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HPLC Profile of Bioactive Compounds Present in *Olea europeae, Myrtus communis* and *Monotheca buxifolia* and their Antibacterial Activities

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Authors' contributions

This work was carried out in collaboration between all authors. Author MZ designed the study, wrote the first draft of the manuscript and supervised the whole work. Author MZ also managed the literature searches. Author NA, Huma and Farzana managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study the aqueous extract of *Olea europeae, Myrtus communis* and *Monotheca buxifolia* 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 identification and quantification of bioactive compounds in selected plants through HPLC.
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 selected plants extracts were determined using agar well diffusion, minimum inhibitory concentration and minimum bactericidal concentration methods. The total polyphenol contents were determined by Follin Ciocalteu 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 *Olea europeae* extract, 1.51,

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2.06, 1.98, 1.79 cm for *Myrtus communis* 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 *Monotheca buxifolia* respectively. Morin, Quercetin and Chlorogenic acid were detected at retention times; 12.518, 10.022 and 6.305 minutes respectively in *Olea europeae* extract. Morein and Hydroxy benzoic acid were detected at 12.858 and 6.079 minutes retention time respectively in *Myrtus communis* extract. Chlorogenic acid, Quercetin, Morin, Pyrogallol and Phloroglucinol were detected in root extract of *Monotheca buxifolia* 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: Oleaeuropeae; Myrtus communis; Monotheca buxifolia; 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 europeae*, 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 europeae*) 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 2.4 m in height with small foliage and deep fissured bark. It is native to Southern Europe, North Africa and West Asia [8]. *Monotheca 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 through High performance liquid chromatography (HPLC) in *Olea europeae, Myrtus communis* and *Monotheca buxifolia*.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Olea europeae 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 *Monotheca 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 powered 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 (0.03 g/15 ml) 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 0.039 gm of DPPH was accurately weighed with the help of digital balance and dissolve in the distilled methanol to give an appropriate solution of 100ml (0.039 gm/100 ml) 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 + 0 ml 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= {(Control solution absorbance_-plant sample solution absorbance×100)/(Control solution absorbance)} (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 H_2O and incubated at room temperature for 3 minute. Then added 10 ml of 20% of Na_2CO_3 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 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.

2.6 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 Fig. 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 Fig. 2. The injection volume was 10 micro liters and lambda max was 320 nm.

3. RESULTS

3.1 Antimicrobial Activities of the Extracts

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

The MIC and MBC of *Oleaeuropeae, Myrtus communis and Monotheca 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 *Oleaeuropeae, Myrtus communis and Monotheca 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.

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

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:

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

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

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

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

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 *Oleaeuropeae,*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 *Monotheca buxifolia,* these are found by formula as

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

Where

C_X = Concentration of unknown
A_S = Peak area of standard
A_x =Peak area of unknown
Cs = Concentration of standard

4. DISCUSSION

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

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
Olea europeae,	*Escherichia coli*	1.82	3.92
	Klebsiella pneumonia	1.52	2.3
	Bacillus cereus	2.46	3.58
	Enterococcus faecalis	1 .72	3.15
Myrtus communis	*Escherichia coli*	1.51	3.66
	Klebsiella pneumoniae	2.06	3.78
	Enterococcus Faecalis	1.98	2.48
	Bacillus cereus	1.79	4.06
Monotheca buxifolia (Stem)	*Escherichia coli*	1.98	2.56
	Klebsiella pneumonia	2.28	1.68
	Bacillus cereus	1.62	2.4
	Enterococcus faecalis	2.36	2.42
Monotheca buxifolia (root)	*Escherichia coli*	1.78	2.8
	Klebsiella pneumonia	2.05	3.7
	Bacillus cereus	1.77	3.54
	Enterococcus faecalis	1.80	2.8

Table 2. MIC and MBC of aqueous extracts of selected plants

Plant Extract	Parameter	*Escherichia coli*	*Klebsiella pneumonia*	*Enterococcus faecalis*	*Bacillus cereus*
Olea europeae	MIC	0.06	0.04	0.08	0.06
	MBC	0.10	0.08	0.10	0.08
Myrtus communis	MIC	0.1	0.06	0.04	0.08
	MBC	0.06	0.08	0.08	0.1
Monotheca buxifolia (Stem)	MIC	0.02	0.06	0.04	0.08
	MBC	0.10	0.10	0.02	0.06
Monotheca buxifolia (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
Olea europeae	53.6	52.0	48.5	57.7
Myrtus communis	45.0	49.0	78.0	89.0
Monotheca buxifolia (Stem)	47.6	46.32	68.0	79.0
Monotheca buxifolia (root)	49.04	53.0	65.0	76.0

It was found that the free radical-scavenging activities of the extracts increased with increasing concentration which is clear from the figure. The present study correlates with the study of Ozen [11] in which he investigate the antioxidant activity of the plant extracts both in vivo and in vitro. According to his study the scavenging effect of water and ethanol extracts of watercress and standards on the DPPH• radical decreased in order of ethanol extract > water extract> BHA which were 87.08, 75.15, and 61.08% at the dose of 500 mg/mL, respectively. These results show that watercress contains a free radical inhibitor, as well as antioxidant components that reacts with free radicals.

The total polyphenol were measured using Follin-ciocalteu method [10], 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], and Mazandarani et al. [12] 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. They found that the total phenolic contents of aerial parts of *Nasturtium officinale were* 8.03 to 9.35 mgGAEg^{-1} in vegetative period and 6.5 to 7.65 mgGAEg^{-1} in generative period. Ozen found that aerial parts of *Nasturtium officinale* had 88.60 ±2.41 and 74.18 ±1.72 µg pyrocatechol equivalent of phenolic compounds in 1000 mg of water and ethanol extracts, respectively [11].

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 extracts showed 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 Figs. 1-5 along with standard. Antioxidants have been identified in a number of medicinal plants through HPLC [14, 15]. According to Cartea et al. [14]. Quercetin and kaempferol were the most flavonoids found in *Nasturtium officinale*. In literature there no such reports about the antioxidants profiles of selected plants.

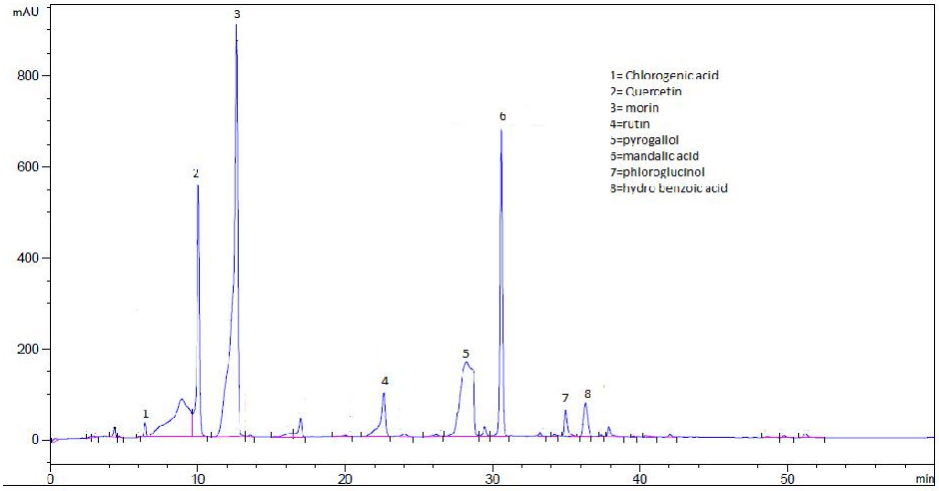

Fig. 1. A representative HPLC-UV chromatogram of the standard phenolic compounds

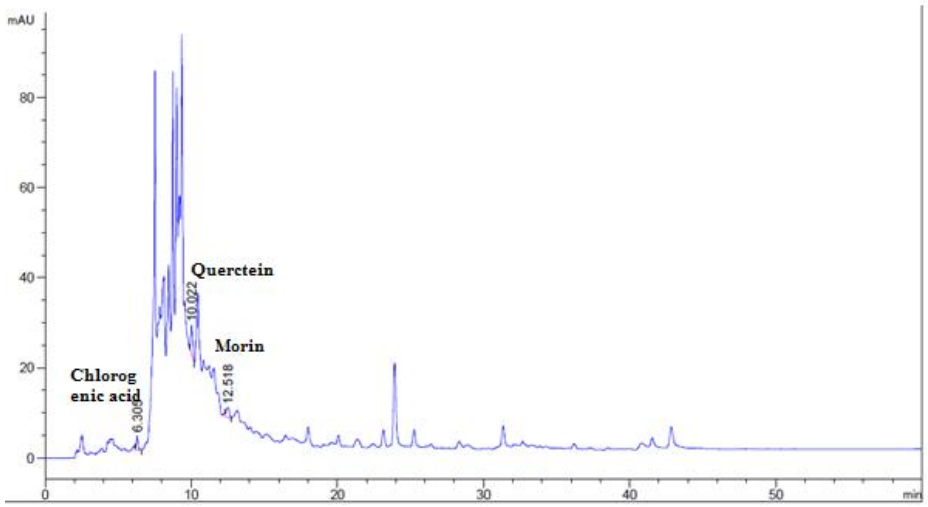

Fig. 2. Chromatograms of *Olea europeae* extract

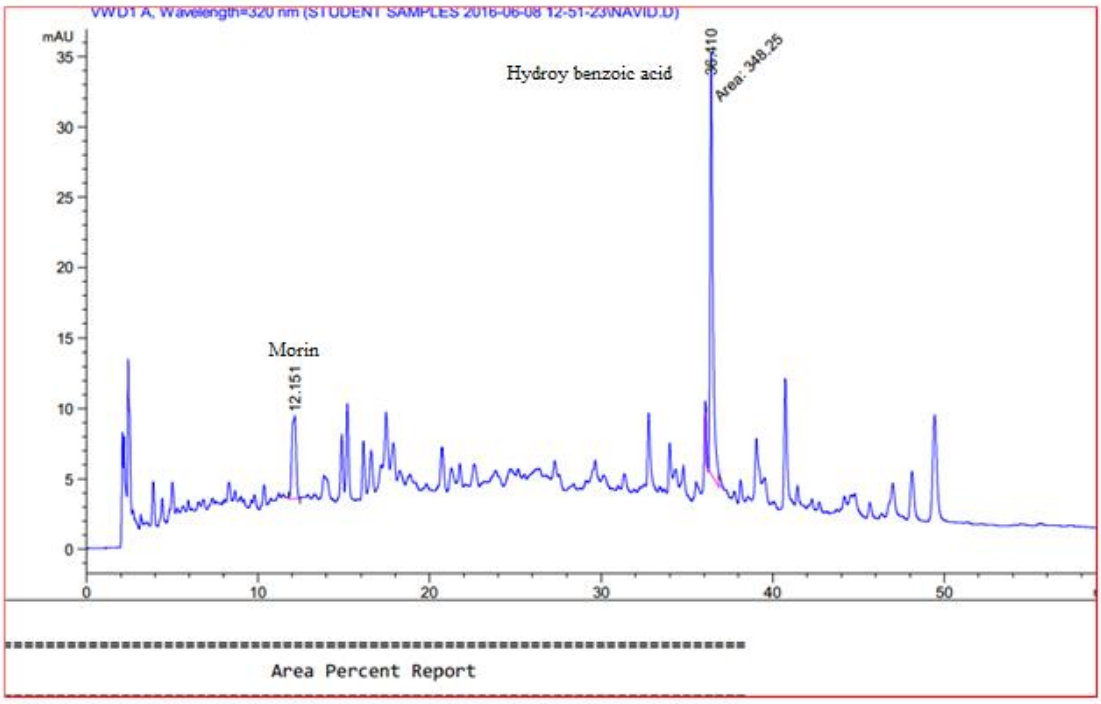

Fig. 3. Typical HPLC-UV chromatogram of *Myrtus communis* extract

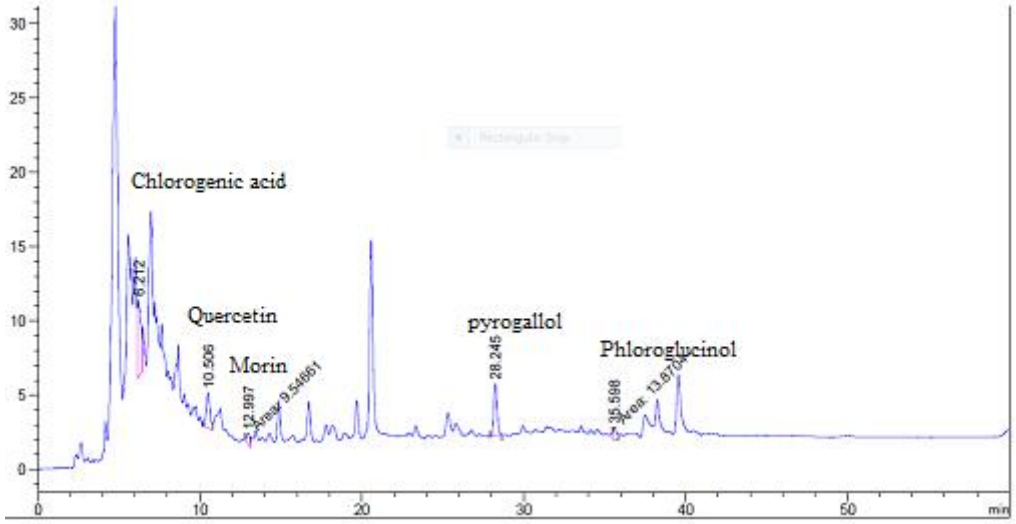

Fig. 4. A representative HPLC chromatogram *Monotheca buxifolia* (root) extract

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

Plants	Total phenolic contents	Total polyphenols
Oleaeuropeae	5.5	5.89
Myrtus communis	5.25	5.21
Monotheca buxifolia (root)	5.0	3.52
Monotheca buxifolia (stem)	7.5	6.72

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

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

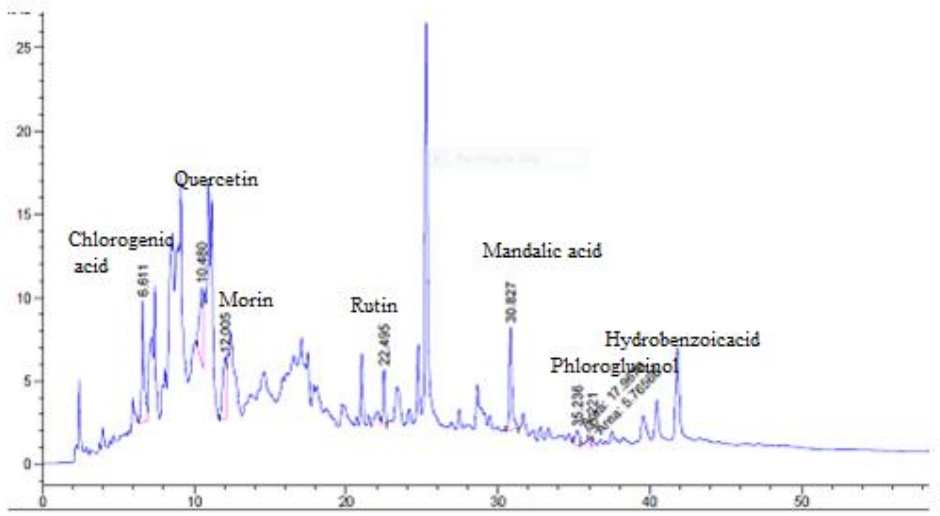

Fig. 5. A representative HPLC chromatogram of *Monotheca buxifolia* (stem) extract

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 *Oleaeuropeae, Myrtus communis* and *Monotheca 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 *Oleaeuropeae,*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 *Monotheca buxifolia.*

The *Olea europeae, Myrtus communis* and *Monotheca 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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