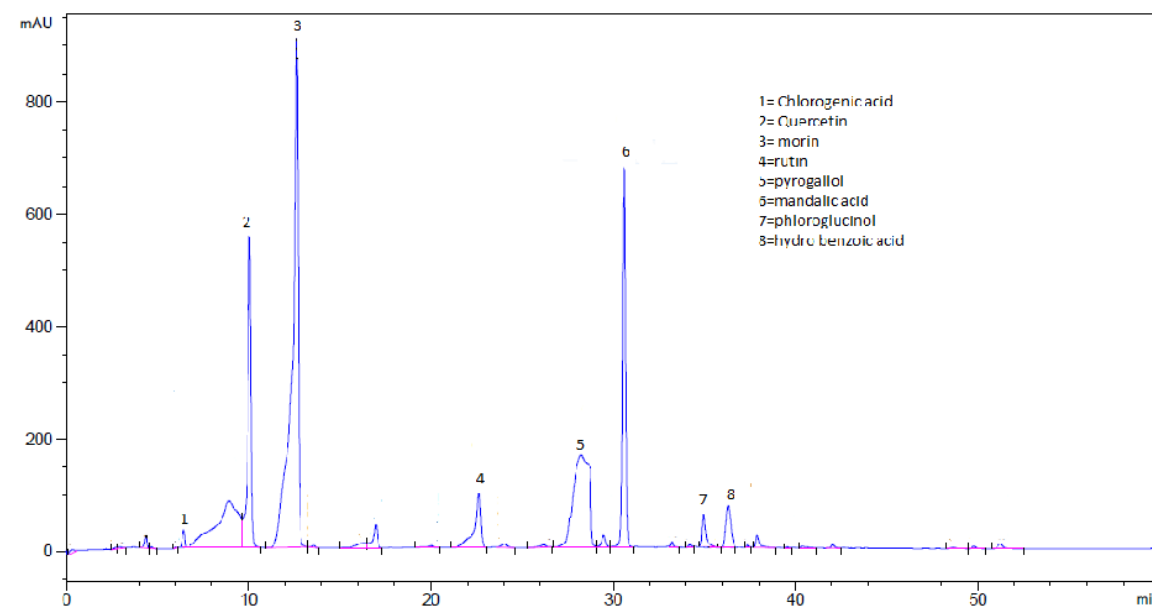


Figure 1: A representative HPLC-UV chromatogram of the standard phenolic compounds

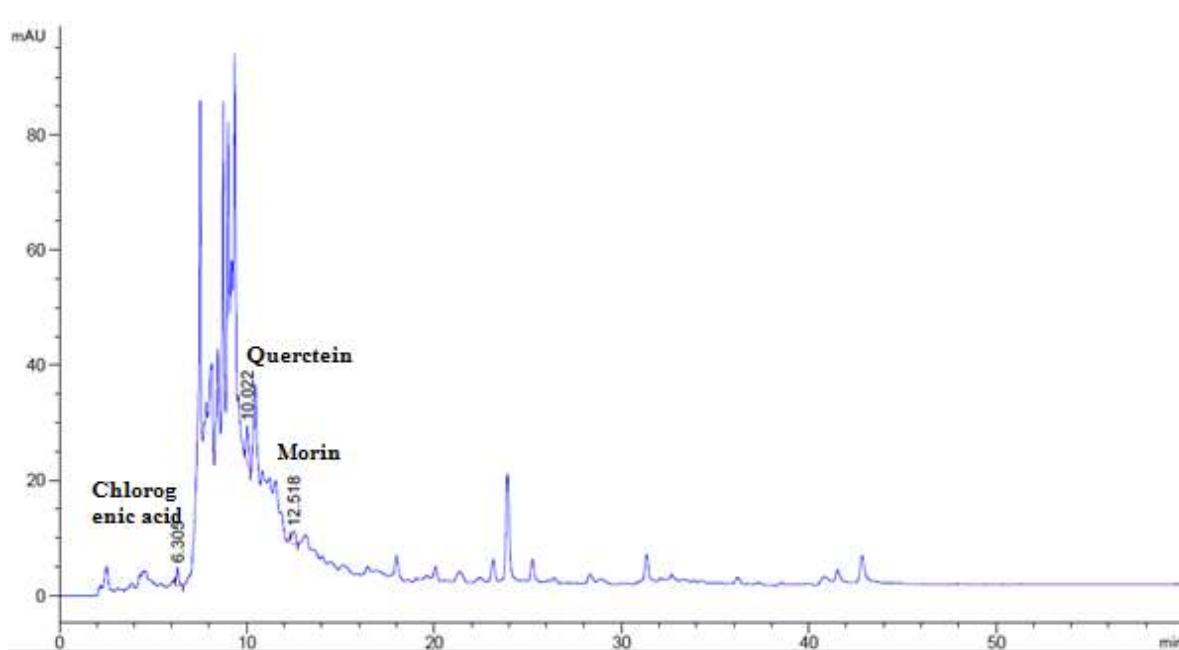


Figure 2: Chromatograms of *Olea europaea* extract

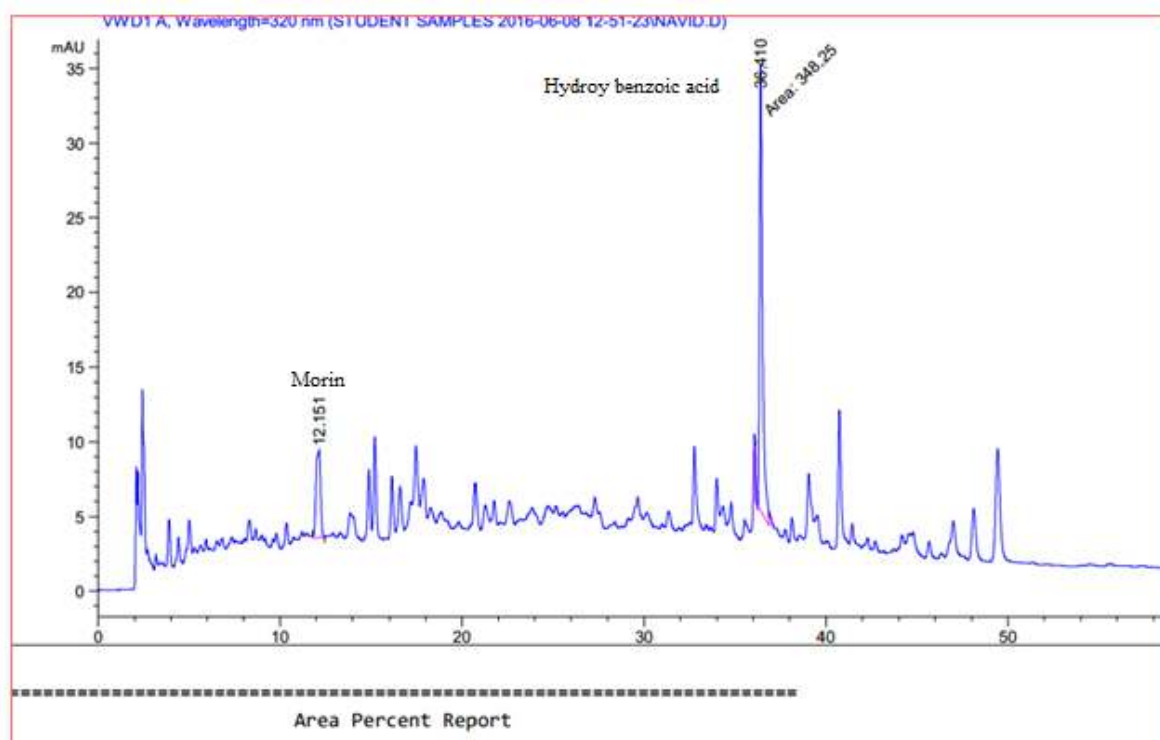


Figure 3: Typical HPLC-UV chromatogram of *Myrtus communis* extract.

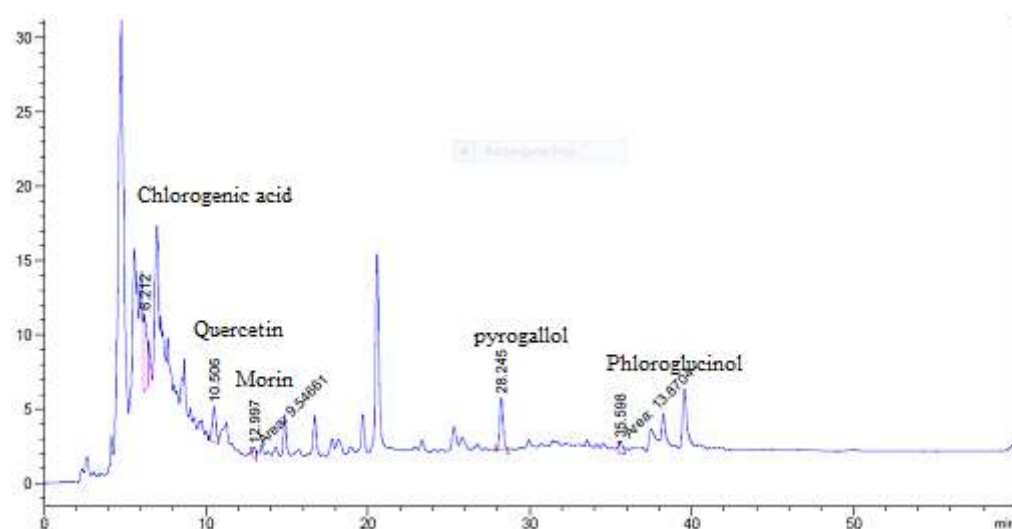


Figure 4: A representative HPLC chromatogram *Monotheca buxifolia*(root) extract

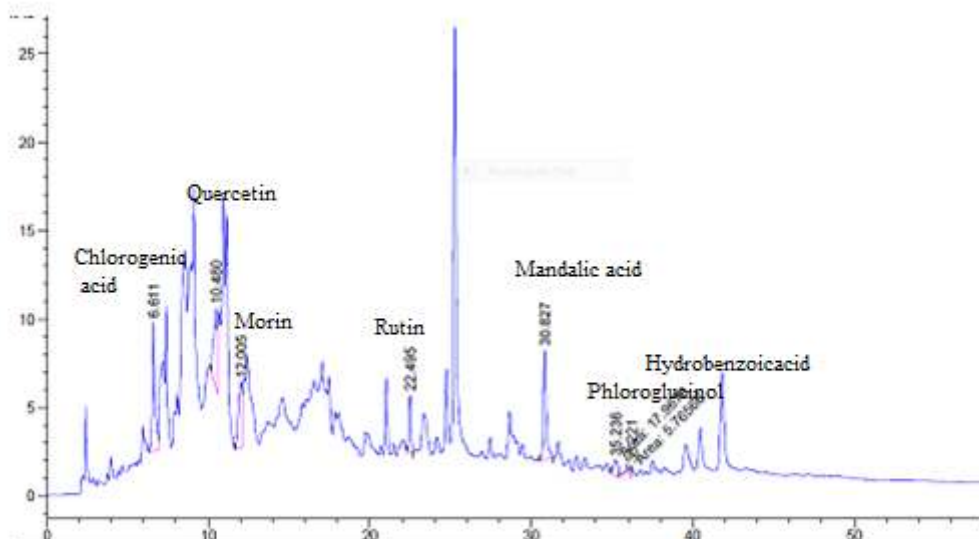


Figure 5: A representative HPLC chromatogram of *Monothecabuxifolia*(stem) extract.