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# Characterization of Olive Oil By-products: Antioxidant Activity, Its Ability to Reduce Aflatoxigenic Fungi Hazard and Its Aflatoxins

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author AGAR designed the study. Authors AGAR and ANB wrote the protocol and wrote the first draft of the manuscript. Authors AGAR and MGS managed the literature searches, where authors MGS and ANB performed the statistical analysis. Author AGAR managed the analyses of the study. All authors read and approved the final manuscript.*

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## ABSTRACT

**The Aim:** The aim of this study is to explore a new application for turning low value byproducts to value added bioactive components using a simple technique.
**Study Design:** The large amounts of byproducts resulted had an environmentally harmful; the phenolic components were extracted using aqueous isopropanol from olive leaves and pomace.
**Place and Duration of Study:** samples were analysed in laboratories of Fats and Oils Department, Toxicology and Food Contaminants; National Research Centre; and in Department of Food Technology, City of Scientific Research and Technological Application during season of 2016.
**Methodology:** A novel extraction method was used to recover the remaining active components from olive processing wastes ($OPW_S$) either from pomace or leaves. The oil residues re-extracted

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from dried pomace using n-hexane; collected as pomace olive oil.
**Results:** Although the $OPW_S$ considered as a harmful environmental byproduct; OPWs extracts showed a highly contents of polyphenols along with antioxidant activity, the best results recorded in pomace olive extract (POE), it had a good character to reduce toxigenic fungal growth and mycotoxins. Antimicrobial property, MIC and MFC of polar and non-polar extracts were determined.
**Conclusions:** It showed a better antimicrobial character in conformity with its total phenolics, total flavonoids and antioxidant activities. The olive pomace extract showed a high content of bioactive components. The extracts had anti-mycotic properties against 4 types of toxigenic fungi, and anti-mycotoxigenic characters that showed a high ability of the olive pomace extracts either on fungal inhibition or reducing aflatoxin ratio.

*Keywords: Olive oil byproducts; phenolics; antioxidants; aflatoxins; antimicrobial.*

## 1. INTRODUCTION

Like any other processes, the food industry has a primary product as the main target along with many secondary products and wastes. The latter one rated as a one-value product of the food industry. Among the food problem which increased recently, according to several factors; the novel vision not only to raise the food bulk amount but to enhance its nutritional properties conjugated with raising its safety and quality. Olive oil processing is a technique to have oil from olive fruits against many disagreements about all kinds of fats and it's found in the diet, olive oil appeared as a healthy edible oil related to its characteristics, it was rich in monounsaturated fatty acid especially oleic acid [1] which has many health features such as reducing inflammation beside its impact on genes binding with concern [2]. Furthermore, olive oil had the modest amount of some antioxidant like vitamin E and vitamin K with several health benefits.

Olive oil processing had a large amount of byproducts, those consisted of leaves, stems, and twigs beside other debris residues may find in olive fruits, it was appeared as an olive oil manufacturing wastes. Otherwise, those by-products must take care of it according to its harmful impact on the environment [3]. Thus, if that by-product turned to be a valuable product it will solve the environmental problem of oil manufacturing side to raise the gain of the olive industry. The challenge of industrial byproducts not depending on its harmful to surrounded environment only, but it needs to turn it from low cost material to high cost one, also by adding value to the new product, this may help to be safe. The environment along with its participate in solving some other problems such as food safety and food mal-nutrition by extracting some bioactive components from by-products and gets it as a new products may add to main product itself and/or to supplement other food products [4,5]. Not just case, it can be used in many other manufacturing materials such as the kinds of industrial microbiology as well as recent bioremediation technology. Olive oil processing wastes (OPWs) generally contains several useful materials and components, along with its amount of little oil residues. The fermentation of the novel carbon source, beside it is property as renewable, to produce many value-added products like microbial-polymer, lipase, some organic acids, and bio-surfactant is very motivating in microbial bio-technological industrial [6].

Poly phenolic compounds, flavonoids, vitamins, minerals and many other bioactive components may present in byproduct of agricultural wastes [7]. The importance of those components was depending on its role, if it is used as food supplement and/or for food preservation. poly phenols along with flavonoids deemed as antioxidant substances play a great role against microorganisms, it acts as an anti-carcinogenic, anti-inflammatory with immune modulatory properties [8]. Polyphenols had ability to chelate pro-oxidant metal, beside it has a function in oxidative stress of food material [9].

The hidden enemy in food materials, food mycotoxins, food free of toxigenic fungi with visual eyes not an indication that food is safe for human consumption. Toxins May excretion of food by fungi before the sporulation or fungi disappeared according to changes in environmental factors while mycotoxin still present on those foods [10]. Many types of mycotoxin may found according to environmental area characters, the most dangerous one was aflatoxin $B_1$ ($AFB_1$) followed by its group like Aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$) and finally aflatoxin $G_2$ ($AFG_2$) [11], all of these toxins

are health hazard materials which need to prevent it to reach foods [12]; some of active components and several antioxidant play a major role to stop the bad effect of those mycotoxin in food and feed chains, likewise poly phenols as well some vitamins had ability to stand against mycotoxin harmful. Mycotoxins may happen in food products and it could contaminate several food types and crops throughout the food and feed chains; the toxigenic fungal growth along with its mycotoxin excretion was deemed as a great problem for foods and feed commodities; according to the carcinogenic impacts of those toxins, there is a need to get a method that able to reduce the carcinogenic risks, many types of strategies are utilized to dominate the fungal growth beside its toxin synthesis in stored food and feed materials, like chemical and physical treatments or by biological methods, it was preferably if it use a natural products without any side effects, the novel strategy is to utilize the natural extracts which lookalikes an ideal material to do this function and provides an occasion to avert chemical preservation methods [13].

The aim of this study is to use a novel extraction method supported by ultrasonic as a highly efficient way that able to regain the most useful component from OPWs as a byproduct valorize and add value based on scientific applications via investigation their ability to reduce aflatoxigenic fungi hazard and its aflatoxins on $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ as well as take into consideration of MIC, MFC and $IC_{50}$. Those by-products may contain many bioactive components which having the ability as antimicrobial, antioxidant, antifungal and anti-toxigenic.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Plant Materials

Olive oil processing byproducts which consists of olive pomace (OP) and olive leaves (OL), beside its pomace olive oil (POO), collected after the process of the olive oil (season 2016), from the farm factory that located around the South Sinai area, Egypt. Each material labeled, numbered, a noted with the date of collection, and their localities were recorded.

### 2.2 Preparation of Plant Extract

One hundred gram of the air dried (at oven, 40℃) of pomace or olive leave samples were extracted using ultrasonic supported techniques (at room temperature for 40 min) with isopropanol: water 1:1 v/v. The aqueous isopropyl solution was added at ratio of 5:1 (v/w), pH was adjusted to 2-3 [14]. The last step was done twice, after that it collected and centrifuged at 4000 Xg for 20 minutes. The supernatant containing the extract was then transferred to a pre-weighed flask and the extract was concentrated by rotary evaporator at 50℃. The crude extract was weighed and dissolved in a known volume of dimethyl sulphoxide (DMSO) to obtain a final concentration of 2 mg/1 µl. POO was also extracted from dried olive pomace with hexane; collected oil-hexane was evaporated under vacuum using rotary evaporator to regain the crude POO.

### 2.3 Selection of Bacterial and Fungal Strains

In order to suggest methodologies for screening the natural extracts antimicrobial activity, two different qualitative methods were evaluated as follows: agar diffusion test, employing two different types of reservoirs (filter paper disc impregnated with extracts–test and wells in dishes). Bacteria and fungi strains were prepared and reactivate from a lyophilized media of each strain, bacterial strains are divided to Gram positive strain (*Staphylococcus aureus* and *Bacillus cereus*) and Gram negative (*Campylobacter jejuni* and *salmonella typhi*) cultured on tryptic soy agar; whereas the toxigenic fungal strain under investigation were *Aspergillus flavus, Aspergillus parasiticus Fusarium solani, Alternaria sp.*, those microorganisms were cultured on potato dextrose agar media.

### 2.4 Antibacterial Assay

Plant extracts were made by isopropanol: water (1:1 v/v), the natural components were dissolved and diluted with solvents as mentioned previously. Same number of subsequent dilutions were performed. The effect of various plant extracts on the several bacterial strains were assayed by the agar well diffusion method and further confirmed by the disc diffusion method. The minimum concentrations of the extract to inhibit the microorganisms were also determined by a micro-dilution method using plant fractions serially diluted in sterile nutrient broth.

## 2.5 Detection of Antimicrobial Activity

### 2.5.1 Determination of minimal inhibition concentration of extracts

The minimum inhibitory concentration (MIC) was determined by a micro dilution method using serially diluted plant extracts according to the NCCLS protocol [15]. The aqueous, methanol and chloroform extracts were diluted to get a series of concentrations from 6.25 mg/ml to 100 mg/ml in sterile nutrient broth. The microorganism suspension of 50µl was added to the broth dilutions. These were incubated for 18 hours at 37℃. MIC of each extract was taken as the lowest concentration that did not give any visible bacterial growth.

### 2.5.2 Determination of minimal fungicidal concentration of extracts

The fungicidal effect of the plant extracts can be assessed by the inhibition of mycelial growth of the fungus and is observed as a zone of inhibition near the disc or the wells. The activity of the plant extracts on various fungal strains was assayed by the agar plug method and spore germination inhibition assay. The commercial potato dextrose agar medium (39 g) was suspended in 1000 ml of distilled water. The medium was dissolved completely by boiling and was then autoclaved at 15 lbs pressure (121℃) for 15 minutes. Agar medium was prepared and poured onto the Petri plates. A fungal plug was placed in the center of the plate. Sterile discs immersed in the four plant extracts were also placed on the plates. Nystatin was used as the antifungal control. The antifungal effect was seen as crescent-shaped zones of inhibition [16].

## 2.6 Total Phenolic Compounds

The total phenolic compounds assay was carried out using the Folin-Ciocalteu reagent, following the method [17], and based on the reduction of a phosphor wolframate-phospho molybdate complex by phenolics to blue reaction products. 1 mg extract was dissolved in 1 ml methanol and 500 µl of the dissolved sample was taken and added to 0.5 ml of the distilled water and 0.125 ml of Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 minutes before adding about 1.25 ml of 7% $Na_2CO_3$. The solution was adjusted with distilled water to a final volume of 3 ml and mixed thoroughly. After incubation in the dark for 30 min, the absorbance at 650 nm was read versus the prepared blank. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml). Total phenolic content was estimated as µg Gallic acid equivalents (GAE)/mg of dry weight.

## 2.7 Total Flavonoid Content

The total flavonoid contents of the extracts were determined by a modified colorimetric method described by Sakanaka [15] with some modifications, using catechol as a standard. Extracts or standard solutions (250 µl) were mixed with distilled water (1.25 ml) and 75 µl of 5% sodium nitrite ($NaNO_2$) solution followed by the addition of 150 µl of 10% aluminum chloride ($AlCl_3$) solution 5 min later. After 6 min, 0.5 ml of 1M sodium hydroxide (NaOH) and 0.6 ml distilled water were added. The solutions were then mixed and absorbance was measured at 510 nm. The results were expressed as mg catechol /g of sample. All determinations were performed in triplicate.

## 2.8 HPLC of Phenolic Compounds

Identification of the phenolic compounds of the extracts was performed by HPLC analyses according to a modification of the McDonald method [18], using a Waters system (Waters Chromatography Division, Massachusetts, MA 01757, USA) equipped with a variable UV/VIS detector set at 280 nm. Separations were achieved on a Shimadzu Pathfinder® AS silica 100, 5.0 µm RP column (150 x 4.6 mm, id 5 µm). The flow-rate was 1.0 µ/min. The mobile phase used was 0.01% acetic acid in water (A) versus methanol : acetonitrile : acetic acid (95:5:1 v/v/v) (B) for a total running time of 80 min and the gradient changed as follows: Solvent B started at 5% for 2 min, then increased to 25% in 8 min, to 40% in 10 min, to 50% in 10 min, to 100% in 10 min, held for 22 min, and returned to initial conditions over 18 min. The data were processed by a Waters Baseline 815 program and the qualification was performed by external standard calibration, [14].

## 2.9 Antioxidant Activity Assays

### 2.9.1 Scavenging activity on DPPH radicals

The OPW$_S$ were determined using DPPH free radicals scavenging assay according the method [19], with some modifications. Briefly, 1 mL of solution containing different concentrations of extract was mixed with 1 mL of 0.078 mM DPPH

in methanol. Concerning of DPPH for POO is based on procedure by [20], using iso-octane to dissolve both DPPH and oil samples. The mixture was shaken and allowed to stand at room temperature in dark for 30 min. The absorbance of the solution at 517 nm was measured using a spectrophotometer. All of the tests were carried out in triplicates. The inhibition of DPPH radical was calculated as follows:

$$\text{The inhibition of DPPH radical (\%)} = [(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$$

Where, $A_{control}$ = Absorbance of the control solution; $A_{sample}$ = Absorbance of the test extract. $IC_{50}$ value (mg extract/mL) is the inhibitory concentration of the test content at which the DPPH radicals were scavenged by 50% and was calculated interpolation from linear regression analysis. The antioxidant activity is expressed in terms of IC50 (concentration of the extract / reference compound required to inhibit DPPH radical formation by 50%).

#### 2.9.2 ABTS radical scavenging assay

To determine ABTS radical scavenging assay, the method of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) was adopted [21]. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.802 ± 0.005 units at 734 nm using the spectrophotometer. The Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity

$$(\%) = [(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$$

Where $Abs_{control}$ is the absorbance of ABTS radical + methanol; $Abs_{sample}$ is the absorbance of ABTS radical + sample extract /standard.

### 2.10 Fatty acid Composition of POO

Methyl esters of fatty acids (FAME) were prepared according to AOCS Official Method Ce 1k-07 [22]. Diluted FAME were separated on a HP 5890 series II (Hewlett Packard, Palo Alto, USA) equipped with an Innowax capillary column (30 m_0.20 mm_0.20 mm) and FID. Hydrogen was used as the carrier gas at flow rate of 1.5 mL/min. The column temperature was isotherm 210℃. Detector and injector temperatures were set at 240℃. Fatty acids were identified by comparison of the retention times with authentic standards and the results were reported as weight percentages after integration and calculation using Chem. Station (Agilent Technologies).

### 2.11 Statistical Analysis

Data were statistically analysed with SPSS software (version16). One-way analysis of variance was used to study significant difference among means, with a significance level at $P$= 0.05.

## 3. RESULTS AND DISCUSSION

The oil content of the olive fruits may be extended to attain more than 20% at ripeness; exceedingly the oil is utilized as major healthy edible oil, with rising the consumed amounts during last decades. Olive farming plays both economic and social importance, olive oil act as a substantial economic product of the Mediterranean countries, that appeared as the leading area in olive oil outputs; otherwise, this manufacturing had plenty of wastes either as water or as pomace, the composition of this pomace had many compounds such as oils, mono and polysaccharides, fibers, protein trace, and polyphenols. The chemical characteristics of $POW_S$ were described as in Table 1. The $POW_S$ (produced by 2-phases extraction system) appeared as the high water content which reached around 59%, as a result of that; all polar components were stagnant in $POW_S$, according to this, polyphenols and antioxidant substances are the major ones that turned to find waste.

The pH value of $POW_S$ was acidic, it was equal to 5.1, and this value may help to preserve the waste against most of microorganism contamination along with its contents of antioxidant and polyphenols. The oil content in $POW_S$ appeared in consideration amount (13.4%) which may highlight the importance of recovering it as low grade oil with many benefits. Otherwise the contents of $POW_S$ from the free fatty acid estimated as oleic was 1.31%, As well as, the moderate value of peroxide number

which explained that the impact of lipase enzymes towards pomace olive oil, which leads to the hydrolysis of tri-acylglycerols producing of free fatty acids and oxidation raising the peroxide value.

**Table 1. Chemical characteristics of $POW_S$ and its extracted POO**

| Characteristics | Values |
|---|---|
| Moisture (%) | 58.61 ±3.62 |
| pH | 5.1 ±0.67 |
| Oil % (dry weight basis) | 13.44 ±0.91 |
| Free fatty acid % (as oleic acid) | 1.31 ±0.22 |
| Peroxide value (meq./kg) | 10.67 ±0.7 |
| Un-saponifiable matter (g/kg) | 27.1±041 |

*Value = Mean ± SD*

The phenolic content and flavonoids for different by products extract was showed in Fig. 1. It has been found that, OPE was the major byproduct part of olive oil production in both polyphenols compounds and total flavonoids, the mean values were recorded at 278.41 mg cat./g and 69.66 mg GAE/g for polyphenols and flavonoid, respectively. Thus, for olive leaf extract (OLE) polyphenols were 195.83 mg cat. Otherwise the POO, which extracted from $OPW_{S,}$ was the lowest one in its contents of polyphenols and flavonoids. Polyphenols are the main combination of natural products which take place in the higher vegetation crops [23]. Out of diverse plants, the olive was familiar as a source of polyphenols [24], and the compendium of most drugs all over the world contain olive oil monographs; likewise, the French pharmacopeia includes the olive leaf monograph [25]. Olive bio-phenol is now known as the prospective objective for food and pharmaceuticals processing [26]. The fruit, leaves, and oil from olive have all attracted considerable attention as sources of polyphenol [27]. The leaf of olive, as a model, is a familiar source of bio-phenols which is be sold on by various trademarks as a nutraceutical.

## 3.1 HPLC Analysis of Phenolic Compounds

Polyphenolic compounds in the byproduct extracts have been carried out by HPLC. Thirteen phenolic compounds were analyzed as standards and determined the phenolic compounds in three by product extracts (Tyrosol, Luteolin-7-rutinoside, Rutin, Dihydro-quercetin, 10-hydroxy-oleuropein, Luteolin-7-glucoside, Verbascoside, Apigenin-7-glucoside, Chrysoeriol-7-*O*-glucoside, Oleuropein glucoside, Oleuropein, Oleoside, Apigenin). All those compounds have been reported in the olive oil phenolic fraction [28]. The structures of the main phenolic compounds found in olive by products are shown in Table (2). The concentrations of the phenolic compounds expressed in (mg/g). OLE contained high value from 10-hydroxy-oleuropein (11.48), Apigenin (15.08) compare with other compounds respectively. While, POO extract contained Oleoside (18.0), Apigenin (20.0) with respect to other compounds (Table 2).

The size of phenolic components is a substantial agent when estimating the goodness of olive oil in order that its involvement in resistance to oxidation and the appearance of pungency and bitterness sensory attributes as a sign of virgin olive oil good quality [29] and for the antioxidant properties attributed to virgin olive oil from the recent scientific literature [1,30]. From both a qualitative and quantitative point of view the three extracts of byproduct: olive pomace, olive leaves and pomace olive oil are different. In all the samples studied, 10-hydroxy-oleuropein, Apigenin-7-glucoside and Apigenin are the major constituents in OLE while Oleoside and Apigenin in POO.

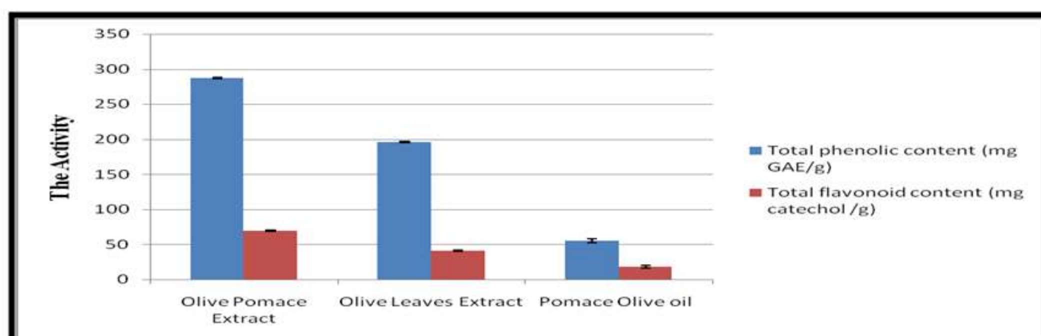


**Fig. 1. Total phenolic and total flavonoid content for aqueous extracts of olive pomace, olive leaves and pomace olive oil**

**Table 2. Phenolic compounds in olive leaves**

| Phenolic compound | Concentration (mg/g)[a] | | |
|---|---|---|---|
| | OLE | OPE | POO |
| Tyrosol | 0.42 | 0.25 | 0.025 |
| Luteolin-7-rutinoside | 0.02 | 0.07 | nd |
| Rutin | 0.14 | 0.12 | 0.01 |
| Dihydroquercetin | 0.08 | 0.21 | 0.02 |
| 10-hydroxy-oleuropein | 11.48 | 2.18 | 2.48 |
| Luteolin-7-glucoside | 0.09 | 0.15 | 0.00 |
| Verbascoside | 0.57 | 0.00 | 1.07 |
| Apigenin-7-glucoside | 7.4 | 0.00 | nd |
| Chrysoeriol-7-*O*-glucoside | 1.05 | 0.28 | nd |
| Oleuropein glucoside | 0.02 | 0.14 | nd |
| Oleuropein | 0.46 | 0.72 | 0.04 |
| Oleoside | 0.07 | 0.03 | 18.00 |
| Apigenin | 15.08 | 0.39 | 20.00 |

*nd: Not detected*

*[a] Expressed as milligrams of compound per gram of dry weight extract. Concentrations were calculated by external calibration against oleuropein using HPLC peak areas at 280nm. At this wavelength all compounds showed an absorption maximum with very similar extinction coefficients*

Olive oil and its by-products are a great sources of phenolic components extracts. These compounds have important roles in oxidative stability of olive oil. Table 2 presents the concentrations of the total phenolic compounds in the analyzed OPWs samples where there were significant differences between the samples. Epidemiological researches have reconditioned the low happening of heart coronary illness, atherosclerosis, and many cancer cases with olive oil consuming in the Mediterranean diet [31]. Olive oil, which is one of the main sources of monounsaturated fatty acid lookalike to play a conservative impact contra breast cancer, furthermore, this explanation not clear cut evidence, whether it was related to the monounsaturated fatty acid or the elevation of phenolic component's content beside tocopherols. Some researches executed in Europe countries recorded that olive oil holds a preservative function versus breast cancer. A number of reports have linked the health benefits of olive oil with its phenolic content [32]. The model of scavenging stable radical DPPH [33] a vastly applied method to estimate the antioxidant capacities of natural products, and it had been utilized for olive oil as well as individual antioxidant polyphenols. In the current study, the antioxidant activity of extracts was estimated for olive pomace, olive leaves and pomace olive oil. The antioxidant activity analysis plays a crucial role to illustrate how the polyphenol contents enhance the ability of those byproduct against the free radicals and carcinogenic materials, It was found that the OPE and OLE showed a better antioxidant capacities parallel to ascorbic acid.

As described in Tables 3 and 4, the values of antioxidant activity estimated as DPPH and ABTS inhibition ratios. The results showed that the radical scavenging activity of the extract from OPE was determined as 6.54, 43.11, 71.72 and 72.24% with different concentrations 25, 50, 75, and 100µg/ml of OPE, respectively; however, the same OLE concentrations amount to inhibition percentages of DPPH equal to 5, 27.36, 65.64 and 66.49% respectively. But, for POO appeared as the lowest component for DPPH inhibition ratio at the same concentrations. The other side of the story, the radical scavenging activity of ascorbic acid (standard) was measured as 88.79, 89.31, 89.53 and 90.40% with the same earlier concentrations. It was respected that; the scavenging capacity of OPE, OLE, and POO was advanced by increasing in extract concentration.

Otherwise, the value of $IC_{50}$ was recorded as 59.95, 57.52, 51.22, and 14.00 for OPE, OLE, POO, and ascorbic acid, respectively. Scavenging of the ABTS derived nitrogen-centered radical cation (ABTS•+) was applied to match the total antioxidant activities of the three types of extracts (OPE, OLE, and POO). As may be seen below in Table (4), the ABTS values were estimated for the three extracts compared to Ascorbic acid, at the sample concentration of 100µl/ml OPE showed the best effective byproduct of antioxidant activity (83.33%) seems close to the standard solution (ascorbic acid)

which had an inhibition ratio of 92.66%. By using the OLE concentration at 100 µl/ml, the ABTS inhibition was recorded as 78.24%, while it was 70.5% at 100 µl/ml of the POO extract comparing to the standard solution. While the $IC_{50}$ values were recorded as 34.92, 35.31, 49.18, and 14.43 for the OPE, OLE, POO, and ascorbic acid, respectively.

Thus, findings obtained in this study demonstrated that olive by-products possess antioxidant/free-radical scavenging properties, which are very likely due to the existence of high purport of phenolic components. The olive oil polyphenols demonstrated to be efficient in several experimental as free-radical scavengers presenting weakly to conservative effectiveness dependent on its thematic lineaments [34]. As predictable, component with the existence of a 3,4-dihydroxy moiety attached to an aromatic ring was more active than those with only hydroxyl group [35]; the glycosidation decreased the antioxidant activity. Our studies confirmed these findings: in fact, oleuropein Apigenin-7-glucoside, Apigenin, a hydroxytyrosol derivative, was more active than Oleuropein glucoside and Oleuropein. Moreover these findings suggest that olive by-products could exert a protective effect against pathogenic bacteria.

## 3.2 Chemical Characteristics of POO

Olive oil is contain triacylglycerols as a major component and consist of small amounts of free fatty acids, pigments, phosphatides, glycerol, microscopic olive bits, flavor compounds, and sterols. Triacylglycerols are the main energy conserve for plants and animals. Triacylglycerols are molecules derived from the natural esterification of three fatty acid molecules with one molecule of the glycerol; the last one can be appeared as a molecule with "E-shaped", resembling longish hydrocarbon chains, varying from about 14 to 24 of carbon chain in length as in olive oil. Crude pomace olive oil is the important by-product in olive oil processing and is actually improving frequently consequent to the technological progress whose the mills being undergone in last year's. Pomace olive oil is obtained by a repeated extraction of the remaining olive oil in press cake [36].

**Table 3. DPPH radical scavenging ability, for aqueous extracts of olive pomace, olive leaves and olive oil**

| Concentration of samples (µg/ml) | % inhibition of DPPH• | | | |
|---|---|---|---|---|
| | OPE | OLE | POO | Ascorbic acid |
| **25** | 6.54±5.16$^{c}$ | 5±4.29$^{c}$ | 19.86 ±1.65$^{b}$ | 88.79± 2.08$^{a}$ |
| **50** | 43.11±9.39$^{b}$ | 27.36±2.56$^{c}$ | 48.80±0.36$^{b}$ | 89.31± 3.23$^{a}$ |
| **75** | 71.72±0.85$^{b}$ | 65.64±6.53$^{b}$ | 61.16 ±1.7$^{c}$ | 89.53 ± 3.48$^{a}$ |
| **100** | 72.24±1.43$^{b}$ | 66.49±2.02$^{c}$ | 65.7± 2.32$^{c}$ | 90.40± 1.77$^{a}$ |
| **$IC_{(50)}$** | 59.95±13.64$^{a}$ | 57.52±5.97$^{a}$ | 51.22 ± 0.38$^{a}$ | 14.00±0.28$^{b}$ |

- *The results are expressed as mean ± SD, each value is the average of three duplicate reading values .*
- *$^{abcd}$ Means in the same raw followed by different lowercase letters are significantly different (P = 0.05)*
- *OPE: olive pomace extract , OLE: olive leaves extract, POO: pomace olive oil*

**Table 4. Antioxidant capacity of the extracts of olive pomace, olive leaves and olive oil as per ABTS•+ radical assay expressed as percentage activity (n = 3) as a function of concentration of extracts**

| Concentration of samples (µg/ml) | % inhibition of ABTS• | | | |
|---|---|---|---|---|
| | OPE | OLE | POO | Ascorbic acid |
| 25 | 34.18±4.19$^{c}$ | 66.9±4.58$^{b}$ | 40.56±1.34c | 86.60±2.37a |
| 50 | 71.81±5.06b | 70.9±3.63$^{b}$ | 50.86 ± 1.79c | 89.39±0.21a |
| 75 | 82.9±1.31$^{b}$ | 77.57±2.77$^{c}$ | 66.5± 0.45d | 92.13±0.29a |
| 100 | 83.33±0.55$^{b}$ | 78.24±4.87$^{c}$ | 70.5 ± 1.13d | 92.66±0.27a |
| $IC_{(50)}$ | 34.92±2.51$^{b}$ | 35.31±1.81$^{b}$ | 49.18±1.71$^{a}$ | 14.43±0.39$^{c}$ |

- *The results are expressed as mean ± SD, each value is the average of three duplicate reading values*
- *$^{abcd}$ Means in the same raw followed by different lowercase letters are significantly different (P = 0.05)*

It is worthy to mention that; crude pomace olive oil is a natural vegetable oil that comes from the olive husks which have a unique fatty acid composition like virgin olive oil. The values of the fatty acid composition for the POO extracted from OPWs recorded in Table 5. As the data shown, the main fatty acid was oleic acid, which percentage at 67.87%, while saturated fatty acids were the palmitic and stearic acids with percentage at 14.8% and 3.3%, respectively. Linoleic acid was the higher percentage polyunsaturated fatty acid with percent at 13.1%, and linolenic acid was recorded at a ratio of 0.92%; otherwise, other fatty acids were appeared as traces. These results are harmonious in opinion with that found in Kiriakoulakis [37].

**Table 5. Fatty acid composition of crude POO**

| Fatty acid | % |
|---|---|
| palmitic acid | 14.8 ± 3.41 |
| stearic acid | 3.3 ± 2.11 |
| Oleic acid (%) | 67.87 ±2.62 |
| Leinoleic acid | 13.08± 2.88 |
| Linolenic acid | 0.92± 0.98 |
| Arachidic acid | 0.03 ± 0.01 |
| Σ S FA | 18.13± 2.73 |
| Σ MUFA | 67.87 ±2.62 |
| Σ PUFA | 14.00± 1.92 |

- *Data are expressed as percent of total fatty acids determined.*
- *Values are the mean ± SD each was done as triplicates.*
- *SFA: Saturated fatty acid;*
- *MUFA: Monounsaturated fatty acids;*
- *PUFA: Polyunsaturated fatty acids*

The antibacterial behavior of byproducts materials extracted from POWs on the bacterial strains can be characterized by some parameters like minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The values of MIC and MBC for OPE, OLE, and POO and they're comparable to classical antibiotics, results showed varied values according to a type of microorganism (Table 6). Generally, the olive waste material extracts showed selective antibacterial characters.

As the data shown in Table 6; against four types of bacteria, two gram negative bacteria strains and two gram positive strains, the two extracts (OPE and OLE) along with the POO were investigated to determine its ability to inhibit the bacteria side to calculate its MIC and MBC values in the presence of standard antibiotic (Chloramphenicol). Out of the three components under investigation the OPE showed the high ability to inhibit the bacterial strains by using a low concentration of the extract either for MIC or for MBC, thus the results indicate also; the activity of the three materials ordered as OPE>OLE>POO.

Furthermore, minimal fungicidal concentration was also estimated for OPE, OLE, and POO. The results reflecting a high ability of OPE to inhibit the fungal growth of the four fungi under the study. One hundred fifty milligram per milliliter of OPE was able to stop the growth of *A. flavus, A. ochraceus*, *Penicillium Sp,* and *Fusarium moniliforme*, but for POO it needed two hundred fifty milligram per milliliter to stop the fungal growth of the four previous fungi. The data were shown as in Table 7.

**Table 6. The values of minimal inhibition concentration and minimal bacterial concentration**

| Strains | MIC and MBC (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chloramphenicol | | Olive pomace extract | | Olive leaf extract | | Pomace olive oil | |
| | MIC (mg/ml) | MBC (mg/ml) | MIC (mg/ml) | MBC (mg/ml) | MIC (mg/ml) | MBC (mg/ml) | MIC (mg/ml) | MBC (mg/ml) |
| *Enterococcus Facium* | 0.22 | *0.22* | 1.70 | 3.40 | 3.30 | 6.70 | 7.20 | 14.40 |
| *Bacillus cereus* | 0.64 | *0*.64 | 1.70 | 3.40 | 3.30 | 6.70 | 7.20 | 14.40 |
| *Psedomonase aeruginosa* | 4.10 | 4.10 | 1.90 | 3.80 | 3.90 | 7.40 | 7.90 | 15.90 |
| *Klebsiella poneumoniae* | 0.13 | 0.13 | 1.70 | 3.40 | 3.30 | 6.60 | 7.20 | 14.40 |

*MIC: Minimal inhibition concentration*
*MBC: Minimal bacterial concentration*

**Table 7. The values of minimal fungicidal concentration of OPE, OLE, and POO**

| Strains | Imidzole (mg/ml) | OPE (mg/ml) | POO (mg/ml) | OLE (mg/ml) |
|---|---|---|---|---|
| *Aspergillus flavus* | 0.041 | 150 | 250 | 200 |
| *A. ochraceus* | 0.023 | 150 | 250 | 200 |
| *Penicillium* sp | 0.027 | 150 | 250 | 200 |
| *Fusarium moniliforme* | 0.030 | 150 | 250 | 200 |

*OPE:- olive pomace extract; POO:- pomace olive oil ; OLE:- olive leaves extract*

In contemporary time; as many investigations cases focused on the effectiveness of olive oil and the identifying active components as antimicrobial activity [38] reported that the virgin olive oil (VOO) had a great bactericidal impact versus a wide shadow of micro-organisms, thus, it was noticed that; Gram-positive was more sensitive than Gram-negative bacteria. Furthermore, the antibacterial efficiency was higher in the case of using the VOO, followed by POO, that was in agreement with its phenolic content. Contraindication, this impact was not shown in the other vegetable oils (sunflower, soybean, corn, and cotton). Medina [37] have connected the antimicrobial performance of VOO by its phenolic components. The isolated phenolic compound from virgin olive oil by HPLC showed a good antioxidant activity and microbicidal characteristics, Medina tested the antibacterial efficiency versus strain *L. monocytogenes.*

The three types olive byproduct extracts were investigated to determine its ability to inhibit toxigenic fungi and reduce its toxin excretion as illustrated in Table 8, toxigenic fungi that used in this study are: *Aspergillus flavus, Aspergillus ochraceus, Penicillium* sp., and *Fusarium moniliforme*. This test was to work out how those byproducts own an ability to use as anti-mycotic and anti-mycotoxigenic substances with positive impact on food material when it may use as a fungal preservation in the presence of Imidzole as a standard antifungal component, so each 200 ppm of one extract was added to a well of media plate that contaminated with fungal strain the value of fungal grew were performed accordingly its recorded as millimeter diameter each two days. Four treatments were applied versus fungal strain, those treatments were: control, olive pomace extract, olive leaves extract, and pomace olive oil. First of all, *Aspergillus flavus* fungi were use in the first group; olive pomace extract was the brilliance extract against fungal growth all over the total days of the experiment, whilst, the impact of olive leaves extract comes next to it, and the power of POO to reduce the growth comes as a last one.

As described in the Fig. 2, OPE had the most efficiency power as antimycotic extract, however; for *A. flavus* fungi, at four days fungal growth, the effect of OLE and POO had no significances different, along with there was no significances between the use of OPE and OLE at six days growth. For *Aspergillus ochraceus*; at two days of fungal growth there was no significances different between PE and LE, but at six days of *A. chraceus* the efficiency of OPE, OLE, and POO to reduce fungal growth with no significant. Notwithstanding, OPE and OLE had the effect without significances of 8 days of fungal growth. Finally, no variation were appeared along with the impact of OPE and OLE of 6 days of fungal growth. Overall the result; the efficiency of the extracts was ordered as OPE>OLE>POO.

As the ability of OPWs were investigated for its antimycotic properties, also anti-aflatoxigenic properties were estimated. The results recorded that; again OPE was the most effective material against aflatoxins contents, otherwise POO was the lowest one in reducing aflatoxins efficiency. The OLE was appeared as a moderately effective material in aflatoxin degradation between OPE and POO. In case of using OPE, the aflatoxin reducing ratio were ranged as 62.7%, 67.9%, 51.9%, and 55.4% for $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, respectively. The total aflatoxins were reduced by a ratio of 60.6%.

Notwithstanding, in case of using OLE, the aflatoxin reducing ratio was 51.9%, 44.3%, 47.1%, and 38.7% for $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, respectively. The total aflatoxins were reduced by a ratio of 47.01%. Finally, in case of using POO to degrade aflatoxins, reducing ratios were 32.6%, 22.1%, 33.4%, and 18.5%, for $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$, respectively; however, The total aflatoxins were reduced by a ratio of 28.72%.

**Table 8. Antimycotic properties of olive byproduct materials against toxigenic fungi**

| Treatments | Diameter of fungal growth (mm) | | | |
|---|---|---|---|---|
| Growth age | 2 days | 4 days | 6 Days | 8 Days |
| ***Fungi 1*** | ***Aspergillus flavus*** | | | |
| Control | 20±2.08 | 38 ± 1.5 | 58± 1 | 82± 2.64 |
| Olive pomace extract | 4±0.21 | 17± 3.64 | 39±3.05[b] | 52± 3.98 |
| Olive leaves extract | 7±0.53 | 22±3.06[a] | 45±2.51[b] | 58±4.12 |
| Pomace olive oil | 15±1.52 | 26±1[a] | 35±2.08 | 43±1.15 |
| ***Fungi 2*** | ***Aspergillus ochraceus*** | | | |
| Control | 19±1 | 35±3.05 | 57±1.53 | 77±2.51 |
| Olive pomace extract | 0±0.57[c] | 9±3.21 | 29±2.08[d] | 53±4.36 |
| Olive leaves extract | 3±2.07[c] | 18±3.21 | 33±3.21[d] | 52±4.33 |
| Pomace olive oil | 15±1.53 | 25±1.15 | 33±1.51[d] | 44±1.73 |
| ***Fungi 3*** | ***Penicillium* sp.,** | | | |
| Control | 21±0.57 | 40±2.01[g] | 49±2.69 | 81±2.52 |
| Olive pomace extract | 6±2.51[f] | 13±3.7 | 34±1.61 | 40±2.01[h] |
| Olive leaves extract | 7±0.58[f] | 23±3.21 | 41±1.44 | 41±3.18[h] |
| Pomace olive oil | 15±1.71 | 38±1.15[g] | 49±1 | 59±1.52 |
| ***Fungi 4*** | ***Fusarium moniliforme*** | | | |
| Control | 17±1 | 34±2.51 | 65±2.07 | 77±2 |
| Olive pomace extract | 0 | 3±1.27 | 17±1.3[k] | 36±1.03 |
| Olive leaves extract | 0 | 9±1.55 | 23±1.71[k] | 41±3.22 |
| Pomace olive oil | 17±1.53 | 36±2.14 | 53±1.81 | 59±2.44 |

- *Fungal growth was represented as millimeter diameter on plate ± SD*
- *The values with the same letters are non-significant.*

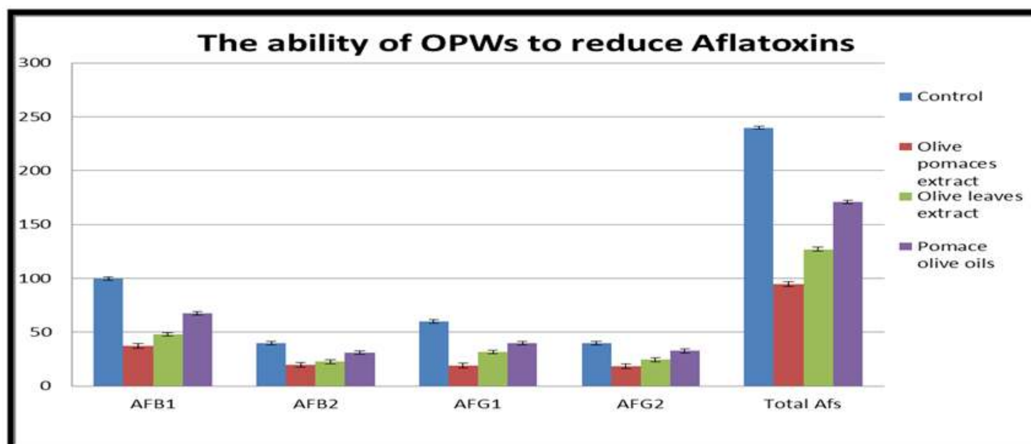


**Fig. 2. The ability of OPWs to reduce aflatoxins**

As a result of a study done by [39,40] on antioxidant protection role on native and sonicated extraction of olive cake residues on liver, kidney, and biochemical parameters of animal bio-fluids against aflatoxin $B_1$; the data recorded that , in case of treated group fed with contaminated aflatoxin $B_1$ diet in the presence of native and sonicated olive cake extracts, this were able to recover the biochemical parameters of blood to near the normal rates with minor changes, otherwise, it was also able to restored the damages in the tissues of liver and kidney which happened by aflatoxin $B_1$ as closed to the normal tissue successfully.

### 3.3 Correlation between Antioxidant Activity, Total Phenolic Content and Reduce Aflatoxins

As many plant phenolic compounds are good sources of natural antioxidants [41], we determined the Pearson correlation coefficients

**Table 9. Bivariate correlation of antioxidant activity, total phenolic content and reduce aflatoxins of olive by-products extracts**

| Relationship | Correlation Coefficient | *p* (2-Tailed) |
|---|---|---|
| Total phenolic content & flavonoid content | 0.983** | 0.000 |
| Antioxidant & total phenolic content | 0.46* | 0.213 |
| Antioxidant & total flavonoid content | 0.424* | 0.255 |
| Antioxidant & **$AFB_1$** | 0.924** | 0.00 |
| Antioxidant & **$AFB_2$** | 0.919** | 0.00 |
| Antioxidant & **$AFG_1$** | 0.787* | 0.012 |
| Antioxidant & **$AFG_2$** | 0.858** | 0.003 |
| Antioxidant & Total aflatoxins | 0.896** | 0.001 |

***. Correlation is significant at the 0.01 level (2-tailed)*
**. Correlation is significant at the 0.05 level (2-tailed)*

between the antioxidant activity , total phenolic content, total flavonoid content and reduce aflatoxins of the extracts (Table 9). There was a moderate significant relationship between antioxidant activity, total phenolic and total flavonoid content (correlation coefficient, r =~0.4, *p* =0.05), indicating that high phenolic content is a significantly important factor for determining the antioxidant activity of olive by-products extracts. The antioxidant activity of olive by-products may be attributed to the phytochemical compounds they contain, especially the polyphenolic compounds, i.e., mainly 10-hydroxy-oleuropein, Apigenin-7-glucoside and Apigenin. Recent studies have also demonstrated that the individual antioxidant activity of phenolic compounds in model systems have mutually synergistic or antagonistic effects [42,43]. In this study, the olive by-products extracts displayed the strongest detoxification effect but moderate antioxidant activity and total phenolic content. The statistical analysis supported these results, where the antioxidant activities of the extracts showed strong correlation with their detoxification effect (r =~ 0.8) and moderate correlation with total phenolic content (r =0.46), and total flavonoid content (r =0.424) (Table 9).

## 4. CONCLUSIONS

Olive oil and its by-products are a great source of nutraceutical, and broadly used in cosmetic and personal care products. These by-products contain many bioactive components which they have the ability as antimicrobial, antifungal, antitoxigenic (to reduce aflatoxigenic fungi hazard and its aflatoxins). Along with that, it may uses in other manufacturing process, like, food supplement, or preservatives. The statistical analysis showed strong correlation with their detoxification effect. Moreover these findings suggest that olive by-products could exert a protective effect against pathogenic bacteria.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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