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Research Article

Phytochemical profile, antimicrobial potential and GC-MS analysis of wild variety of *Olea Europaea* (Olive) cultivated in Pakistan

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Abstract

Olive plant produces a variety of bioactive molecules and thus has important medicinal value in folk medicine. In this study, different leaf and fruit extracts of Pakistani wild variety of *Olea europaea* was tested for their phytochemical content, antimicrobial activity and mass spectrometric analysis. Olive leaves and fruit samples were extracted with five different solvents to obtain the crude extract and screened for various kinds of phytochemicals. Phytochemicals were further confirmed through Fourier Transmission Infra-red Spectroscopy (FTIR). The plant extract showed significant antimicrobial activity against all the strains tested. Methanol, ethanol and ethyl acetate extracts were found more effective against most of the pathogenic bacteria with high zone of inhibition. Gas Chromatography-Mass Spectrometry (GC-MS) analysis revealed that olive fruits have Oleic acid, Palmitic acid, Linoleic acid, Octadecadienoic acid, Stearic acid, Palmitoleic acid and Tridecanoic acid as oil contents. In this work, the antimicrobial potential and phytochemical contents were explored which may further pave the way for the bio-industrial applications.

Keywords: *Olea europaea*; Phytochemical content; Antimicrobial activity; GC-MS analysis

Introduction

Plants have been used as medicines since the dawn of civilization for thousands of years [1]. About 80% of population in developing countries uses traditional medicine for curing different diseases. So, the investigation of these plants is helpful in understanding their characteristics,

usefulness and effective nature [2]. Chemical constituents of plants have been exploited for the discovery of therapeutic agents as well as new sources of such economical materials as tannins, oils, gums, forerunners for the production of complex chemical substances. The olive tree is amongst the oldest known cultivated trees in the world that has been an important source of nutrition and medicine [3]. Olive is a broad-leaved, evergreen tree of the family Oleaceae that is present in native coastal areas of the Mediterranean region. The olive has been used generally in customary medications in European Mediterranean islands and countries such as Spain, Israel, Morocco, France and Greece where it is cultivated mainly as edible oil and table olives [4].

Bacteria are serious pathogens and cause a wide variety of human diseases including cholera, leprosy, bacterial pneumonia, whooping cough, and diphtheria. Bacterial pathogens are also a serious threat to the food industry [5]. Antibiotics provide main basis for the therapy of microbial infections. However, the emergence and spreading of bacterial resistance has made the treatment of infectious diseases more problematic [6]. The antimicrobial activity of plants is highly related to secondary substances that are synthesized and produced by plants [7]. The olive tree naturally possesses strong antimicrobial activity which has been utilized in traditional medicine to fight fever and overcome infections [8].

It is desirable to get knowledge about the bioactive constituents of plants like fatty acids because of their nutritional value, diagnosis of definite diseases and pharmacology. Various analytical techniques like Spectrophotometry, HPLC and gas chromatography (GC) have been applied for the analysis of fatty acids [9-12]. GC-MS is a useful method for the determination of fatty acids due to its high speed, resolution and sensitivities [13].

The aim of the study was to investigate the antimicrobial activities and phytochemical contents utilizing different methods in order to compare with the reported literature. The data produced here may be used in production of biomolecules of medical importance.

Materials and methods

Collection of plant materials

The fresh leaves of olive plant were collected from district Dir of Khyber Pakhtunkhwa, Pakistan. The rinsed olive leaves of collected samples were air dried under shade and then converted to fine powder by crushing in electronic grinder. The fine powdered form of the plants was then kept in airtight glass containers to be protected from different contaminants until used for further analysis and screening.

Preparation of leaves extracts

The olive leaves were air dried and ground into powder form and then 100 (g) of the powder was extracted with 350 mL of ethyl acetate, ethanol, methanol, distilled water and hexane each (Technical grade- Merck) and boiled water in 1000 mL conical flasks. Flasks were vigorously shaken at 400 rpm overnight in a Labotec model 20.2 shaking machine. After shaking, the supernatant was decanted into pre-weighed, labeled flasks. The process was repeated three times to exhaustively extract the leaves materials. The solvents were removed under vacuum by rotary evaporator at 40°C and the extraction efficiency was quantified by determining the weight of each of the extracts [14, 15].

Phytochemical analysis

Olive leaves extracts were screened with five different solvents like distilled water, hexane, ethanol, methanol and ethyl acetate for various kinds of phytochemical constituents e.g. alkaloids, steroids, flavonoids, saponins, tannins, carotenoids,

cyanogenic glycosides, phenolic compounds, carbohydrates, proteins and riboflavin. The phytochemicals in each extract were qualitatively and quantitatively determined for the presence of biologically active components according to the method reported in literature [16, 17].

Antimicrobial assay

The extracts obtained from leaves were tested for antimicrobial activity against six Gram positive bacterial strains, three Gram negative bacterial strains and one fungal strain (Table 1) by disc diffusion method as described by Aida et al. (2001) [17-19].

The results were based on the measurement of minimum zone of inhibition (ZOI) that was shown in millimeter (mm). The experiments were performed in triplicate and the average diameter of the inhibitory zone was measured by using standard deviation method.

Table 1. List of microorganisms used in this study for Olive (*Olea europaea)* antimicrobial activity

S. No	Species	Type	Details of the microbial strains used
1	*Escherichia coli*	Gram negative	ATCC25922
2	*Salmonella typhi*	Gram negative	Hayatabad Medical Complex Peshawar, Pakistan
3	*Pseudomonas aeruginosa*	Gram negative	ATCC9721
4	*Klebsiella pneumonia*	Gram negative	Microbiology lab. Quaid-e-Azam University Islamabad, Pakistan
5	*Agrobacterium tumefaciens*	Gram negative	Microbiology lab. Quaid-e-Azam University Islamabad, Pakistan
6	*Erwinia carrotovora*	Gram negative	Microbiology lab. Quaid-e-Azam University Islamabad, Pakistan
7	*Staphylococcus aureus*	Gram positive	ATCC6538
8	*Bacillus subtillus*	Gram positive	Hayatabad Medical Complex Peshawar, Pakistan
9	*Bacillus atrophus*	Gram positive	Microbiology lab. Quaid-e-Azam University Islamabad, Pakistan
10	*Candida albicans*	Fungus	Hayatabad Medical Complex Peshawar, Pakistan

Identification of phytochemicals by GC-MS analysis

About 100 g olive seed material was extracted through soxhlet extraction apparatus with 250 ml hexane. The extract was concentrated by removing solvent under reduced pressure in a rotary evaporator. According to AOAC standard reference method, derivatization was performed prior to GC-MS analysis [16]. About 25 mg fat was mixed with 0.1 ml internal standard (1.37 mg) and 1.5 ml of sodium hydroxide solution in methanol (0.5 N), preserved and warmed in boiling water bath for 5 min. The hydrolyzed sample was chilled and mixed with 2.5 ml of boron triflouride solution in methanol (10%). The solution was then conserved and warmed in boiling water bath for 30 min and chilled. The esterified solution was mixed with 5 ml saturated sodium chloride solution and extracted two times with 1 ml n-hexane. The filtration of n-hexane extract is done with 0.45 µm

membrane filter and injected 1 µl to GC-MS through auto injector system.

GC-MS analysis was performed on GC-MS Model QP 2010 plus (Tokyo, Japan) equipped with an auto-sampler (AOC-20S) and auto-injector (AOC-20i). TRB-FFAP Technokroma capillary column (30 m × 0.35 mm, 0.250 µm film thicknesses) was used under the following conditions:

oven temperature programmed from 50°C to 150°C at 15°C/min, and the final temperature was raised to 220°C and kept constant for 3 min; injector temperature 240°C; carrier gas He, flow rate 1ml/min; the volume of injected sample was 1µl ; ion source temperature 200°C; scan mass range of m/z 85-380 and interface line temperature 300°C [13].

Components were identified by comparing the mass spectra obtained during GC-MS analysis with those of standard mass spectra from the National Institute of Standard and Technology (NIST) library.

Results and discussion

In this study, phytochemical analysis of local wild variety of olive leaves extracts were investigated using standard protocols [16, 17]. The results of qualitative phytochemical tests showed the presence of important phytochemical components. It is clear from qualitative screening that alkaloids, phenols, tannins, flavonoids, saponins, steroids, terpenoids, carbohydrates and proteins were present in almost all solvent extracts (Table 2). The phytochemicals reported in this study has been reported in the previous literature [11, 12].

Table 2. Qualitative analysis of local olive plant for phytochemical contents

S. No	Plant extracts	A	G	S	F	P/T	S	T	C	P	R
1	Aqueous	+	++	+++	++	-	++	+	++	++	+
2	Hexane	+	+	+	+	-	++	+++	-	-	+
3	Methanol	++	+	++	+++	+	++	+	++	++	++
4	Ethanol	++	++	++	+++	+	+++	++	++	++	+
5	Ethyl Acetate	++	-	++	+	+	++	++	+	+	+

+ = present; ++ or +++= abundant - = absent; A = Alkaloids; G = Glycosides; S = Saponins; F = Flavonoids; P/T = Phenols/Tannins; S = S teroids; T = Terpenoids; C = Carbohydrates; P = Proteins; R = Riboflavin

Further confirmation about phytochemicals was done by spectra obtained using Fourier Transmission Infra-Red Spectroscopy (FTIR) model (IRPrestige-21, Shimadzu Corporation Kyoto Japan) (Figure 1) which revealed that polar phytochemicals were separated in high amount with polar solvents and non-polar constituents with non-polar solvents. In this spectra, the phenols, tannins, carbohydrates and proteins were absent in hexane extract due to non-polar nature of hexan. While methanol and ethanol extracts had highest amount of flavonoides and terpenoids due to the polar nature of the solvent which is according the like dissolve like concept of solvents. These results are supported by the previous literature where different extracts were used for phytochemical study of various plants [11, 12, 16, 17]. The quantitative phytochemical estimation (Figure 2) revealed that ethanollic and methanollic extracts have high content of flavonoids 16.36±0.03% and 14.0±0.25%, respectively. Aqueous and ethanollic extracts showed higher saponins percentage 13.50±0.25% and 13.80±0.25%, respectively. Similarly, ethanollic and methanollic extracts contained high Alkaloids percentage 13.20±0.17% and 12.50±0.15%, respectively. It has been reported that alkaloids, saponins and tannins are

important to be used as antibiotic agents against known pathogens [20, 21]. Chemically, flavonoids are hydroxylated phenol rich compounds which are reported for antimicrobial activities [22]. These phytochemicals are also proved useful antioxidant agents and possessing effective anticancer characteristics [23]. Saponins are capable of precipitation and coagulation of erythrocytes. Some of the activities of saponins are leather formation in aqueous solutions, hemolysis, binding with cholesterols [24].

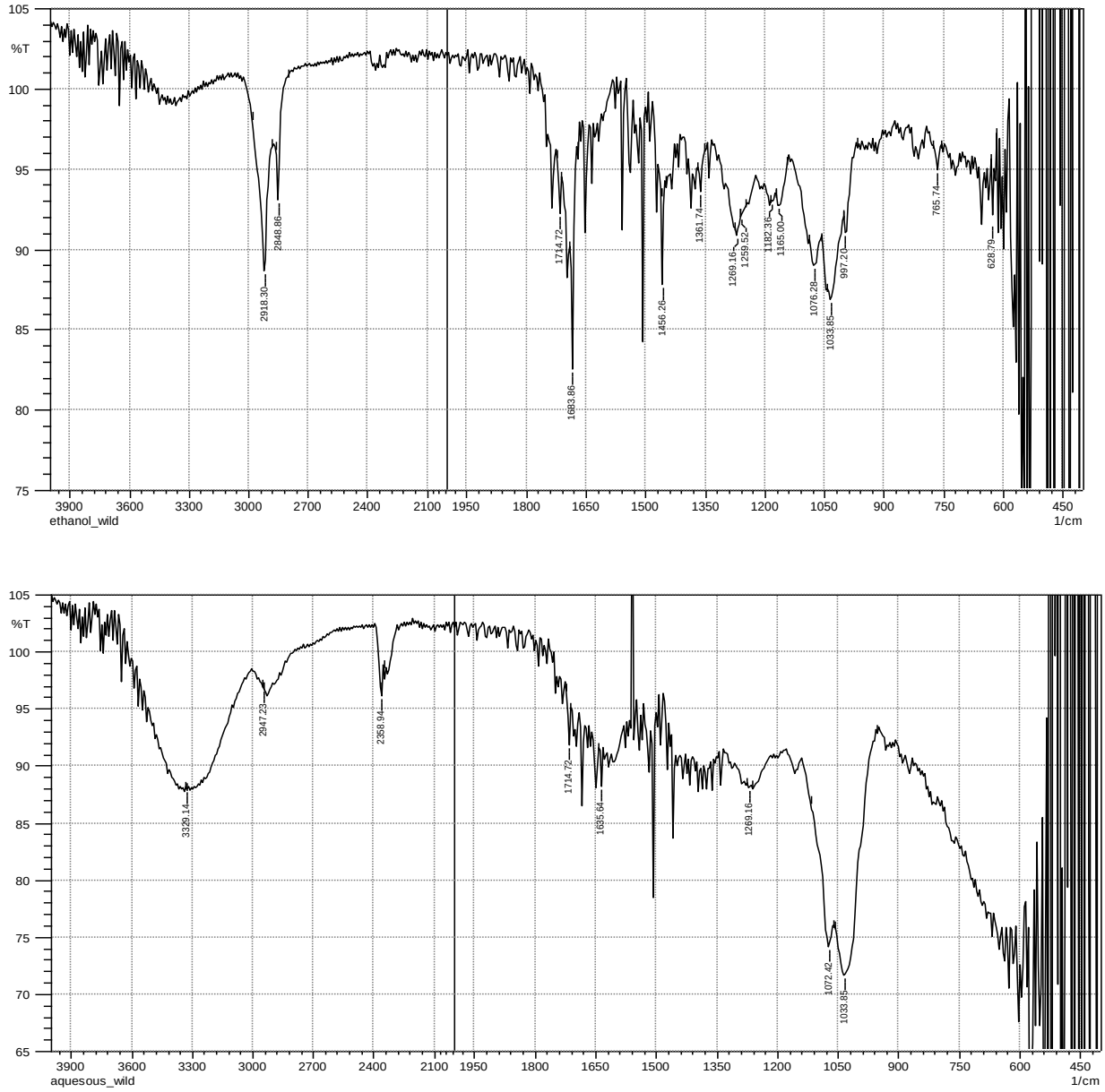

Figure 1. FTIR spectra of olive plant leaves for phytochemicals

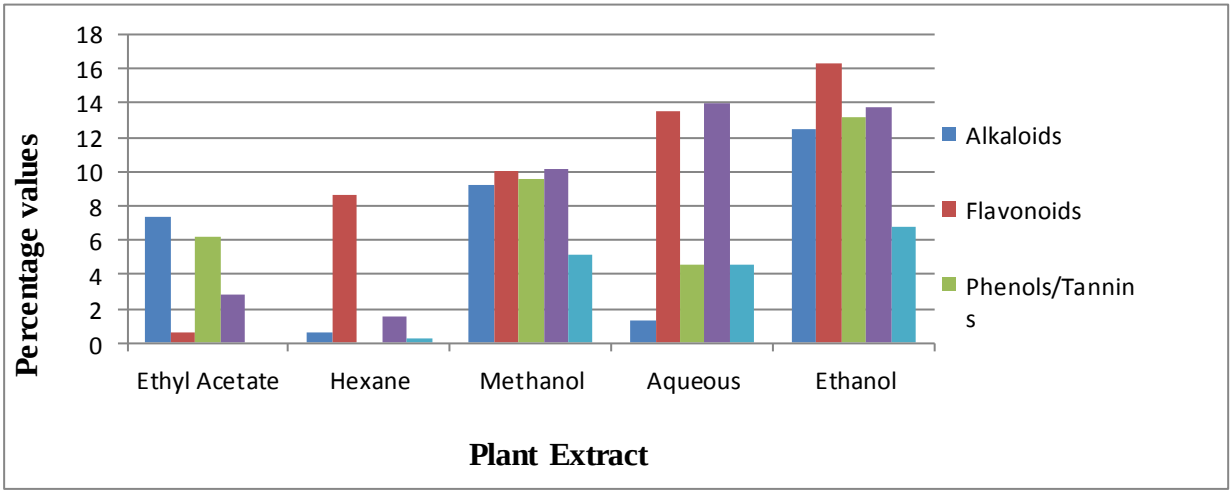

Figure 2. Quantitative phytochemical estimation (percentage) of olive leaves extracts (The obtained results are the mean of triplicate experiments)

The antimicrobial activities revealed that ethyl acetate and methanol extracts of olive exhibited maximum activity while minimum antimicrobial activities were observed for hexane extracts (Figure 3). It is because of the fact that alcohol and ethyl acetate extracts give rise to flavonoids and phenolic phytochemicals as compared to hexane which were confirmed from the Fourier transmission Infra-red Spectroscopy (FTIR) spectra (Figure 1).

The results from the disc diffusion method, followed by measurement of ZOI, indicated that the methanol extract showed strong inhibitory activity against *Bacillus Atrophus*, with the highest inhibition zones (10±0.40mm) which is in the range with its antibiotic counterpart. (16±0.21mm) (Figure 3). The inhibitory activity of these extracts confirmed the antimicrobial activity and its potential use in the treatment of microbial diseases. The antimicrobial activities of Olive leaves have been reported by Sudjana et al. against different bacteria [19]. Antimicrobial activities of different plant extracts obtained were appeared to be very different in the sense of effectiveness as some bacterial species are found more resistant and some other are found more susceptible to the extracts in comparison with their respective antibiotics used in the study. All the bacterial strains showed fewer susceptibilities to both plant extracts as compared to standard antibiotics used which shows that both the standard antibiotics and plant crude extracts have higher antibacterial functions to Gram positive bacterial strains as compared to those of Gram negative bacterial strains. Similar antimicrobial activities of the olive plant have been reported in different countries [25, 26].

The GC-MS chromatogram of *Olive* fruit oil contents revealed the presence of saturated and unsaturated fatty acids as shown in Figure 4. The fragmentation patterns obtained from GC-MS analyzer was compared with that of National Institute of Standard and Technology (NIST) library through which 13 different fatty acids were identified. The different fatty acids identified based on GC-MS are given in Table 3. Oleic acid was found in highest concentration (30.00%), while the other fatty acids were; Palmitic acid (11.65%), Linoleic acid (6.50%), Octadecadienoic acid (5.98%), Stearic acid (5.30%), Palmitoleic acid (1.66%) and Tridecanoic acid (1.38%).

The rest of the fatty acids were present in less than 1% concentration (Table 3).

Similar chromatographic analysis has been done for olive plant of different countries and various biomolecules were evaluated for their biopharmaceutical application [9, 11, 12, 19, 25, 26]. GC-MS analysis revealed that olive oils contain different biologically active compounds like fatty acids thus olive plant, besides its antibacterial and antifungal activities can also be used to produce different pharmaceutical products to cure diseases including cancer.

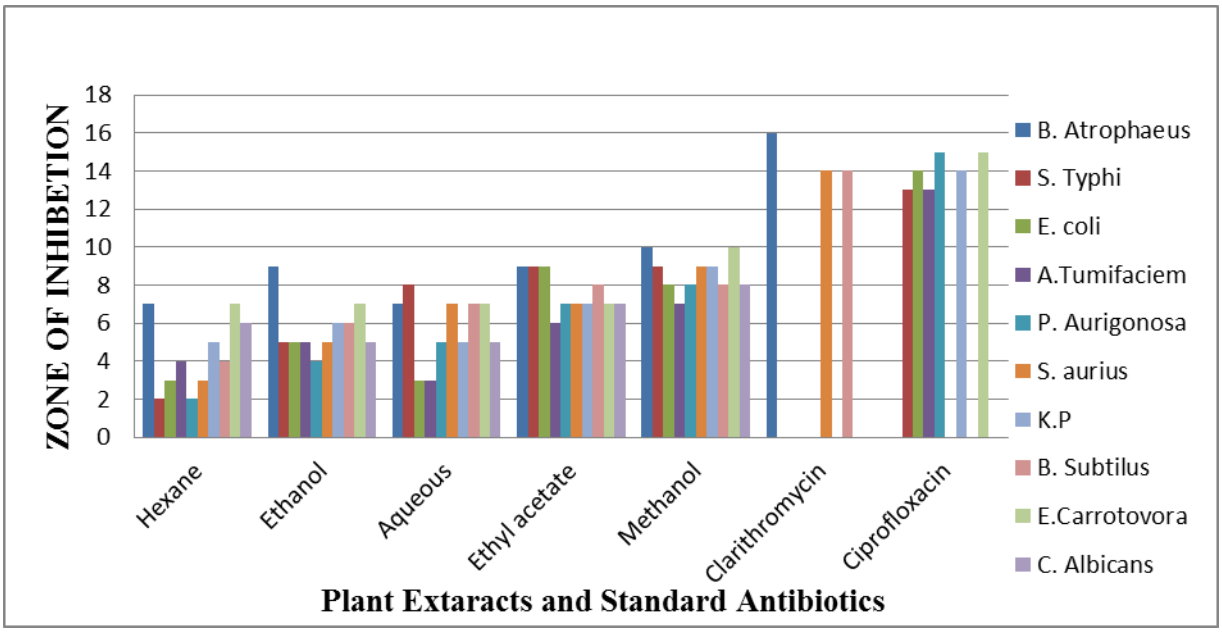

Figure 3. Antimicrobial activity of olive leaves extracts along with standard antibiotics

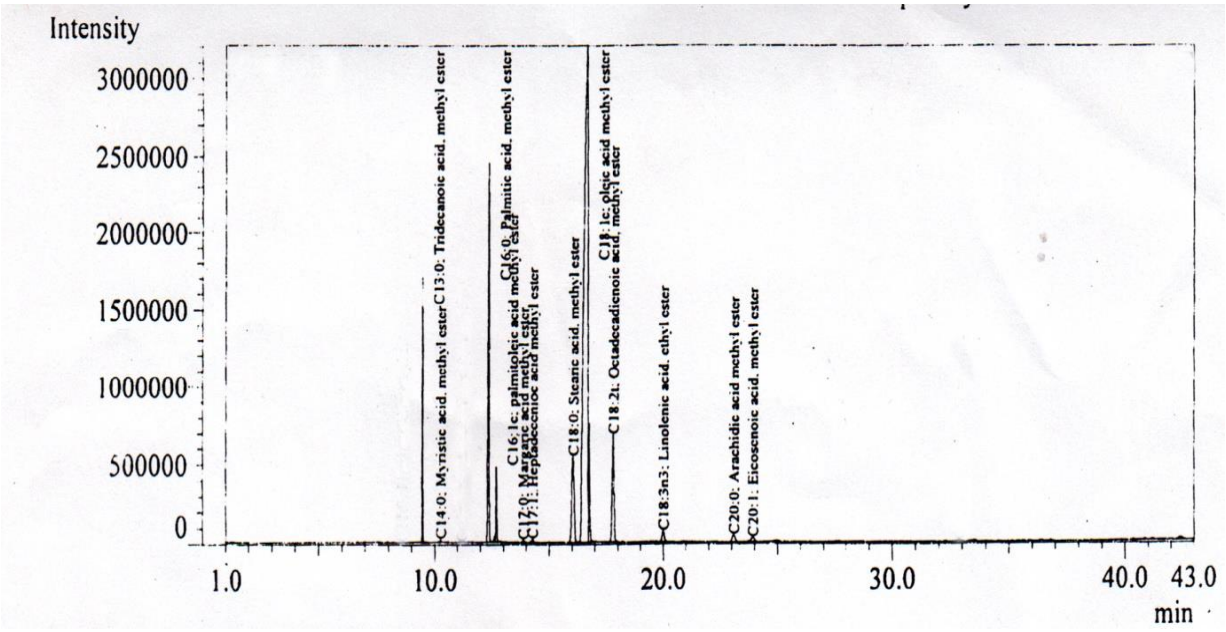

Figure 4. GC-MS Chromatogram of fatty acids detected in olive fruits oils

Table 3. Quantitative results of fatty acids in olive fruit

S. No	Name of Fatty Acid	Area	Conc. (%)	R. Time	m/z
1	C13:0; Tridecanoic acid, methyl ester	1193235	1.38	8.583	87.0
2	C14:0; Myristic acid, methyl ester	13168	0.06	10.280	87.0
3	C16:0; Palmitic acid, methyl ester	2754967	11.6	12.365	87.0
4	C16:1c; Palmitoleic acid, methyl ester	93844	01.6	12.694	97.0
5	C17:0; Magaric acid, methyl ester	12045	0.05	13.865	87.0
6	C17:1; Heptadecenoic acid, methyl ester	3779	0.06	14.272	97.0
7	C18:0; Stearic acid, methyl ester	1212389	05.3	16.056	87.0
8	C18:1c; Oleic acid, methyl ester	2853178	30.0	16.667	97.0
9	C18:2c; Linoleic acid, methyl ester	455104	6.50	17.806	95.0
10	C18:2t; Octadecadienoic acid, methyl ester	455104	5.98	17.806	95.0
11	C18:3n3; Linolenic acid, methyl ester	49070	0.90	19.998	95.0
12	C20:0; Arachidic acid, methyl ester	102881	0.43	23.089	87.0
13	C20:1; Eicosenoic acid, methyl ester	9410	0.14	23.932	87.0

Conclusions

It is concluded from the study that local olive plant extract contains different phytochemicals such as alkaloids, phenols, tannins, flavonoids, saponins, steroids, terpenoids, riboflavin, carbohydrates and proteins. Olive plant revealed broad spectrum antimicrobial activities against bacterial and fungal strains. It is obvious from GC-MS analysis that Olive fruit oils contain highest concentration of Oleic acid and other related fatty acids which may further be evaluated for bio-pharmaceutical applications.

Author's contributions

Conceived and designed the experiment: A Waqar, SA Muhammad & A Nawab, Performed the experiments: A Waqar, Analyzed the data: A Waqar, A Nawab, SA Muhammad, R Hazir & A Muhammad, Contributed reagents/ materials/ analysis tools: U Nimat, M Uzair & I Muhammad, Wrote the paper: A Waqar & A Nawab.

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