

Original Research Article

HPLC profiling 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 anti-bacterial activity, total phenolic content, polyphenol, chlorophyll a, chlorophyll b, β -carotene and lycopene.

Design of the study: The study is composed of HPLC profiling 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 method, minimum inhibitory concentration and minimum bactericidal concentration. The total phenolic contents and polyphenol were determined by FolinCiocalteu 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 of *Oleauropeae*, 1.51, 2.06, 1.98, 1.79 cm of *Myrtuscommunis*, 1.98, 2.28, 2.36, 1.62 cm of *Monotheabuxifolia* (stem) and 1.78, 2.05, 1.80, 1.77 cm *Monotheabuxifolia* (root) respectively. Morin, Quercetin and Chlorogenic acid were detected at retention times were 12.518, 10.022 and 6.305 respectively in *Oleauropeae* extract. Morein and Hydroxy benzoic acid were detected at 12.858 and 6.079 retention time respectively in *Myrtuscommunis* extract. Chlorogenic acid, Quercetin, Morin, Pyrogluconic acid 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 basis of finding it was concluded that selected plants could be used medicinally.

30 Keywords

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

33 1. Introduction

34 Prehistoric men have used medicinal plants for the treatment of various types of diseases [1].
35 Medicinal plants may be defined as those plants which are sources of some drugs or
36 precursors used to treat, mitigate or to prevent a disease or to bring about a change in
37 physiological and pathological processes. Medicinal plants produced their effect on society of
38 developing country, both as a source of money and for improving the quality of life and
39 health as 80% of human population prefer herbal remedies [2].

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

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

56 In the present study, the antioxidant activity, anti-bacterial activity, total phenolic content,
57 polyphenol, pigments that include chlorophyll a, chlorophyll b, β -carotene and lycopene were
58 determined for the selected medicinal plants. Certain phenolic compound were
59 determined through High performance liquid chromatography (HPLC) in *Olea europaea*,
60 *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. powdered plants were dissolved in methanol and kept it for one week with constant shaking 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 and was thus converted into solid form. The crude extracts were kept in a refrigerator at a temperature of 4°C.

2.2. Antibacterial activity

Amoxicillin 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 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

93 five extract solution to the test-tube were added and one type of bacterial strain to these five
 94 test tube were also added. The test tube were placed in incubator for 24 hours and after this
 95 minimum inhibitory concentration against selected bacteria were found. The same tubes were
 96 used for the determination of MBC and after 3 days MBC was estimated from changes in
 97 turbidity in the test tubes.

98 **2.3.Determination of free radical scavenging activity**

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

102 1 gm of each extract was accurately weighed and dissolved separately in the distilled
 103 methanol to give the required solutions of 20 ml to get the required concentrations. Then this
 104 solution was stored as plant extract stock solutions. One solution of 5ml pure distilled
 105 methanol + 0ml plantsolution was also taken which was termed as control solution. Then 1
 106 ml of stock solution of DPPH was added to the control solution and diluted solution of extract
 107 solution. Then all these solutions were kept in dark place for 30 minutes. After this their
 108 absorbance reading was taken at 517 nm with the help of spectrophotometer by the given
 109 formula;

$$110 \quad \%RSA = \frac{(\text{Control solution absorbance} - \text{plant sample solution absorbance}) \times 100}{(\text{Control solution absorbance})} \quad (1)$$

112 **2.4.Total phenolic acid content**

113 The total phenolic of each plant was estimated by FolinCiocalteu reagent method using Gallic
 114 acid as standard phenolic compound. 1g of plant extract was added into a flask containing 9
 115 ml of distilled water. Then 1 ml of FolinCiocalteu reagent was added and the mixture was
 116 mixed thoroughly. After 5 minute of incubations, 10ml of 7% Na₂CO₃ were added. Then the
 117 mixture was diluted to 25 ml with the addition of 4 ml of distilled water. Then the mixture
 118 was incubated at room temperature for 90 minute. Finally the absorbance was measured using
 119 UV/Visible spectrophotometer at 750 nm. The total phenolic acid content was expressed as
 120 mg GAE/g sample.

2.5.Total Polyphenol Assay



Total polyphenol was measured using FolinCiocalteu method. The 1 g of each extract was mixed with 2 ml of FolinCiocalteu 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.

2.6.Determination of Pigments Contents

Pigments such as Chlorophyll α, Chlorophyll b, 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.

2.7.Determination of phenolic contents through HPLC

For the preparation of extracts for each plant, 10 mL of methanol and water were mixed with 1 gram from the powdered sample. These mixtures were then heated on hot plat on 70 °C for 1 hour. After cooling, the samples were filter with Whatman filter paper then these filter samples were centrifuged at 6000 rpm for 15 minutes, after centrifugation it is filter through syringe filtration or micro filtration 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 plantis shown in the figure 3. Only polyphenolic compound was identified. The detailed identification of compound with his peaks position in chromatogram and retention time (RT) of the standard phenolic compounds is given in figure 2. The injection volume was 10 micro litres and lambda max was 320 nm.

3. Result

3.1.Antimicrobial activities of the extracts

The extract of *Olea europaeae*, *Myrtjuscommunis* and *Monothe cabuxifolia*were tested for its antimicrobial activity and their values are shown in the table 1.

The MIC and MBC of *Oleauropeae*, *Myrtuscommunis* and *Monotheabuxifolia* 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 *Oleauropeae*, *Myrtuscommunis* and *Monotheabuxifolia* are shown in table 3.

3.3. Total phenolic acid content

The total phenolic of *Oleauropeae*, *Myrtuscommunis* and *Monotheabuxifolia* were estimated by FolinCiocalteu reagent method using Gallic acid as standard phenolic compound. The total phenolic acid content was expressed as mg GAE/g sample and are given in table 4. The following formula was used for the determination of total phenolic contents.

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

3.4. Total Polyphenols

Total polyphenol was measured using FolinCiocalteu 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.

3.5. 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:

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

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

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

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

3.6. 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, Pyrogluconic acid 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 *Monothe cabuxifolia*, these are found by formula as

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

186

187 Where

188 C_x = Concentration of unknown

189 A_s = Peak area of standard

190 A_x = Peak area of unknown

191 C_s = Concentration of standard

192 4. Discussion

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

198 It was found that the free radical-scavenging activities of the extracts increased with
199 increasing concentration which is clear from the figure. The present study correlates with the
200 study of Ozenet *al.* [10] in which he investigate the antioxidant activity of the plant extracts
201 both in vivo and in vitro. 

202 The total phenolic acid content was determined by FolinCiocalteu reagent method [11], the
203 absorbance was measured using spectrophotometer at 750 nm. The total phenolic acid
204 content was expressed as mg GAE/g sample values. This study correlates with the total
205 phenolic acid contents determined Mazandarani *et al.*, [12] using calorimeter method.



The total polyphenol were measured using FolinCiocalteu method, 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. This study correlates with Silverira *et al.*[6], in which the total polyphenol contents were determined for the extract obtained after homogenizing 1 g of frozen watercress leaves with 3 mL of methanol/water solution.

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

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

5. Conclusion

In this study, anti-bacterial 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*, *Myrtuscommunis* and *Monotheabuxifolia*. 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 *Myrtuscommunis*, Chlorogenic acid, Quercetin, Morin, Pyrogluconic acid 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 *Monotheabuxifolia*.

The *Olea europaea*, *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

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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>Myrtuscommunis</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>MonotheCabuxifolia(Stem)</i>	<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</i>	2.36	2.42

	<i>faecalis</i>		
<i>Monothecabuxifolia</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

279

280 **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 europaea</i>	MIC	0.06	0.04	0.08	0.06
	MBC	0.10	0.08	0.10	0.08
<i>Myrtus communis</i>	MIC	0.1	0.06	0.04	0.08
	MBC	0.06	0.08	0.08	0.1
<i>Monothecabuxifolia</i>(Stem)	MIC	0.02	0.06	0.04	0.08
	MBC	0.10	0.10	0.02	0.06
<i>Monothecabuxifolia</i>(root)	MIC	0.06	0.02	0.04	0.08
	MBC	0.10	0.08	0.02	0.06

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

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

282

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

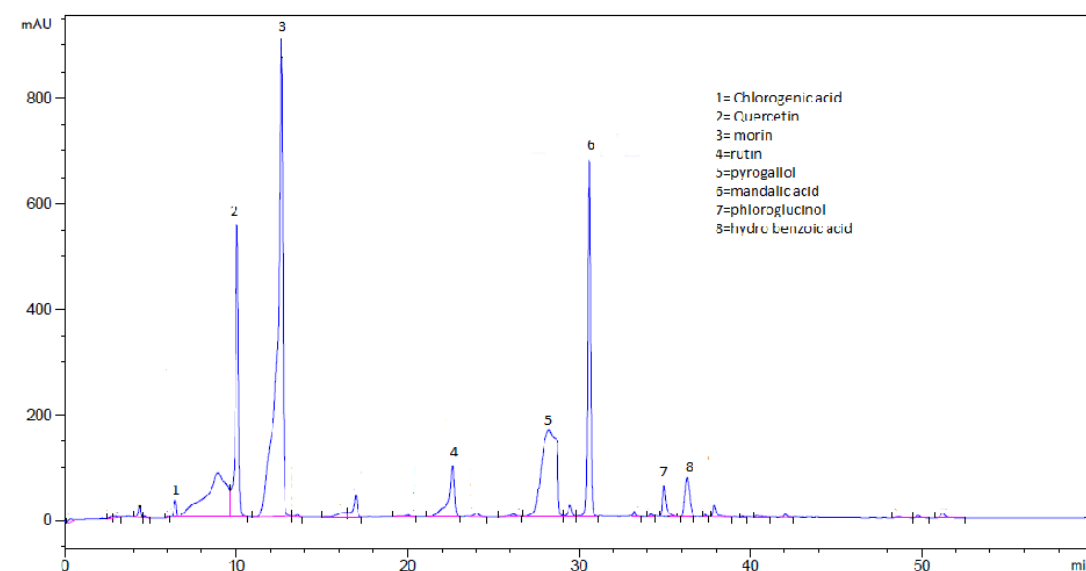
Plants	Total phenolic contents	Total polyphenols
<i>Olea europaea</i>	5.5	5.89
<i>Myrtus communis</i>	5.25	5.21
<i>Monothecabuxifolia</i> (root)	5.0	3.52

<i>Monotheabuxifolia</i> (stem)	7.5	6.72
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284

285 **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>Myrtuscommunis</i>	0.899	0.274	0.5004	0.0889
<i>Monotheabuxifolia</i> (stem)	0.262	0.0732	0.1910	0.1731
<i>Monotheabuxifolia</i> (root)	0.254	0.0543	0.132	0.1543



286

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

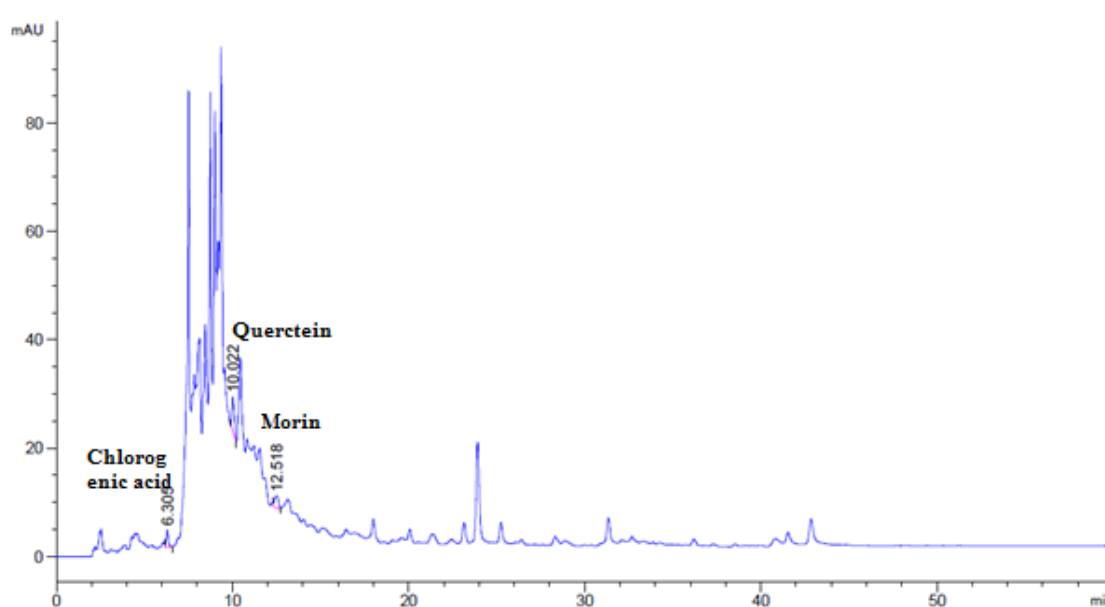


Figure 2: Chromatograms of *Olea europaea* extract

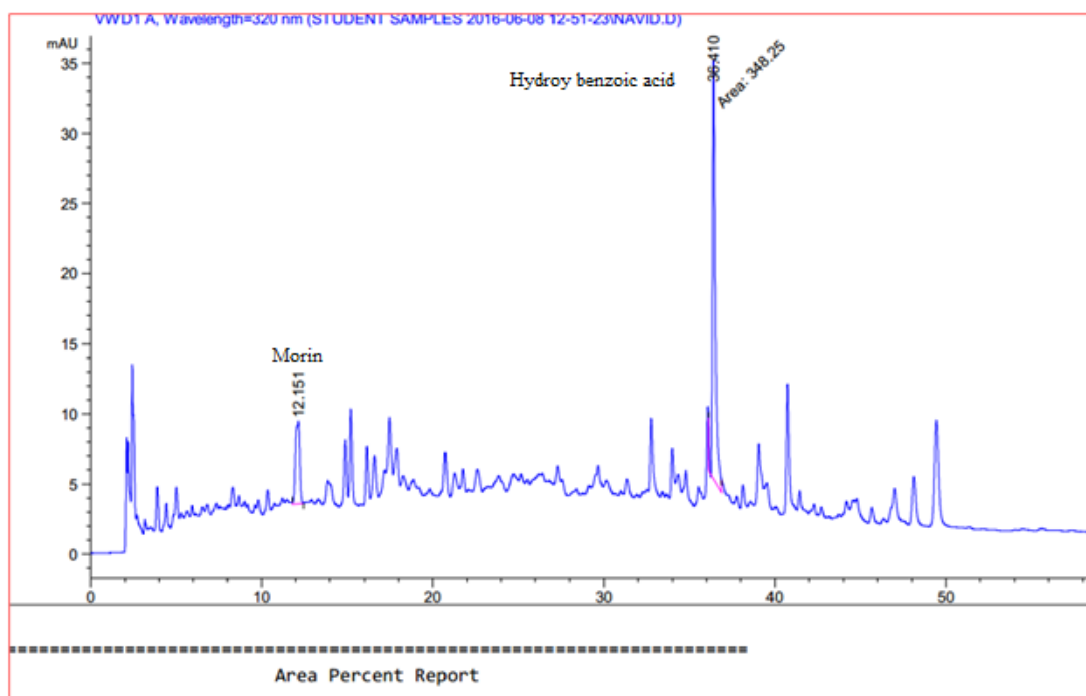


Figure 3: Typical HPLC-UV chromatogram of *Myrtus communis* extract.

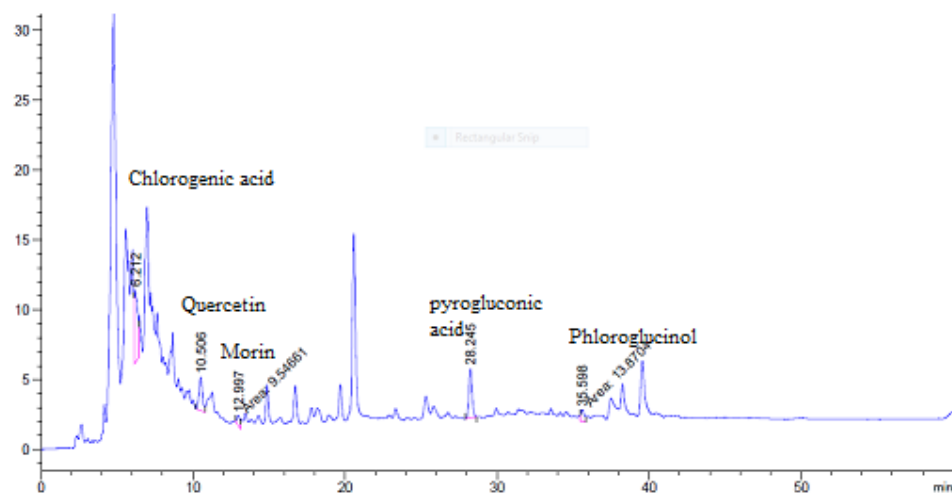


Figure 4: A representative HPLC chromatogram *Monothecabuxifolia*(root) extract

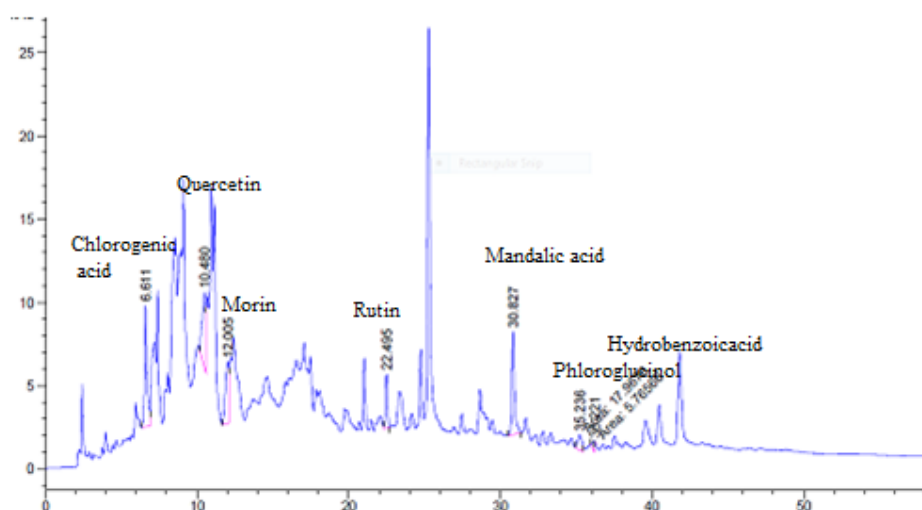


Figure 5: A representative HPLC chromatogram of *Monothecabuxifolia*(stem) extract.