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In Vitro Antioxidant and Antibacterial Activity of Olive Leave Extract

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Abstract: Antioxidants present in herbs and spices could be an effective tool to prevent the non-communicable diseases like cancer, diabetes and myocardial infarction as they have the capacity to stabilize the free radicals which are one of the causative factors of these diseases. In last decade, there is an increasing interest in researches for production of biologically active compounds from natural sources. Olea europaea L. is used in traditional medicine in the Mediterranean areas. The aim of the current study was to investigate the content of phenol compounds and flavonoids extracted from "Olea europaea" olive leaf followed by the assessment of in vitro antioxidant and antimicrobial activities, crude extracts from olive leaves were screened for its in vitro antibacterial activity by using well diffusion method against Gram-negative organisms Escherichia coli bacteria, neomycin (30) was used as a standard for the study of antibacterial activity. Phytochemical screening revealed the presence of some active substances phenolic and flavonoids, to express the desired activities. Total phenols and total flavonoids were measured using the Folin- Ciocalteu and aluminum chloride colorimetric methods, respectively. The antioxidant properties have been determined by Reducing power ability where tested by $FeCl_3$, in this procedure the Fe^{3+} reduced to Fe^{2+} by donating an electron. The ethanolic and water extract of green leaves showed good antibacterial activity with zone of inhibition (15mm, 13mm), respectively. These results suggest that leaves of olive have interesting antibacterial activities due to Polyphenol-rich extracts.

Keywords: Olea europaea, antioxidant activity and antibacterial activity.

1 Introduction

Olive is one of the most important crops in the Mediterranean countries. More than eight million of olive trees are cultivated worldwide among which the Mediterranean basin presents around 98% of them. Oleaeuropaea L. is widely studied for its alimentary use, the fruits and the oil are important components in the daily diet of a large part of the world's population. Both the cultivation of olive trees and olive oil extraction generate every year substantial quantities of products generally known as "olive byproducts" and having no practical applications. Olive leaves, available throughout the year, are one of the byproducts of olive farming; they accumulate during the pruning of the olive trees and can be found in large amounts in olive oil industries after being separated from fruits before processing (about 10% of the weight of olives). Several reports have shown that olive leaves have antioxidant activity, anti-HIV properties, anti-proliferative and apoptotic effects, protective effect against human leukemia, lipid-lowering activity (Leila et al., 2015).

In pharmaceutical industry, the olive leaf extracts were used as natural antibacterial, and antioxidant in non-sterile pharmaceutical dosage forms instead of conventional chemical preservatives which have toxicity to human beings even at low concentrations especially when used in pediatric/children preparations. Results showed that the extracts work as natural antibacterial and antioxidants for the prepared pediatric syrup and potentially can substitute the chemical preservatives. Regarding food applications of the extracts, they were added into meat fillets as antioxidants and compared to chemical preservatives that are used usually in meats, and results indicated significant decrease in the amount of secondary oxidation products (represented as malonodialdehyde) of the tested meat

samples treated with different concentrations of olive leaves extracts, indicating that this extract can be used as natural antioxidants in meat substituting synthetic ones (Fuad et al., 2014).

Recently, the medicinal properties of olive products have focused on its polyphenols (particularly oleuropein and hydroxytyrosol), which according to animal and in vitro studies have antioxidant, hypoglycemic, antimicrobial and anti-atherosclerotic properties (Barbaro et al., 2014).

Olive phenolic are much more concentrated in the leaves compared with olive fruit or olive oil: 1450 mg total phenolic /100 g fresh leaf vs. 110 mg/100 g fruit and 23 mg/100 ml extra virgin olive oil (Lockyer et al., 2017).

The primary active compounds in unprocessed olive leaf are oleuropein and hydroxytyrosol as well as polyphenols and flavonoids, including luteolin, rutin, caffeic acid, catechin and apigenin (Herrero et al., 2011). Elenolic acid is a component of olive oil and olive leaf extract. It can be considered as a marker for maturation of olives. The phenolic composition of olive leaf extract varies according to plant variety, harvesting season and method, leaf maturity, storage conditions and extraction method.

Phenolic compounds are becoming increasingly popular because of their potential role in contributing to human health. Experimental evidence obtained from human and animal studies demonstrate that phenolic compounds from Oleaeuropaea leaves have biological activities which may be important in the reduction in risk and severity of certain chronic diseases (Leila et al., 2015).

Olive leaves are an important source of antioxidants, such as phenolic compounds and flavonoids, which display effective antioxidant activity when various methodologies are used. OLE inhibits the action of reactive species that participate in cellular biochemical processes and protects human erythrocytes against oxidative damage. These results show that olive leaves are effective antioxidant in biological systems, suggesting that their intake may be related to prevention of oxidative stress in vivo, with consequent health benefits. Moreover, OLE have the potential to be used as natural antioxidants in preservation of food products, pharmaceuticals and cosmetics, in which chain reactions mediated by free radicals result in oxidative alterations (Mariza et al., 2018).

The antioxidant capacity of olive leaves is attributed mainly to the presence of phenolic compounds. Several of these compounds were evaluated individually and the antioxidant effects were related to functional groups characteristic, amount and hydroxyl position in their structures, which gives them redox properties. In addition, some studies suggest that phenolic compounds have a synergistic effect on antioxidant capacity when they are together (Zhang et al., 2015).

The antioxidant and antimicrobial efficiency of the OLE are directly related with its polyphenols. There are some studies in literature revealing that polyphenols can inhibit the sporulation of Bacillus cereus and growth of Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Vibrio parahaemolyticus and Staphylococcus aureus, that all known as food pathogens (Erdohan and Turhan, 2011).

2 Materials and Methods

2.1 Plant Preparation

The fresh plant parts (leaves) were collected washed with tap water, some of them was air dried in shade at room temperature. The leaf petioles were carefully manually separated and dry leaves were pulverized into powder and stored protected from light until further use.

2.2 Extraction

Ten grams of green leaves (fresh sample) and powdered sample extracted by Soxhlet apparatus with 100 ml ethanol and water. Extract was filtered concentrated under vacuum by rotary evaporator and stored at 7°C until used.

2.3 Total phenolic Content

The total phenolic content of all extract was determined by using Folin- Ciocalteu method . A standard pyrogallol curve was constructed by preparing the dilutions of (0.1, 0.5, 1.0, 2.5 and 5 mg/ml) in methanol. 100 µl of each of these dilutions were mixed with 500 µl of water and then with 100 µl of Folin-Ciocalteu reagent and allowed to stand for 6 minutes. Then 1ml of 7% sodium carbonate and 500 µl of distilled water was added to the reaction mixture. The absorbance was recorded after 90 minutes at 760 nm spectrometrically. The same procedure was repeated with ethanolic and water extracts of green and powder leaves . The total phenolic content of the extract was calculated as pyrogallol equivalents (mg PYG/g). All the experiments were performed in triplicate (Odabasoglu et al.,2004).

2.4 Total Flavonoids Content (TFC)

Aluminum chloride complex forming assay was used to determine the total flavonoid content of the extracts . Quercetin was used as standard and flavonoid content was determined as quercetin equivalent. A calibration curve for quercetin was drawn for the (0.1, 0.5, 1.0, 2.5 and 5mg/ml) concentrations were prepared in methanol. 100 µl of each of the quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% Sodium nitrate and allowed to stand for 6 minutes. Then 150 µl of 10% Aluminum chloride solution was added and allowed to stand for 5 minutes after which 200 µl solution of 1M Sodium hydroxide was added sequentially. The absorbance of this reaction mixture was recorded at 510 nm on UV spectrophotometer. The same procedure was repeated with the water and ethanolic extracts, flavonoid content was calculated as quercetin equivalents (mg QE/g). All the

procedures were performed in triplicate (Piyanete et al., 2009).

2.5 Reducing Power Assay (RPA)

The reducing power was determined according to the (Naznin and Hasan, 2009). 100µl of different extract was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide then mixture was incubated in water bath at 50 C0 for 20 minutes and 2.5 ml of trichloroacetic acid was added to the mixture which was then centrifuged at 3000 rpm for 10 minutes. Finally 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 1 ml $FeCl_3$, substances, which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferricyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm by UV-Visible spectrophotometer. Quantification was done with respect to stander calibration curve of ascorbic acid the results were expressed as ascorbic acid "µg/ml".

$$K_3Fe(CN)_6 + FeCl_3 \xrightarrow{\text{antioxidant}} K_4Fe(CN)_6 + FeCl_2.$$

2.6 Antibacterial Activity Assay

Antibacterial activity of aqueous and ethanolic extracts was determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). bacterial culture to be tested was spread on Mueller-Hinton agar plates with a sterile swab moistened with the bacterial suspension. Subsequently, wells of 10 mm diameter were punched into the agar medium and filled with (20ml, 40ml, 60ml, 80ml) of each plant extract and allowed to diffuse at room temperature. The plates were then incubated in the upright position at 37° for 24 h. Wells containing the solvent employed to dissolve the plant extract served as negative controls while standard antibiotic discs of neomycin (30µg) were used as the positive controls. After incubation, the diameters of the growth inhibition zones were measured in mm.

3 Results

3.1Total Phenolic Content (TPC)

Table 1. Total phenolic content of pyrogallol (standard).

Concentration of pyrogallol (µg/ml)	Absorbance (765nm) Mean ± S.D.
100	0.410 ± 0.032
200	0.799 ± 0.022
300	1.333 ± 0.004
400	1.828 ± 0.011
500	2.105 ± 0.022

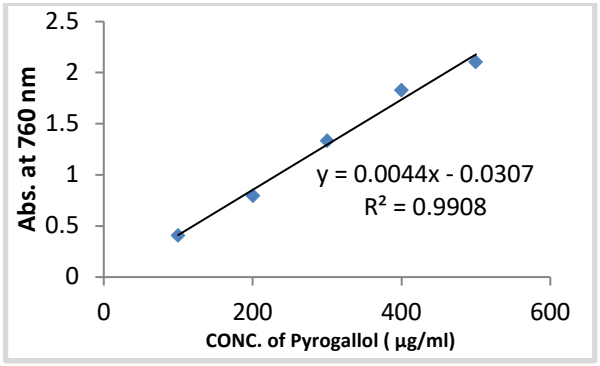

Fig.1: Total phenolic content of pyrogallol (standard).

Table 2. Results of total phenolic content of Olive Leaf (*Oleaeuropaea* L.) Extracts.

Extracts	Total phenolic contents (mg/g) Mean ± S.D.
Eth.ex.GL	6.27 ± 0.05
W.ex.GL	5.23 ± 0.01
Eth.ex.PL	5.61 ± 0.02
W.ex.PL	5.52 ± 0.02

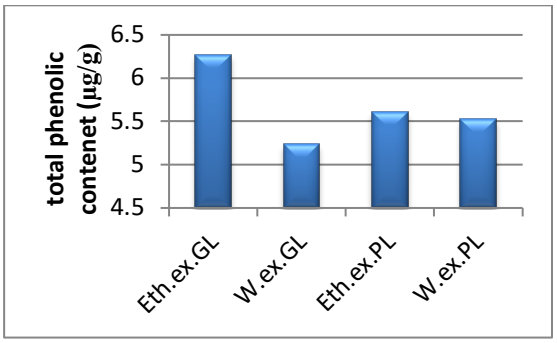

Fig.2: Total phenolic content of different extract type (µg of pyrogallol /10g of sample).

3.2 Total Flavonoids Content (TFC)

Table 3: Total flavonoids content of quercetin (standard)

Concentration of quercetin (µg/ml)	Absorbance Mean ± S.D.
100	0.279 ± 0.09
200	0.560 ± 0.03
300	0.834 ± 0.03
400	1.066 ± 0.09
500	1.300 ± 0.06

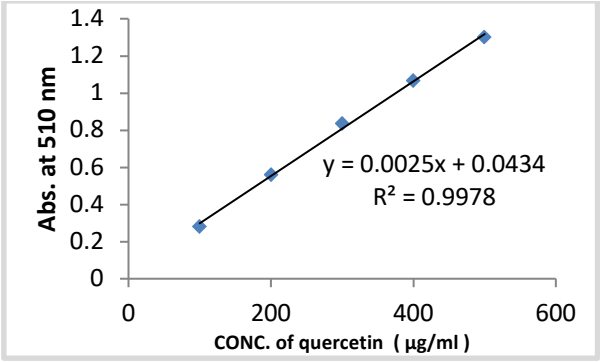

Fig.3: Total flavonoids content of quercetin (standard).

Table 4: Results of total Flavonoids content of Olive Leaf (Olea europaea L.) Extracts.

Extracts	Total flavonoids contents (µg/g)
Eth.ex.GL	10.02 ± 0.02
W.ex.GL	10.22 ± 0.10
Eth.ex.PL	9.70 ± 0.01
W.ex.PL	9.94 ± 0.02

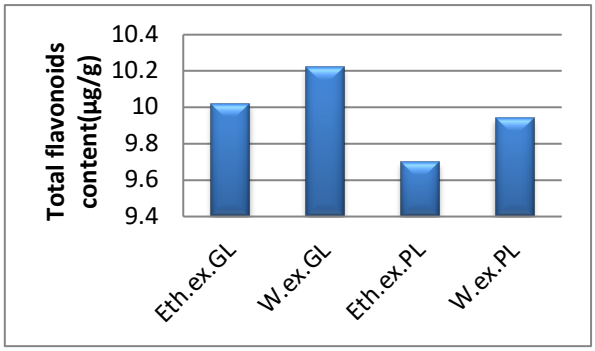

Fig.4: Total flavonoids content of different extract type (µg of quercetin /10g of sample).

1- Reducing power assay (RPA):

Table 5: Reducing power assay of ascorbic acid (standard).

Concentration of vitamin C (µg/ml)	Absorbance Mean ± S.D.
100	0.201 ± 0.02
200	0.495 ± 0.03
300	0.697 ± 0.08
400	0.992 ± 0.07
500	1.201 ± 0.03

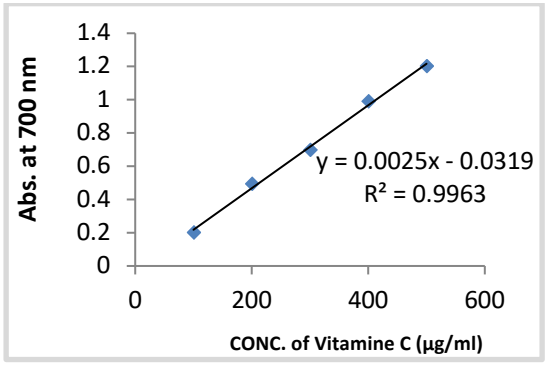

Fig.5: Reducing power assay of ascorbic acid (standard).

Table 6: Reducing power assay of Olive Leaf (Olea europaea L.) Extracts.

Extracts	Reducing power assay (µg/g)
Eth.ex.GL	8.28 ± 0.05
W.ex.GL	7.96 ± 0.01
Eth.ex.PL	7.23 ± 0.02
W.ex.PL	7.24 ± 0.02

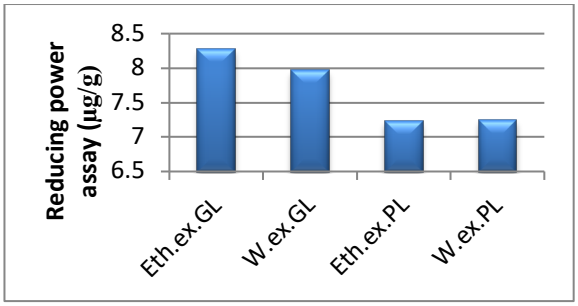

Fig.6: Reducing power assay of different extract type (µg of Vit C /10g of sample).

2- Antibacterial activity assay:

Table 7: The inhibition zone diameters of O .L .Exs against E.Coli.

O. L. Exs	Inhibition zone
Eth.ex.GL	15 mm
W.ex.GL	13 mm
Eth.ex.PL	8 mm
W.ex.PL	8 mm

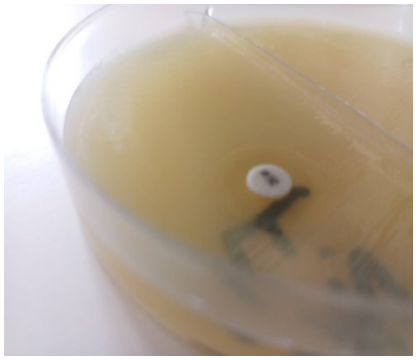

Fig.7: Neomycin (30µg).

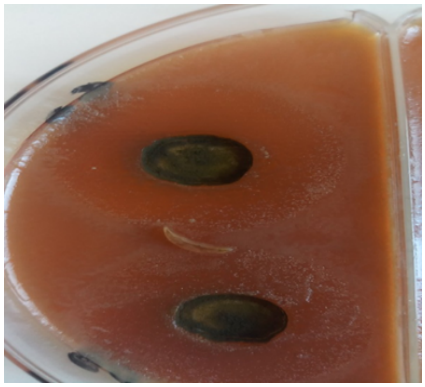

Fig.8: Eth.ex.GL inhibition zone.

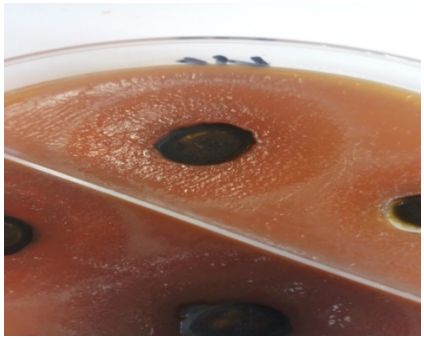

Fig.9: W.ex.GL inhibition zone.

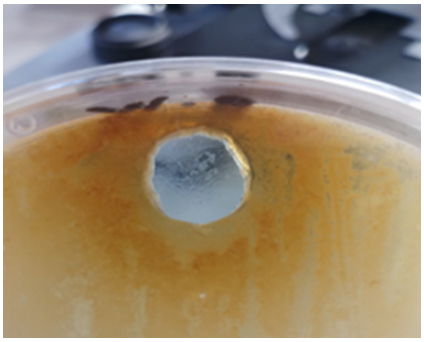

Fig.10: Eth.ex.PL inhibition zone.

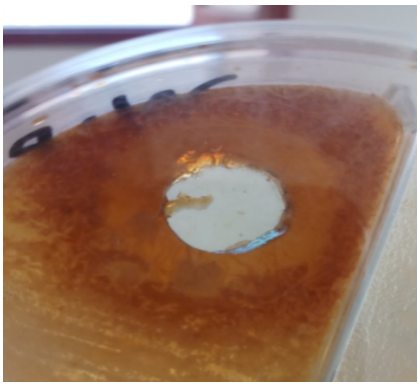

Fig.11: W.ex.PL inhibition zone.

4 Discussion and Conclusion

4.1 Total Phenolic Content

Phenolic compounds in plants constitute a major class of secondary plant metabolites with bioactive potential attributed to antioxidants activity. The total phenolic content estimated by the Folin-Ciocalteu method is expressed in terms of pyrogallol equivalent (the standard curve equation: $y = 0.0044x - 0.0307$, $R^2 = 0.991$). The obtained result revealed that the highest total phenolic content was found in ethanolic extract of green leave (6.27 ± 0.05 µg PYR/10g sample) followed by ethanolic extract of dried leave (5.61 ± 0.02 µg of PYR/10g sample), the lowest content was registered in water extracts. In general, the results indicate that the solvent of extraction influenced the phenolic content, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material **(Bimlesh Kumar *et al.*, 2010).**

4.2 Total Flavonoids Content

The flavonoids results expressed as µg quercetin equivalents (que)/ 10g sample with reference to a standard curve ($Y = 0.0025x + 0.0434$, $R^2 = 0.998$), established their presence in all olive leave extracts with a highest value in water green leave extracts and lowest value in ethanolic powder leave extracts, The total flavonoids contents in plant extracts of the depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction, **Prashant Tiwari, *et al.*, (2011)** found The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased.

4.3 Reducing Power Assay

The ability of extract to reduce Fe^{+3} to Fe^{+2} (reducing effect) was determined according to the method described

by **(Naznin and Hasan, 2009).** The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Dietary antioxidant such as ascorbic acid was used for comparison. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates, so that they can act as primary and secondary antioxidants. The results were expressed as µg ascorbic acid /10g sample. The Eth.ex.GL have strong reducing capacity (8.28 ± 0.051 µg/g). The reducing capacity of antioxidant be due to their electron transfer property such as polyphenols flavonoids. Many studies demonstrated that the plants extract possess a strong reducing capacity. In other hand, many researchers has been widely reported the relationship between polyphenol structure and their and ferric reducing capacity. Phenolic compounds are called antioxidants because of their ability to scavenge free radicals, lipid peroxidation inhibition, and reducing effect **(Abderrahim and Abdenassar, 2016).**

4.4 Antibacterial Activity Assay

In the present study, effectiveness of different leaves extract of *Olea europaea* was also confirmed by well diffusion assay and growth inhibitions zone diameters were measured in presence of each extract. Results are presented in Table **7**.

It is evident that the Gram-negative organisms *Escherichia coli* showed a slightly higher sensitivity to ethanolic and water extract of green leave compared to the powder leave, while control discs neomycin possessed significantly higher antimicrobial activity compared with the extracts (16mm).

The high content of phenolic and flavonoids compounds identified in the extract might contribute for its antimicrobial properties. It was reported that an antimicrobial action of phenolic compounds was related to inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes **(Aicha and Mohamed, 2017).** Increased membrane permeability is a major factor in the mechanism of antimicrobial action, where compounds may disrupt membranes and cause a loss of cellular integrity and eventual cell death.

In conclusion, the data obtained in this study demonstrate that the use of olive leaves as nutraceuticals may lower the risk of microbial infections, mainly due to the protective action provided by its phenolic compounds. The use of extracts is recommended to achieve health benefits due to the additive and synergistic effects of phytochemicals present in whole extract. However, further detailed studies are required to determine the active ingredients responsible for these effects and to determine the mechanism of action of these compounds in the anti-microbial activity.

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