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# OPTIMIZATION OF RAPID PROTOCOL FOR EXTRACTION OF PHYTOCHEMICALS FROM *OLEA EUROPAEA* L. – OLEACEAE

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**Abstract**

The purpose of this study was to optimize a rapid protocol for the extraction of phytochemicals, using a microwave-assisted extraction technique (MAE) from *Olea europaea* L. For this purpose, fruits, seeds, and leaves of *O. europaea* var. *Earlik* were extracted by MAE. MAE parameters including power level (300-1000 W), extraction time (1-10 min), and polar (Ethanol and Acetone) and Non-polar solvents (n-hexane) ratios (8.65 -10 mL) for maximum phenolic and flavonoid content were optimized using central composite design (CCD). Extracts with non-polar solvent (n-hexane) and esterified olive oil were analyzed by GC-MS. After that, the extracts with maximum phenolics were analyzed and compared for antioxidant and antifungal activities. Results showed that leaf extracts in 4.5 minutes, 1000 W, and 10 mL ethanol contained higher phenolics and flavonoids. Leaf extract also showed higher antioxidant potential and higher antifungal potential against *Aspergillus flavus*. Same biological and phytochemical assays were repeated for microwave-assisted extracts of olive seeds and fruits with promising results. Parameters that showed the optimized results were power level (600 W), solvent (10 mL) and solvent (ethanol). Overall leaf extract showed higher antifungal and antioxidant potential compared to seed and fruit extract using the optimized extraction parameters with leaves. GC-MS analysis showed 20, 21, 16, and 8 components from fruit, seed, leaf and olive oil respectively. The results of the study demonstrated that MAE was a fast and energy-efficient method for extracting both polar and non-polar components from olives and can be used as an extraction method for olives which can lead to savings in time, energy, and cost. This rapid method of extraction can be used to analyze the phytochemicals from various other varieties of olives.

**Key words:** Antioxidants, Flavonoids, GC-MS, Microwave assisted extraction, *Olea europaea*, Phenolics, Response surface methodology.

## Introduction

Olive (*Olea europaea* L.), is an ancient and extensively grown tree. It belongs to the family Oleaceae (Olive family) and is grown in the world, mainly in the Mediterranean region (Italy, France, Portugal, Greece, Saudia Arabia, Yemen, Lanan, Bahrain, and Spain). In Arabic and English, it is called Zaitoon and Olive respectively (Farhangi *et al*., 2014). Olive trees are evergreen and can live up to 1000 years (Filippou *et al*., 2007). All over the Mediterranean region, olive tree leaves are a source of traditional medicine. Olives and their numerous components are recognized for their health-promoting functional dietary components and bioactive elements (Ghanbari *et al*., 2012). Both olive fruits and their derivatives contain a diverse variety of phenolic constituents. Phenolic alcohols, secoiridoids, flavonoids, and phenolic acids (common phenolic compounds) have been discovered in olives (in almost whole of the fruit's tissues) but their amount varies in various parts (Ryan *et al*., 2002). Antimicrobial, antioxidant, anti-carcinogenic, antiviral, and health-promoting activities are found in number of plant polyphenols and bioactive compounds (Sanchez-Moreno, 2002).

3.17 million hectares of land in Pakistan has the potential to produce olives. Pakistan's total edible oil consumption is estimated to be around 2.9 million tonnes. Imports account for around 67 % of this consumption. Annually 38 billion rupees are spent for this purpose (Azmat *et al*., 2020). Various wild olive varieties are being transformed into commercial (economic) variants in the native environment and the plantation of these variants play a substantial role in people's economic prosperity. *Olea ferruginea* Kahu (wild olive) is wild in the Pothohar and Hazara region of Pakistan; its presence indicates that it can be successfully grown here. Pothohar area has been designated as the "Olive valley" by Punjab government and they have launched various incentives to promote olive production in the area (Khanum *et al*., 2020). To enhance the production of olives, it is needed to check the quality of olives not only to meet the requirements of the country but also to compete in the international market.

For the extraction of various phytochemicals and determination of antioxidant and antifungal potential of Pakistani varieties of *O. europaea*, the basic step is extraction. A precise method of extraction of phytochemicals can save many intermediate steps, energy, and cost of quality check procedures. Among various types of extraction, microwave-assisted extraction (MAE) is one of the rapid, reliable methods and its solvent consumption is also low. It can separate the active portions or chemical constituents from plants in less time. Microwaves, which fall within the wavelength range of 300 Megahertz to 300 Gigahertz, are a promising option for targeted extraction due to their numerous advantages, such as efficient heating within a closed vessel, minimal solvent loss, no leakage of metabolites, and reduced extraction time. Furthermore, the yield of plant metabolites can be increased by

lowering the temperature and increasing the pressure within the vessel, particularly for heat-sensitive compounds (Kosar *et al*., 2007; Akhtar *et al*., 2019). As olives possess many interesting biological properties, so various bioassays can be utilized to check the quality of extracted phytochemicals from the olives grown in Pakistan. Olives and their derivatives are recognized for their health-promoting functional dietary and bioactive elements. During the development and ripening process, the phenolic compounds present in the fruit and leaves of *O. europaea* L. vary qualitatively and quantitatively (Hashmi *et al*., 2015). The phenolic components have shown a wide range of pharmacological activities (*In-vivo* and *In-vitro*) like anti-diabetic, gastroprotective, anti-oxidant, anti-convulsant, anti-inflammatory, anti-cancer, analgesic, anti-microbial, antinociceptive antiviral, and anti-hyperglycemic, and therefore, may be considered as the quality indicator for final food or medicinal product for the market (Ghanbari *et al*., 2012).

The main objective of this study was to optimize the extraction of phytochemicals (phenols, and flavonoids) using microwave-assisted techniques from fruits, seeds, and leaves of *O. europaea* var. *Earlik* (grown in Pakistan). A comparison was made on the amount of phytochemicals extracted from fruits, seeds, and leaves to evaluate the antioxidant and antifungal potential of optimized microwave-assisted extracts of *O. europaea* L. To date, not much work is conducted on the phytochemical analysis of Earlik variety of *O. europaea* of the Olive valley of Pakistan by using MAE. Also, very less data is available on the antioxidant and antifungal activity of *O. europaea* (Earlik) of Olive valley.

## Material and Methods

The present study was conducted in the Nano-Biotechnology and Biochemistry Lab. of the Botany Dept. Lahore College for Women University, Lahore Pakistan. For this study, fruits, seeds, and leaves of the Earlik variety of *O. europaea* were used.

**Plant material:** *O. europaea* was collected from Barani Agriculture and Research Institute Chakwal, Pakistan. The plant sample was washed thoroughly in running tap water. The fruit was stored at 4°C until further use. Seeds and leaves were separated from the fruit and dried under shade. After drying, it was ground into a fine powder and stored in airtight jars for future experiments. Phytochemicals were extracted using 3 solvents differing in polarity i.e., ethanol, acetone, and n-hexane.

**Microwave-assisted extraction:** The extraction was carried out by using the model MDS-6G of Sineo Microwave-Assisted Extractor. The extraction system was equipped with 6 Teflon extracting vessels which were covered with protectors. 5 vessels were for sample loading while 6th one was to maintain a temperature and pressure inside the vessels and was equipped with temperature and pressure sensor. This vessel contained solvent only, to avoid overheating and bursting. In other vessels, 1 g of plant material was taken with 10 mL of respective solvent (according to experimental design). The vessel was tightly closed with its provided lid, tightened properly, and was placed on a central moving or turning frame.

All the parameters were selected from the main panel of the extractor. Power, time, and temperature values were set according to the experimental design, and the sample was run under given conditions. MAE parameters selected for the present modeling and optimization of MAE of *O. europaea* were solvent type, time of extraction, power level, and concentration of solvent. Vials were labeled and extracts were procured from plant material, filtered in these vials, and dried under shade for further analysis.

**Total phenolic content:** The phenolic compounds were estimated by using the spectrophotometric method. In 1 mg of dry plant extract, 1 mL of respective solvent was added and shaken very well. 125 µL of this sample was taken in a test tube. Folin-Ciocalteu (FC) assay was the principle technique used in the present study to analyze the phenolic content of extracts. In a test tube, 125 µL of FC reagent and 500 µL of d.$H_2O$ (distilled water) were added to dissolve 125 µL of sample extract. The mixture was thoroughly mixed before adding 1.25 mL of 7% sodium carbonate ($Na_2CO_3$) that was combined with d.$H_2O$ (distilled water) to make a final volume of 3 mL. Test tubes were incubated for 90 minutes in the dark. The absorbance at 760 nm was measured in comparison to the prepared blank. Through a calibration curve with Gallic acid, the phenolic levels were reported as mg of Gallic acid which was equivalent to per gram of dry weight (mg GAE/g) (Rebey *et al*., 2012).

**Total flavonoid content test:** Flavonoids were estimated by the spectrophotometric method. In 1 mg of dry plant extract, 1 mL of respective solvent was added and shaken very well. 250 µL of this sample was taken in a test tube. In a test tube, 1.25 mL of d.$H_2O$ (distilled water) and 75 µL of 5% sodium nitrite ($NaNO_2$) were added. After 5 minutes, 150 µL of 10% aluminum chloride (AlCl3) was added. After 6 minutes the solution was prepared in the test tube using 500 µL of 1 molar (1 M) sodium hydroxide (NaOH) and 275 µL of distilled water. The solution was homogenized, and the absorbance pattern for all samples was measured at 510 nm. The standard curve was calculated using catechin, and the results were presented in µg of Catechin equivalent (CEs) per mg of extract (Patel *et al*., 2010).

**Research design:** In this study, the Design Expert software (Version 11) was utilized for the implementation of Response Surface Methodology (RSM). Central Composite Design (CCD) was applied to generate a design comprising 60 experimental runs. It was used to optimize MAE parameters and their effect on TPC and TFC. The parameters/factors selected for extraction were (A) time (1-10 minutes), (B) power level (300 - 1000 W), (C) solvent ratio (8.65-10 mL), and (D) solvent type (Polar and Non-polar) with plant material. The relationship of these dependent and independent variables was established by applying a quadratic polynomial regression model as explained in the following equation where "γ" is the predicted response:

$$\text{Equation 1: } \gamma = \beta_0 + \beta_{1A} + \beta_{2B} + \beta_{3C} + \beta_{4D} + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC$$

Predicted values are related to intercept ($\beta_0$), linear coefficients ($\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$), squared coefficients ($\beta_{11}$, $\beta_{22}$, $\beta_{33}$, $\beta_{44}$) and interactive coefficients ($\beta_{12}$, $\beta_{13}$, $\beta_{23}$) by Equation 1. The quality of fit for the model was checked by Analysis of Variance (ANOVA) at a 5% level of significance.

**Antioxidant activity:** The antioxidant activity of the optimized MAE extract was measured using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay technique with minor modifications of the protocol of Alhakmani *et al*., (2013). Microwave-assisted fruit, seed, and leaf extracts of *O. europaea* var. *Earlik* which contained maximum phenolics were checked for antioxidant activity. For that purpose, 1mL of extract was mixed with 1 mL of 1 mM DPPH solution. The test tubes were wrapped in aluminum foil and set aside for 30 minutes in the dark. A double-beam spectrophotometer was used to measure the optical density (color intensity) of each solution at 517 nm. A blank solution of DPPH with methanol was prepared similarly. The percentage of DPPH inhibition was calculated by using the equation.

$$\% \text{ DPPH inhibition} = (Ac - As/ Ac)\ 100$$

"Ac" refers to the absorption of methanol + DPPH, As refers to the absorption of sample + DPPH. This assay was done to compare the optimized MAE extracts with standard antioxidants.

**Antifungal activity:** Antifungal activity of MAE of fruits, seeds, and leaves with maximum amount of phenolics was determined against *Aspergillus flavus*. Malt extract agar (MEA) (2%) was made in a 250 mL flask by adding 2 g of ME (malt extract) in 60 mL of $d.H_2O$ (distilled water) and autoclaved for 30 minutes at 121°C. 0.5% w/v concentration of ethanolic olive extract was made by adding 10 mL stock solution in 95 mL of MAE medium. Dilutions were made with the strengths 0.5, 1.0, and 1.5%. There was no plant extract in the control treatments. To prevent bacterial contamination, each concentration was given an Amoxil capsule 125 mg per 100 mL. All the concentrations were replicated twice. The inoculum of *A. flavus* was placed separately in the center of each flask. After 7 days, the fungal growth was measured by filtering the solution of each concentration through pre-weighed Whatman no. 1 filter paper (Anum *et al*., 2021). The test fungal biomass was measured by using this formula:

$$\% \text{ Age growth inhibition} = \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \text{ x } 100$$

**GC-MS Analysis:** Hexane fractions of MAE of fruit, seed, leaves, and esterified oil of *O. europaea* var. *Earlik* were subjected to GC-MS analysis. GC-MS was performed to detect the existence of non-polar components on Agilent technology GC-7890B, fitted with Agilent DB 5MS column of 30 m length, 0.25 mm dia, and 0.25 µm film thickness, coupled with Agilent MS 5977A. As a carrier gas, which flows at a rate of 1.0 mL per minute, ultra-high pure helium gas (99.9999%) was utilized. Acquisition General method, 280 °C MS transfer line temperature, 230°C Ion source temperature, Ionization mode was EI, Temperature: Initial 50°C hold for 1 min, Ramp at 25°C to 120°C for 5 mins. Total Run time was 51.133 mins (a slightly modified protocol of Abdelrahman *et al*., (2019).

The GC retention indices, mass spectra, and retention times of non-polar components in n-hexane fractions of MAE extracts and esterified olive oil were analyzed. Comparison with published literature and a library search of mass spectra were also performed to identify these components.

**Statistical analysis**

Results of experimental work were collected. Design Expert software 11th version was used to apply Response Surface Methodology (RSM). To assess the significance of the results, Analysis of Variance (ANOVA) was conducted at a 5% significance level, followed by Duncan's New Multiple Range Test. 3D contour graphs for amounts of phenolics and flavonoids were obtained to analyze the interactive effects of various parameters on amounts of phenolics and flavonoids.

**Results and Discussion**

**Phenolic and flavonoid content:** Four key elements include solvent type, solvent ratio, power level, and time. RSM was employed to optimize the selected factors using Central Composite Design (CCD). RSM is used to optimize various parameters in experiments. The fruit extraction was done on all 60 experimental runs generated by RSM (Table 1). As the experimental work had different levels of factors, these factors showed different responses according to the level of factor.

In Graphs, factors are coded as A = power level, B = time, C = solvent ratio, and D = solvent type. Model F-value for phenolics was 11.04 and for flavonoids was 15.50, which showed the significance of the model. A P-value of model for both phenolics and flavonoids was (0.0500) which was significant. The significance of the coefficients was evaluated, and factors or combinations of factors that had a P-value less than 0.05 were considered significant for the dependent variable's yield. The model was not able to describe our response if values were greater than 0.1000.

Furthermore, the value of Adjusted $R^2$ for phenolics was 0.8315 and for flavonoids was 0.8769 meaning that model could describe our experiment by 83% and 87% respectively, and the value of Predicted $R^2$ were 0.6469 for phenolics and for flavonoids 0.6807 meaning that this model could predict by 64% and 68% accuracy, which was good for applied statistics. The predicted and adjusted $R^2$ values were nearer to each other. Adequate precision value is 17.8534 for phenolics while for flavonoids it was 18.4756 which is a ratio of signal to noise, the signal-to-noise ratio was calculated to assess the model's ability to navigate the design space, and a high ratio was observed, indicating that the model was reliable. Additionally, the

CV % indicated good reproducibility of the results. Three-dimensional (3D) contour graphs were utilized to investigate the impact of each factor on the response variables, namely phenolic and flavonoid content of *O. europaea* fruits (Figs. 1 & 2) Power, extraction time, solvent ratio, and solvent type, these factors had a notable impact on the phenolic yield as depicted from figure 1. Each contour graph depicted the impact of two independent variables on the dependent variable's response, including the maximum response.

Optimized conditions for the maximum amount of phenolics (275.79 µg) were found to be at run no. 5 i.e., power level as 600 W, time as 10.3 min, 9.5 mL solvent ratio, and ethanol as a solvent. Whereas the minimum amount of phenolics (62.78 µg) was found at run # 9 i.e., 300 W, 4.5 min, 9.5 mL solvent ratio, and n-hexane as a solvent. Additionally, the results were found to be close to the predicted values, indicating that the model was reliable (Table 1). Optimal conditions for a maximum amount of flavonoids (209.895 µg) were found to be a power level of 600 W, time as 4.5 min, 9.5 mL solvent ratio, and n-hexane as a solvent. Whereas minimum amount of flavonoids (12.705 µg) were found to be power level as 300 W, time as 4.5 min, 9.5 mL solvent ratio and n-hexane as a solvent. Additionally, the results were found to be close to the predicted values, indicating that the model is reliable (Table 1).

Optimized MAE parameters of fruit extracts from Run # 5 were applied to seed and leaf extract. Fig. 3(a) showed a comparison between the amount of phenolics extracted from fruits, seeds, and leaves of Earlik variety with 3 different solvents (ethanol, acetone, and n-hexane). The amount of phenolics was higher in the ethanolic extract of leaves (331.56 µg) compared to other solvents and plant parts. The amount of phenolics in MAE of fruits, seeds, and leaves with non-polar solvent (n-hexane) in the lower compared to polar solvents (ethanol, and acetone). Same trend was also observed phenolic content of plant parts i.e., shown in Fig. 3(b). It showed a comparison between the amount of flavonoids extracted from fruits, seeds, and leaves of Earlik variety with 3 different solvents (ethanol, acetone, and n-hexane). The amount of flavonoids was higher in the ethanolic extract of leaves (335.65 µg) compared to seeds (279.63 µg), and fruits (208.95 µg). Overall, the maximum amount of flavonoids was higher in ethanolic extract of leaves was compared to fruits and seeds.

**Table 1. Report of results of phenolics extracted from fruit of *O. europaea* var. *earlik*.**

| Run order | Phenolics (GAE) | | Flavonoids (QE) | | Run order | Phenolics (GAE) | | Flavonoids (QE) | |
|---|---|---|---|---|---|---|---|---|---|
| | Actual value | Predicted value | Actual value | Predicted value | | Actual value | Predicted value | Actual value | Predicted value |
| 1 | 220.00 | 223.93 | 200.97 | 190.46 | 31 | 157.32 | 160.23 | 140.33 | 127.05 |
| 2 | 150.00 | 171.31 | 193.83 | 197.16 | 32 | 169.36 | 176.61 | 70.770 | 54.070 |
| 3 | 168.06 | 172.48 | 167.16 | 157.49 | 33 | 161.11 | 171.31 | 180.39 | 197.16 |
| 4 | 156.89 | 160.23 | 158.24 | 127.05 | 34 | 169.00 | 160.23 | 134.61 | 127.05 |
| **5** | **275.79** | **271.70** | **136.81** | **143.50** | 35 | 254.19 | 255.10 | 175.66 | 164.53 |
| 6 | 130.99 | 134.09 | 56.00 | 72.710 | 36 | 140.22 | 133.40 | 174.72 | 161.88 |
| 7 | 172.26 | 165.30 | 140.28 | 151.70 | 37 | 167.33 | 167.55 | 133.00 | 144.76 |
| 8 | 153.99 | 160.29 | 196.56 | 208.440 | 38 | 164.00 | 163.42 | 100.00 | 105.32 |
| **9** | **62.780** | **74.66** | **12.710** | **16.210** | 39 | 247.00 | 258.04 | 176.93 | 187.94 |
| 10 | 149.93 | 163.42 | 120.96 | 105.32 | 40 | 167.33 | 163.42 | 91.350 | 105.32 |
| 11 | 130.00 | 132.91 | 129.36 | 137.05 | 41 | 179.22 | 169.53 | 101.850 | 102.47 |
| 12 | 202.71 | 204.56 | 122.11 | 126.27 | 42 | 236.00 | 225.19 | 190.00 | 177.88 |
| 13 | 153.99 | 142.37 | 98.910 | 106.27 | 43 | 157.18 | 171.41 | 90.410 | 91.59 |
| 14 | 171.97 | 160.18 | 50.510 | 58.990 | 44 | 153.00 | 162.23 | 145.00 | 140.29 |
| 15 | 160.51 | 163.42 | 120.86 | 105.32 | 45 | 163.13 | 160.23 | 114.45 | 127.05 |
| 16 | 150.00 | 141.03 | 143.64 | 145.09 | 46 | 155.88 | 160.23 | 108.47 | 127.05 |
| 17 | 260.27 | 266.93 | 150.57 | 133.05 | 47 | 230.00 | 218.53 | 154.00 | 169.56 |
| 18 | 111.65 | 125.04 | 145.22 | 145.42 | 48 | 158.78 | 160.23 | 108.05 | 127.05 |
| 19 | 145.72 | 158.72 | 100.00 | 81.920 | **49** | **181.54** | **171.31** | **207.59** | **197.16** |
| 20 | 213.00 | 201.49 | 99.220 | 110.00 | 50 | 155.00 | 163.67 | 201.81 | 190.44 |
| 21 | 156.75 | 163.42 | 98.810 | 105.32 | 51 | 177.19 | 172.11 | 119.91 | 129.75 |
| 22 | 150.07 | 160.96 | 55.230 | 64.780 | 52 | 241.00 | 235.79 | 134.19 | 137.14 |
| 23 | 169.50 | 175.53 | 113.40 | 113.28 | 53 | 183.13 | 163.42 | 101.95 | 105.32 |
| 24 | 152.00 | 147.46 | 139.00 | 131.07 | 54 | 161.76 | 171.31 | 187.43 | 197.16 |
| 25 | 124.12 | 120.35 | 91.880 | 104.36 | 55 | 149.35 | 154.49 | 119.49 | 110.65 |
| 26 | 214.02 | 220.81 | 164.96 | 154.33 | 56 | 129.34 | 115.84 | 108.99 | 100.94 |
| 27 | 153.26 | 142.69 | 105.73 | 97.640 | **57** | **221.00** | **171.31** | **209.90** | **197.16** |
| 28 | 179.22 | 174.76 | 80.010 | 82.080 | 58 | 193.33 | 199.45 | 182.81 | 183.06 |
| 29 | 198.00 | 186.58 | 40.00 | 50.410 | 59 | 169.00 | 178.39 | 61.840 | 65.330 |
| 30 | 155.00 | 171.31 | 203.91 | 197.16 | 60 | 180.38 | 172.58 | 83.890 | 94.330 |

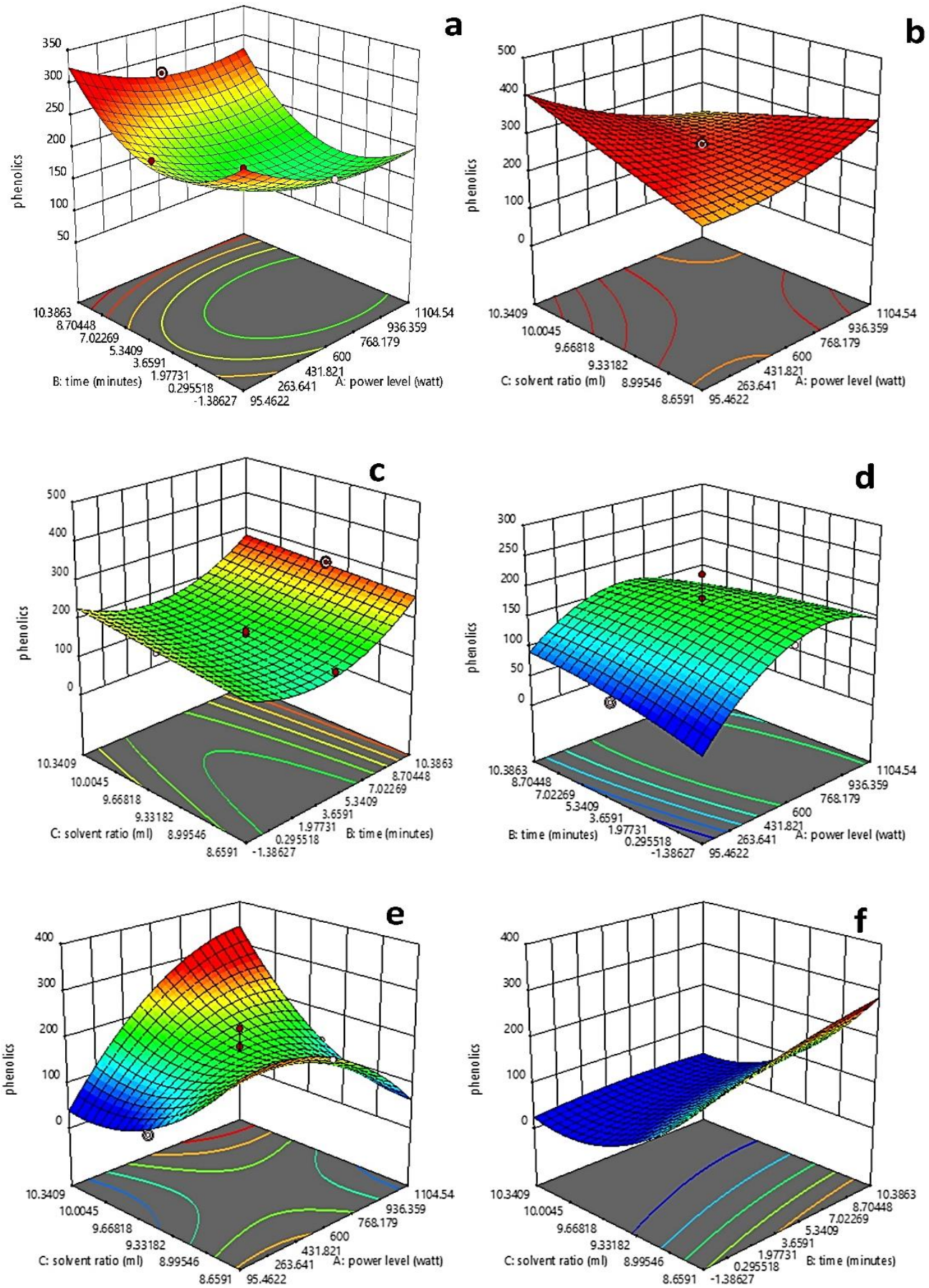



Fig. 1. 3D contour graph for reduced cubic model of phenolics extracted from fruit of *O. europaea* var. Earlik Interaction between time and power level b) Interaction between level and solvent ratio c) Interaction between solvent ratio and time for run # 5 d) interaction between time and power level e) Interaction between solvent ratio and power level f) Interaction between solvent ratio and time for run # 9.

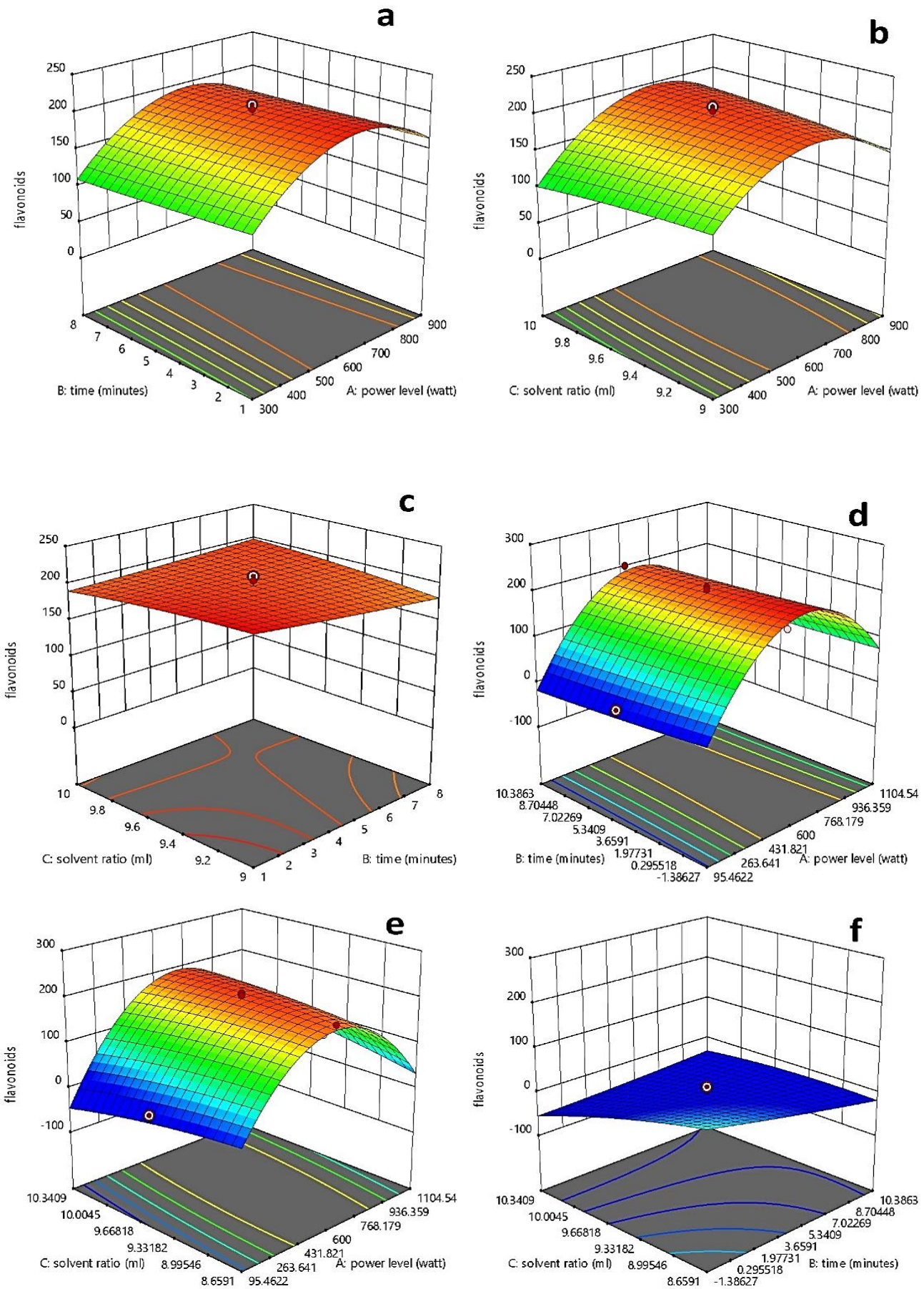



Fig. 2. 3D contour graph for reduced cubic model of flavonoids extracted from fruit of *O. europae*a var. Earlik Interaction between time and power level b) Interaction between power level and solvent ratio c) Interaction between solvent ratio and time for run # 5 d) interaction between time and power level e) Interaction between solvent ratio and power level f) Interaction between solvent ratio and time for run # 9.

**Table 2. ANOVA for MAE of olives.**

| Source | Phenolics | | Flavonoids | |
|---|---|---|---|---|
| | F-value | p-value | F-value | p-value |
| **Model** | 11.04 | < 0.0001* | 15.5 | < 0.0001* |
| A-power level | 2.28 | 0.1417 | 2.53 | 0.1223 |
| B-time | 7.95 | 0.0085* | 0.0188 | 0.8919 |
| C-solvent ratio | 5.24 | 0.0293* | 5.64 | 0.0241* |
| D-solvent type | 12.58 | 0.0001* | 50.98 | < 0.0001* |
| AB | 0.2146 | 0.6465 | 16.72 | 0.0003* |
| AC | 2.24 | 0.1453 | 4.71 | 0.0381* |
| AD | 19.48 | < 0.0001* | 14.11 | < 0.0001* |
| BC | 1.27 | 0.2693 | 2.5 | 0.1242 |
| BD | 7.23 | 0.0027* | 1.43 | 0.2548 |
| CD | 3.95 | 0.03* | 1.72 | 0.1957 |
| ABD | 1.25 | 0.3003 | 6.03 | 0.0063* |
| ACD | 40.58 | < 0.0001* | 10.79 | 0.0003* |
| BCD | 0.3661 | 0.6965 | 10.67 | 0.0003* |
| Lack of Fit | 0.7185 | 0.7351 | 1.23 | 0.3454 |

*Indicates significance at 5% p, whereas values of Adjusted $R^2$ =0.8315, predicted $R^2$= 0.6469, Adequate precision = 17.85 for phenolics and Adjusted $R^2$ =0.8769, predicted $R^2$= 0.6807, Adequate precision = 18.47 for flavonoids are described

It is clear from the results that the amount of phenolics and flavonoids vary along with variation in factors and their combinations. In MAE due to microwaves, there was an internal pressure of cell sap and metabolites on the cell wall and cell membrane on optimized temperature and pressure (Masood *et al*., 2021; Khanum *et al*., 2020; Jiangseubchatveera *et al*., 2017). Moisture of plant extract also provides aid to the entire process. This led to a rapid rupture of cells at a certain power and temperature which caused an earlier release of plant metabolites such as phenolics into the surrounding solvent than in conventional methods. These results were confirmed by other research works (Akhtar *et al*., 2020; Ghaffar *et al*., 2020; Hayat *et al*., 2009). The whole process was carried out in the close vessels so there was higher contact of solvent and plant matrix, which helped in softening of plant tissues and cell walls. Results indicated that on increasing the power level, temperature, and time of extraction, the amount of extract was increased but up to a certain level. Previous research also demonstrated the enhanced efficiency of microwave-assisted extraction for the extraction of plant-based metabolites. (Liazid *et al*., 2011). In microwave-assisted extraction, the interaction of power level and timing of exposure was a very significant factor. As shown by the present study (Table 2), that it was a significant combination for the amount of flavonoids, while an interaction of power and solvent type was significant for both responses. The power level is responsible for providing energy to the molecules present inside and outside of plant cells. Too much power level can cause the burning of the plant sample or heat degradation of the phyto-constituents. Higher power levels used in microwave-assisted extraction can cause the abrupt rupture of cells, which is ideal for extracting targeted compounds. The moisture inside the cells absorbs the microwaves, which generates internal heat and pressure within the cell environment. This added pressure can cause the cell walls to burst, resulting in the early release of the targeted compounds. This is in contrast to conventional heating methods that rely on surface heating only (Akhtar *et al*., 2019). Therefore, an optimized combination of power and time can give higher phenolics and flavonoids. It has been endorsed by the results of Alu'datt *et al*., 2011, Shahid *et al*., 2021 and Kishimoto 2022 on MAE of seeds of olive, fruits of watermelon, and olives respectively.

The solvent type was also significant for both responses for the MAE of olives. Solvent and its ratio to the plant material was another significant factor as reported by Zhang *et al*., 2008 and Hayat *et al*., 2009, in earlier studies for MAE. Each solvent has a specific dielectric constant that is responsible for its heating in the MAE. Polar solvents can cause too much heating, whereas non-polar solvents have lower dielectric constants and they cannot absorb too much microwave energy. Therefore, they may be even transparent to microwave energy as well. Therefore, in the present study, it can be related as the highest phenolics and flavonoids were obtained in ethanol whereas the minimum was obtained in n-hexane extract (Table 1).

Thus conditions of runs # 5 and 9 were also provided to other olive plant samples i.e., leaves and seeds to get the extracts from them as well. Microwave-assisted extraction has demonstrated its effectiveness in extracting plant metabolites due to its simplicity, cost-effectiveness, and reduced energy and power consumption without any damage (Zheng *et al*., 2009).

**Antioxidant activity:** Antioxidant potential of MAE (fruits, seeds, and leaves) of *O. europaea* var. *Earlik* with maximum phenolics with 3 solvents (ethanol, acetone, and n-hexane) to determine the quality of phytochemicals. Fig. 4 shows a comparison between antioxidant activities of the MAE of fruit, leave, and seed samples with standard scavenging activity of Ascorbic Acid on DPPH. According to Figure 4, ethanolic Microwave-assisted extracts of fruit and leaves of olives showed the highest antioxidant activity that is not significantly different from that of standard. Percent DPPH inhibition of MAE for the fruit sample is 40%, acetone is 34% and n-hexane is 32 %. Similar trend was seen for seed sample and leaf sample. This analysis was carried out to check whether phenolics and flavonoids extracted from olive samples through microwave extraction were biologically active or not. Debib & Boukhatem, 2017 revealed that the main source of antioxidants in olive leaves (gathered from west Algeria) are their phenolic components. It is clear from the results that phytochemicals extracted from MAE are not damaged they are playing their role actively. It was also evident from present research work that leaves contained more phenolics and showed more antioxidant potential. The phenolic compounds can enhance the antioxidant activity by several potential pathways. The principal method is free radical scavenging in which the phenolic molecules can break the free radical chain reaction. MAE helps to extract phenolic in a shorter period which in turn enhance antioxidant potential. The high antioxidant potential of MAE olive fruits, seeds, and leaves can be well utilized as a functional food and its high medicinal use in diseases is due to its antioxidants (Cosme *et al*., 2020; Lee & Lee, 2010).

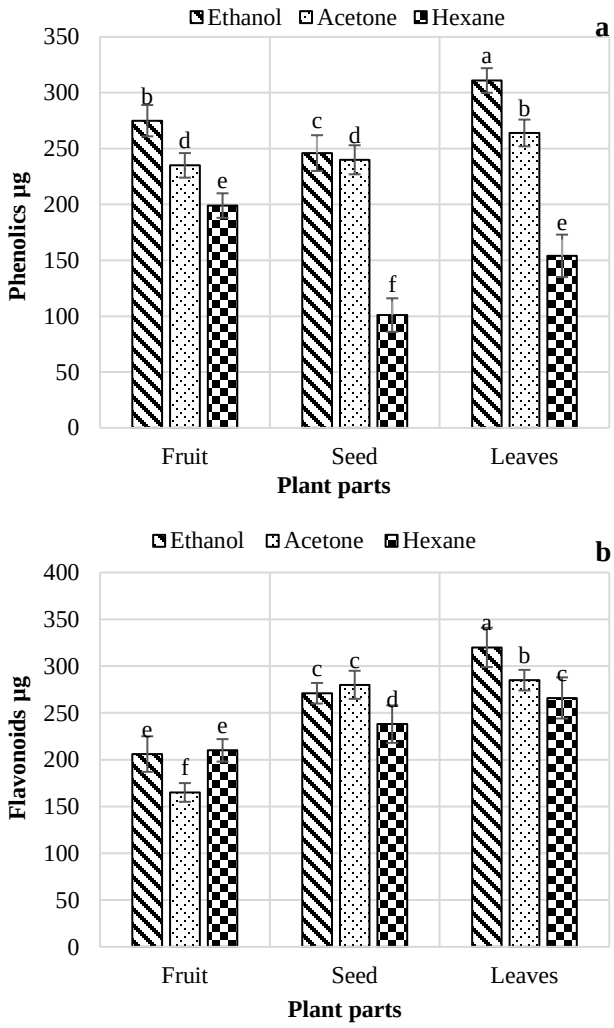



Fig. 3. Comparison of maximum a) phenolics b) flavonoids extracted from *O. europaea* var. Earlik.

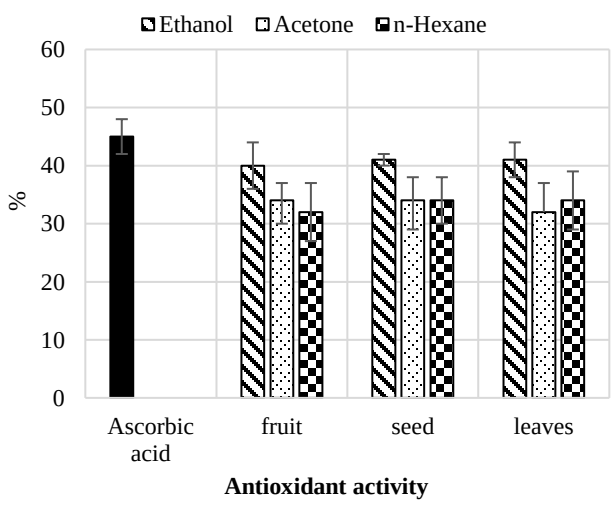



Fig. 4. Antioxidant potential of MAE (fruits, seeds, and leaves) of *O. eruopaea* var. earlik using DPPH.

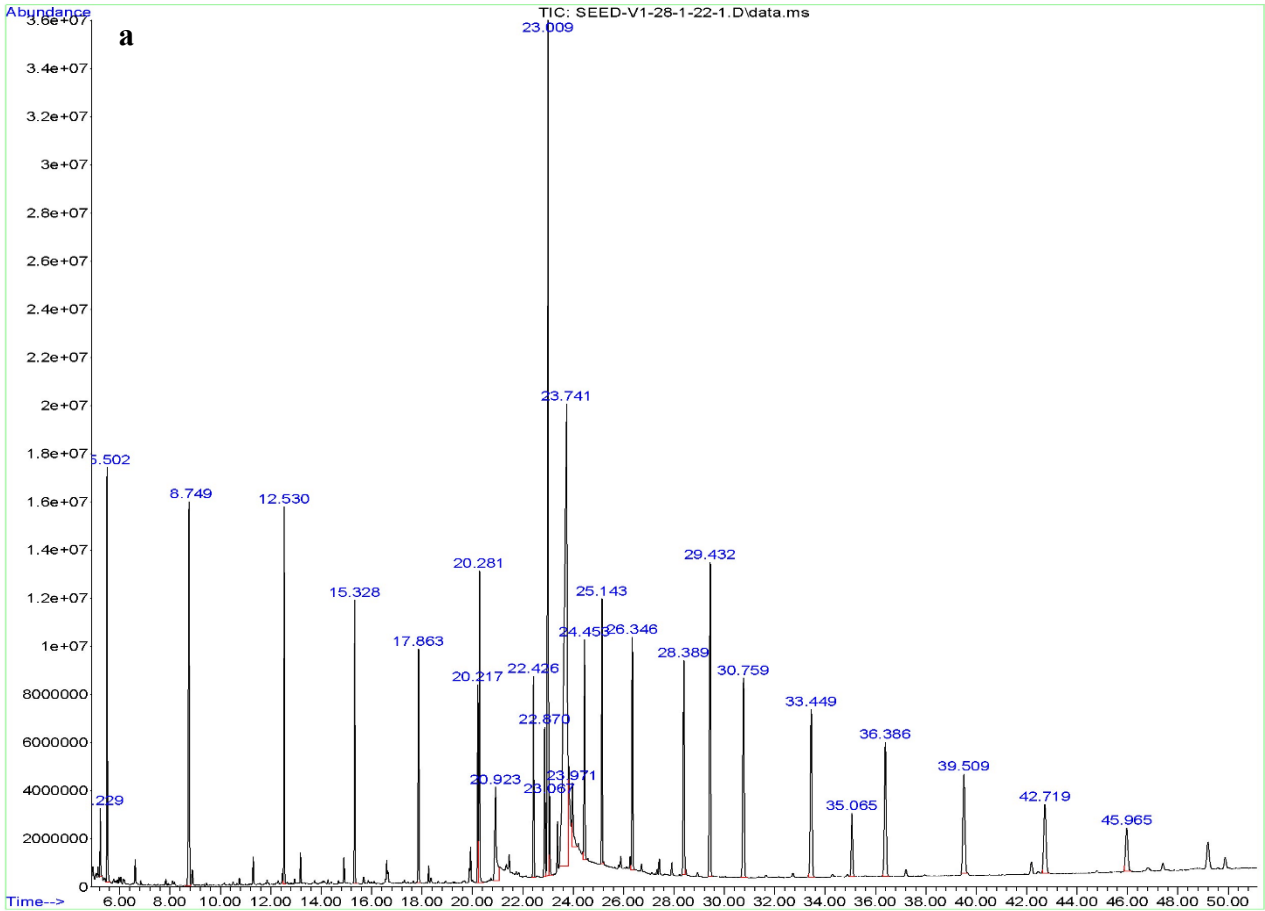
**Antifungal activity:** Antifungal potential of microwave-assisted fruit, seed, and leaf extract of *O. europaea* var. Earlik was measured against *A. flavus*. As the concentration of extracts increased, their antifungal activity was also increased, maximum antifungal activity was seen in the case of 1.5% MAE leaves (31.8%) compared to seed (21.5%), and fruit (11.3%) (Fig. 5). While in 0.5 % MAE of fruit, seed, and leaves of *O. europaea* var. Earlik inhibited the growth of *A. flavus* by 5.6%, 7.9%, and 11.3%. The MAE of fruit showed the least antifungal potential while MAE of the leaf showed the highest antifungal potential. This could be related to the fact that leaf extract has shown more phenolics. Korukluoglu *et al*. (2008) reported that pure phenolic compounds of olive leaf extract had strong antifungal potential against *A. wentii.* The antifungal resistance depends on the active components present in the extracts and also depends on the genus, species, strain, and source of isolation. MAE of fruit, seeds and leaves contained phenolic and flavonoids as active constituents which enhanced the antifungal potential of the extracts. The results suggested that olive leaf extract, as a natural preservative, could be an alternative to synthetic antifungal substance (Korukluoglu *et al*., 2006).

MAE helps to extract more phytochemicals in less time than any other conventional method and these phytochemicals contribute vigorously to antifungal activity as shown in the results. It also shows that fruits have not much antifungal activity. Therefore, it can be claimed that the MAE of olives can be used for rapid quality checks of olives.

**GC-MS analysis:** The primary chemical constituents obtained from the n-hexane fraction of MAE (fruit, leave, and seed) and extra virgin olive oil of *O. europaea* var. Earlik are given in Table 3. Retention time and MS peaks were compared to identify the various compounds. In GC-MS analysis of n-hexane fraction of fruit, seed, leaf extract, and extra virgin olive oil (EVOO), 20, 21, 16, and 8 compounds were identified that represented their composition in *O. europaea* var. Earlik. The major constituents of the extract were identified as tetracosamethyl-cyclodedacsiloxane, Tetracosane, Oleic acid, 9, Octadecanoic acid, methyl ester, Squalene, Hexadecanoic acid, and methyl ester. Other compounds were present but in trace amounts (Table 3 and Fig. 6). The results of the present study demonstrated that MAE was highly effective in extracting non-polar components, even from olive seeds. Non-polar components are typically volatile and can be easily degraded or evaporated under high heat. MAE offers a solution to these issues with its closed-container process and controlled temperature options, which prevent the boiling of the solvent. Additionally, the shorter extraction time in MAE helps to avoid the degradation of key components (Akhtar *et al*., 2019). Similar chromatographic analysis had been done for olive plants of different countries and various biomolecules were evaluated (Goldsmith *et al*., 2015, Zoric *et al*., 2016). GC-MS analysis revealed that olive oils contained different biologically active compounds thus olive plants, besides their antioxidant and antifungal activities could also be used to produce different pharmaceutical products to cure diseases including cancer (Ahmad *et al*., 2017). GC-MS is a useful method for the determination of non-polar components due to its high speed, resolution, and sensitivities (Sudjana *et al*., 2009, Khlif *et al*., 2015).

**Table 3. Comparison of non-polar compounds of hexane fraction from MAE extract of fruit, seed, leave and oil of *Olea europaea* var. Earlik.**

| RT | RI | Compound | Percent in | | | |
|---|---|---|---|---|---|---|
| | | | Leaf | Seed | Fruit | Oil |
| 5.229 | 915 | Undecane | 0.52 | 0.62 | 0.86 | -- |
| 5.497 | 927 | Cyclopentasiloxane, decamethyl | -- | -- | -- | 2.32 |
| 5.502 | 928 | Cyclopentasiloxane, decamethyl | 3.22 | 3.66 | 4.14 | -- |
| 8.743 | 1040 | Cyclohexasiloxane, dodecamethyl | 4.85 | 5.53 | 5.85 | 2.97 |
| 12.525 | 1052 | Hexasiloxane dodecamethyl | 3.15 | -- | -- | -- |
| 12.53 | 1143 | 3-Butoxy-1,1,1,7,7,7, hexamethyl-3,5,5-tris tetrasiloxane | -- | 3.54 | 3.86 | 2.42 |
| 15.322 | 1211 | Cyclooctasiloxane, hexadecamethyl | 2.49 | 2.85 | 3.09 | -- |
| 17.863 | 1271 | Cyclononasiloxane, octaadecamethyl | 2.27 | 2.64 | 2.94 | -- |
| 19.895 | 1319 | 9-Hexadecanoic acid, methyl ester | -- | -- | --- | 2.15 |
| 20.211 | 1327 | Cyclononasiloxane, octaadecamethyl | 1.97 | 2.24 | 2.45 | -- |
| 20.27 | 1328 | Hexadecanoic acid, methyl ester | 0.86 | -- | 1.96 | -- |
| 20.28 | 1328 | Hexadecanoic acid, methyl ester | -- | 3.38 | -- | 14.8 |
| 20.923 | 1343 | n-Hexadecanoic acid | -- | 2.28 | -- | -- |
| 22.414 | 1378 | Cyclooctasiloxane, hexadecamethyl | 2.01 | 2.32 | 2.55 | -- |
| 22.864 | 1388 | 9,12 Octadecanoic acid, methyl ester | -- | 1.8 | 0.75 | -- |
| 22.971 | 1391 | Octadecanoic acid, methyl ester | 2.73 | -- | -- | -- |
| 22.982 | 1391 | 9,Octadecanoic acid, methyl ester | -- | -- | 5.66 | -- |
| 23.003 | 1391 | 9,Octadecanoic acid, methyl ester | -- | 11.6 | -- | -- |
| 23.062 | 1393 | cis-13-Octadecanoic acid, methyl ester | -- | 0.77 | -- | -- |
| 23.548 | 1402 | Oleic acid | 0.43 | -- | 1.23 | -- |
| 23.741 | 1409 | Oleic acid | -- | 19.8 | -- | -- |
| 23.816 | 1410 | 1-Propene-1,2,3-tricarboxylic acid | 0.94 | | 1.03 | -- |
| 23.971 | 1414 | cis Vaccenic acid | -- | 1.13 | -- | -- |
| 24.442 | 1427 | Cyclononasiloxane, octaadecamethyl | 2.34 | 2.76 | -- | -- |
| 24.442 | 1427 | Morphine,2TMS derivative | -- | -- | 2.99 | -- |
| 25.137 | 1443 | Butyl citrate | 3.49 | 2.93 | 3.63 | -- |
| 25.806 | 1459 | Glycidyl palmitate | -- | -- | 1.38 | -- |
| 26.346 | 1472 | Cyclooctasiloxane, hexadecamethyl | 2.52 | 2.98 | 3.33 | -- |
| 27.416 | 1497 | Phenol,2,-2' methylene bis | -- | -- | 0.71 | -- |
| 28.389 | 1520 | Tetracosamethyl- cyclododecasiloxane | 2.95 | 3.43 | 4.21 | -- |
| 28.491 | 1522 | 9-Octadecanoic acid(2)-, Oxiran | -- | -- | 4.03 | -- |
| 29.432 | 1548 | 2 Bis (2-ethylhexyl) pthalate | -- | 5.04 | 6.12 | -- |
| 29.437 | 1549 | 1 Bis (2-ethylhexyl) pthalate | 6.48 | -- | -- | -- |
| 30.753 | 1581 | Tetracosamethyl- cyclododecasiloxane | 3.34 | -- | 4.41 | -- |
| 30.759 | 1592 | Tetracosamethyl- cyclododecasiloxane | -- | 3.91 | -- | -- |
| 30.887 | 1584 | Tetracosamethyl- cyclododecasiloxane | -- | -- | -- | 2.35 |
| 31.657 | 1603 | 1-Cyclohexyldimethylsiloxy-2-methylpropane | -- | -- | 0.61 | -- |
| 32.716 | 1633 | Tetraconose, Heptaconose, Octadecane | 0.54 | -- | -- | -- |
| 33.444 | 1651 | Tetracosamethyl- cyclododecasiloxane | 3.37 | 3.82 | 4.28 | 2.81 |
| 35.075 | 1693 | Squalene, Supraene | 3.3 | 1.28 | -- | -- |
| 35.112 | 1694 | Squalene, Supraene | -- | -- | 18.3 | 70.2 |
| 36.386 | 1730 | Tetracosamethyl- cyclododecasiloxane | -- | 3.4 | -- | -- |
| 36.391 | 1727 | Tetracosamethyl- cyclododecasiloxane | 2.88 | -- | -- | -- |
| 36.391 | 1807 | Tetracosamethyl- cyclododecasiloxane | -- | -- | 3.9 | -- |
| 37.262 | 1754 | Heneicosane, Tetracosane, Tricosane | 7.57 | -- | -- | -- |
| 39.509 | 1814 | Tetracosamethyl- cyclododecasiloxane | 2.42 | 2.71 | 3.27 | -- |
| 39.659 | 1818 | Triacontane, Tetraacosane, Hexadecane, 1-iodo | 1.06 | -- | -- | -- |
| 42.312 | 1896 | Tetracosane, Octadecane, Docosane, 11 butyl | 13.9 | -- | -- | -- |
| 42.504 | 1901 | Vitamin E | 3.84 | -- | -- | -- |
| 42.719 | 1907 | Tetracosamethyl- cyclododecasiloxane | -- | 2.16 | 2.45 | -- |
| 44.799 | 1971 | Heneicosane, Eicosane, Tetratetraconate | 1.9 | -- | -- | -- |
| 45.959 | 2005 | Tetracosamethyl- cyclododecasiloxane | 1.38 | -- | -- | -- |
| 45.965 | 2057 | Tetracosamethyl- cyclododecasiloxane | -- | 1.4 | -- | -- |
| 46.805 | 2030 | Sitosterol | 1.28 | -- | -- | -- |
| 47.505 | 2055 | Tricosane, Octadecane, Tritriocontane | 9.99 | -- | -- | -- |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17.863
28.389
20.217
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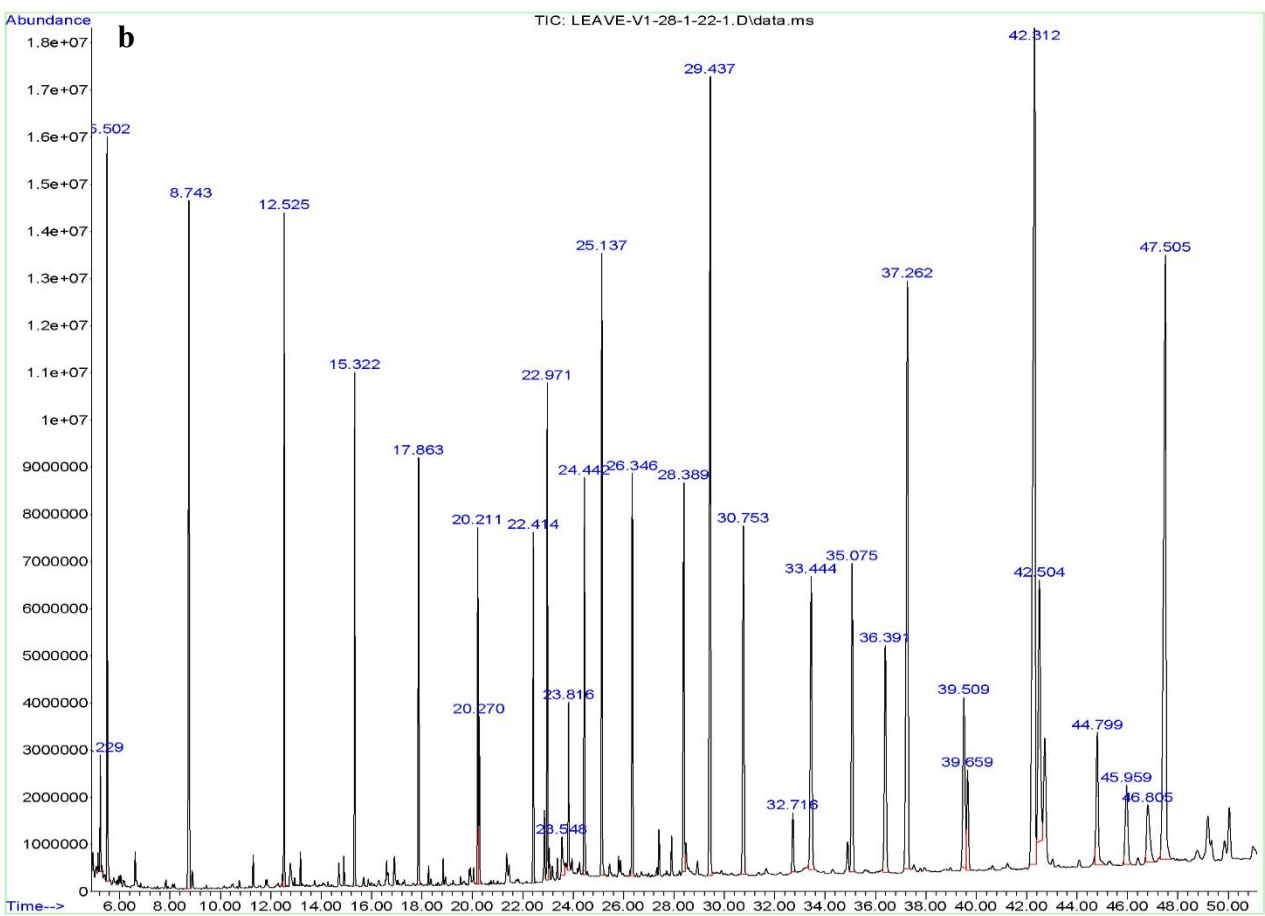
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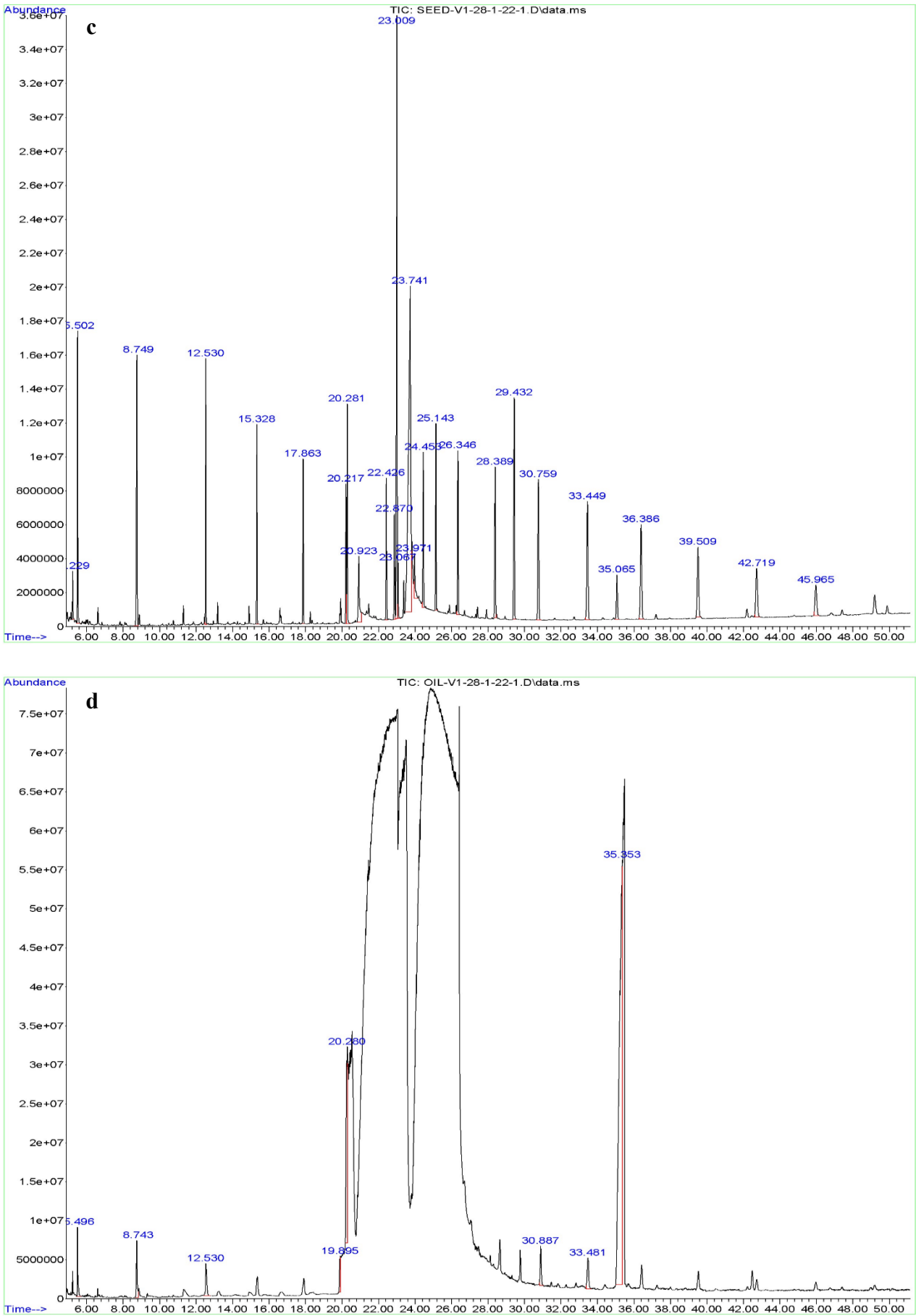


Fig. 6. GC-MS analysis of n-hexane fraction of MAE of (a) fruit (b) leave (c) seed, and (d) of esterified oil of *O. europaea* var. Earlik.

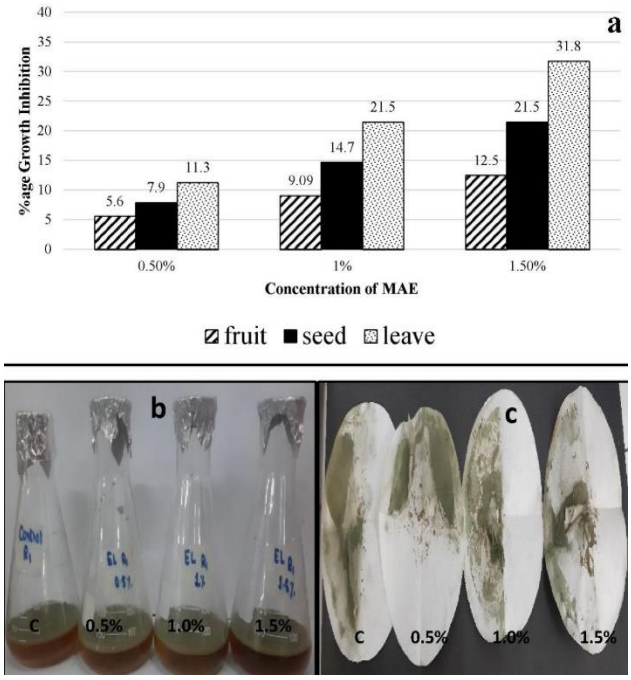


Fig. 5 a-c. Antifungal potential of MAE (fruits seeds, and leaves) of *O. europaea* var. Earlik against *A. flavus.*

## Conclusion

The results of the present study have proved that various plant parts of *O. europaea* have significant amounts of phenolics, flavonoids, and non-polar constituents that can be extracted efficiently using MAE. An optimized protocol for extraction of olive parts using MAE has been established that requires just 3 minutes of microwave heating. This protocol was used with three solvents and produced the same results/trend for all parts of plants used. Therefore, it is concluded that MAE is an efficient and rapid protocol for extraction of phytochemicals from *O. europaea* var. Earlik. In future, these optimized parameters can be utilized to analyze phytochemicals and their role in biological activities in other varieties of *O. europaea*. The present study suggested that by cultivating Pakistani olive varieties, the economy of Pakistan would be strengthened by exporting these olives and their products and to check the quality of olives MAE is the best method.

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