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# ANTIBACTERIAL ACTIVITIES OF OLD AND YOUNG LEAVES OF OLEA *EUROPAEA* (OLIVE TREE) OF ALJOUF REGION, SAUDI ARABIA

A.Z. El-Bahrawy [1], M. Sarwar [*2] and Mazin O. Mohager [2]

Department of Biology [1], College of Science, Aljouf University, Al-Jawf, Saudi Arabia
Department of Medicine, College of Medicine [2], Aljouf University, Al-Jawf, Saudi Arabia

**Keywords:**

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**Correspondence to Author:**
**M. Sarwar**

Professor of Biochemistry and Head of Pathology department. College of medicine, Aljouf University, Al-Jawf Kingdom of Saudi Arabia.

**E-mail**: dr.sarwarbmu@hotmail.com

**ABSTRACT:** Antibacterial activities of different solvent extracts of young and old leaves has been evaluated against *Pseudomonas aeureginosa* (ATTCC10145) an opportunistic bacteria by Agar Well Diffusion Method. Extracts of olive leaves have been prepared by using standard extraction methods and DMS was used as vehicle for all the extracts. Amikacin was used as standard drug to which *P. aeruginosa* was found sensitive. Augmentin to which this bacteria is resistant was used to check combine effect (if any) with olive leave extracts. Ether, Chloroform and Alcoholic extracts of old and young leaves with or without Augmentin supplementation were used to evaluate the antibacterial activities against *P. aeruginosa* and all of these extracts were found active. The effect was displayed as concentration dependent for all the extracts tested in this study. The ether extract showed maximum activity which was more than the alcohol, chloroform extracts and Amikacin used as standard in this study. Augmentin supplementation to these extracts does not exert any additional inhibitory effect in the activities of olive leave extracts. Minimum inhibitory concentration was also determined and it was found that extracts of both old and young leave have inhibitory effect of which was found concentration dependent. There was no significant difference between antibacterial activities of old and young leaves. The outcome of present study suggests that these extracts have every potential to be used as neutraceuticals.

**INTRODUCTION:** Eradication of bacterial infections to overcome the infectious diseases remains uphill task in the human history as a considerable portion of morbidity and mortality is caused due to this menace for the mankind health. In the past decades a numerous wonderful advances has been made in developing the antibiotics to fight with the bacterial infections yet some of them like *Pseudomonas aeruginosa* defy and escape the antibiotic due to the ability of this opportunistic organism to develop an effective drug resistance against the antibiotics used to stop its virulence. *P. aeruginosa* is a problematic drug resistant pathogen implicated in many clinical situations.

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It has potential to be a part of community acquired infection as well as serious infections which are predominantly hospital acquired infections. Statistically representative colonization rates of *P. aeruginosa* for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples [1]. However, colonization rates of this pathogen may exceed 50% during hospitalization, especially among patients who have suffered from trauma or a breach in cutaneous or mucosal barriers [2].

The intrinsic resistance of *P. aeruginosa* to antibiotics and its ability to modify/ acquire genes responsible for encoding resistance determinants. Mechanism of resistance adopted by this pathogen is not only complicated but also diversified. For instance production of β-lactamases and aminoglycoside-modifying enzymes, diminished expression of outer membrane proteins, mutations in topoisomerases, and up-regulation of efflux

pumps may play an important role in its antibiotic resistance mechanism [3]. *P. aeruginosa* is naturally resistant to many antibiotics due to the permeability by virtue of its Gram-negative outer membrane. In addition, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of the antibiotics. In nature *P. aeruginosa* is found in the soil along with the bacilli, actinomycetes and molds, due to its co existence with these organisms it developed resistance to a variety of naturally-occurring antibiotics which they produce. This bacterium is intrinsically resistant to a number of antibiotics and can acquire resistance during therapy. Some strains are multi-drug resistant, ie, they are resistant to three or more classes of antibiotics.

These features limit the choices of antibiotics for *P. aeruginosa*. Only a few antibiotics are effective against *P. aeruginosa*, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains. [4] Therefore, there is an urgent need to search new compounds characterized by having effective arsenal against the resistance of bacteria. In this connection the use of different natural products as antibacterial and antifungal agents is an interesting strategy to overcome the bacterial resistance phenomena. This may culminate into the discovery of effective therapeutic tools against the dreadful bacteria for human health.

In the last decades medicinal plants have attracted the attention of scientists to explore about the antimicrobial plant-derived substances having promising potential against the resistant bacteria. In this regard natural gums and extracts of the whole resins, as well as specific extracts, fractions, essential oils and isolated compounds from the plants have revealed antibacterial activity [5, 6]. Natural antibacterial substances are not easy for bacteria to become resistant to. As natural antibacterial herbs are typically composed of a number of complex substances all working together to produce a result, it is not possible for simple bacteria to resist them[7]. Olive leaves have resisted bacterial attack on themselves for thousands of years which is a good indicaror of this property[8]. The active ingredients (including oleuropein) of this natural antibacterial, break down the cell walls of a wide range of pathogenic bacteria so destroying them [9]. Plants including olive tree are rich in a wide variety of secondary metabolites, such as flavonoids, alkaloids, tannins and terpenoids, which have been found *in vitro* to have antimicrobial properties[10]. With years of research scientists have now isolated the unique molecule, oleuropein responsible for the anti-bacterial effects of olive leaves. Oleuropein, the key nutrient, is most concentrated in the leaves than any other part of the plant [11].

Olive leaves are rich in phytonutrients and antioxidants, of which – oleuropein and hydroxytyrosol are bioactive. Other bitter phytonutrient compounds in olive leaves – caffeic acid and verbascoside – work together to resist bacterial damage [12], the leaves are important for their secondary metabolites such as the secoiridoid compounds oleacein and oleuropein, the former responsible for hypotensive activity [13] and the latter also for hypoglycemic activity [14]. Several reports have shown that olive leaf extract has the capacity to lower blood pressure in animals [15] and increase blood flow in the coronary arteries [16], relieve arrhythmiaand prevent intestinal muscle spasms [17]. A variety of antibacterial actions of oleuropein and its associated compounds have been demonstrated in vitro [18]. Six major phenolic compounds from green olives have been isolated; one particular compound, possibly a hydrolysis product of oleuropein, was much more inhibitory than oleuropein itself to the lactic acid bacterium Leuconostoc mesenteroides FBB 42.

Later on, the oleuropein aglycone and elenolic acid were found to strongly inhibit the growth of three further species of lactic acid bacteria, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, and *Lactobacillus brevis*. Since the aglycone is composed of elenolic acid bound to b-3,4-dihydroxyphenylethyl alcohol, and the latter compound was not inhibitory, the investigators concluded that elenolic acid was the inhibitory part of the aglycone molecule [19]. Oleuropein itself was not inhibitory to these bacteria, but did inhibit three species of non-lactic acid bacteria, *Staphylococcus aureus*, Bacillis *subtilis* and *Pseudomonas solanecearum* [20]. Inspite of these exquisite discoveries there is insufficient evidence of olive

leaves polar solvent extracts on the survival of *P. aeruginosa*. Therefore, plan of present work has set down the ambitions to check the activity difference between young and old olive leave extracts of Ethanol, Chloroform and Ether.

## MATERIAL AND METHODS:

### Extraction of Leaves:

The old and young healthy leaves from olive trees found in Aljouf region Kingdom of Saudi Arabia were collected in the month of March, 2014. The leaves were identified by Department of Botany, College of Science, Aljouf University. Both kind of leaves were washed and dried under shadow. The dried leaves were ground with mechanical grinder and passed through sieve to obtain powder of uniform size particles. 100 g of the ground powder of uniform particles of each of the old and young leaves were soaked separately in 500 ml of 95% ether in a round bottom 1Lflask and kept at a shaker at low speed for seven days at room temperature.

The samples were then put in a fine cloth and squeezed to transfer the liquid in a cleaned and dry flask. The solutions were then double filtered using Whatman paper No.1. All the residues were pooled in a round bottom flask and sequentially extracted by the polar solvents in the order of increasing polarity i.e. with chloroform following by Alcohol (95%).The extracts were then concentrated by using rotary evaporator at low temperature ($50^0$C) and pressure. Crude extracts were stored at $4^0$C till further experimentation [21].

### Identification of bacteria:

*P. aeruginosa* ATCC 10145 was subjected to standard microbiological technique for identification. This include motility, Gram staining, growth at temperature range of 5-42$^o$C, pyocyanine production, oxidase reaction, glucose, lactose fermentation on kilgler iron agar and hydrogen sulphide production [22]. *P. aeruginosa* ATCC 10145 also was tested for β –lactamase production using nitrocefin disks (Sigma- Aldrich, Germany) according to the instructions in the kit [23]. Development of a red color within 5 min in the area of the disk where the culture was applied implied β-lactamase production.

### Preparation of Inocula:

The susceptibility tests were performed by Mueller Hinton agar-well diffusion method [24]. The bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in a saline solution (0.9%, w/v)NaCl and adjusted to a turbidity of 0.5 Mac Farland standard ($10^8$CFU/ml). To obtain the inocula, these suspensions were diluted 100 times in Muller Hinton broth to give $10^8$colony forming units (CFU)/ml.

### Antimicrobial agents:

The isolated *P. aeruginosa* ATCC 10145 was tested for their sensitivity to some clinical used antibiotics such as augmentin 30µg and amikacin 30µg were used to determine the antibiotic sensitivity by the disk diffusion method on Mueller-Hinton (MH) agar. The antimicrobial agents were purchased from Oxoid Ltd.,UK . The results were interpreted according to the CLSI (formerly NCCLS) standard [25].

### Isolation of DNA:

The bacterial samples were inoculated in 5 ml LB broth and persude for incubation at 37°C for 24 hours. After centrifugation at 8000 rpm for 10 mins the supernatant was collected carefully so that the pellet should not be dislodged. The Pellet so obtained was resuspended in 500µL of TE buffer and incubated at -20°C for 1 hour. After bring it to room temperature, incubated at 37°C for 1 hour. The lysation of the cells was carried out by adding 5µL proteinase K enzyme and 30µL 10% w/v SDS. phenol:chloroform in1:1 was added to the suspension and centrifuged at 8000 rpm for 10 minutes at 4°C.The upper aqueous layer was again extracted by adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 8000 rpm for 10 minutes at 4°C.

After careful collection of upper aqueous phase double volume of 70% ethanol was added to the suspension along with 1/10th volume of 3M sodium acetate and left it stand on -20°C for 20 minutes. After centrifugation alcohol was discarded and the pellet was air dried without dislodging and 50µL of TE buffer was added to it. The quantification of total DNA was done by measuring the absorbance at 260 nm and 280 nm [26].

**Minimum inhibitory concentration**:
Olive extracts of ether, chloroform and alcohol were reconstituted in DMS to varying concentrations of 900, 800, 700, 600, 500, 400, 300, 200, 100µg/ml. To each set of these preparations 9 ml of autoclaved molten media (Muller Hinton agar) were added, after shifting on water bath at $50^0$C thoroughly mixed. The final seeded concentration was obtained as, 90, 80,70,60,50,40,30,20 and 10µg/ml for each of ether, chloroform and alcohol extract. These mixed media were poured into nine Petri dishes and the tenth plate was used as control without extracts, then pseudomonas suspensions were inoculated separately on the surface of solidified media on all the plates and were left at room temperature to dry and incubated at 370c for 24hours. The concentration at which no visible growth of bacteria appeared was regarded as minimum inhibitory concentration [27].

**Antibacterial activity by Agar Well Diffusion Method:**
*P. aeruginosa was* grown in Muller-Hinton media (Oxoid), Inoculum of bacteria was prepared by growing pure isolate in nutrient broth at 37˚C for overnight. The overnight broth cultures were sub-cultured in fresh nutrient broth. The agar plates were prepared by pour plate method using 20ml M-H medium. The sterile M-H agar medium is cooled to 45˚C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x $10^8$ cells) and then poured into the sterile petri dishes and allowed to solidify. Wells of 10 mm size were made with sterile cork borer and test extracts were added [28].

**RESULTS AND DISCUSSION:**
Antibacterial activities of old and young leaves of olive tree from Aljouf, Kingdom of Saudi Arabia against *P.aeruginosa* were analyzed. Antibacterial activities were determined by using a technique known as agar diffusion cavity method. Polar solvents i.e. Ether, chloroform and Alcoholic crude extracts of leaves mentioned above were poured in the 10mm wells and inhibition zone around the well were measured in millimeter. As depicted in **Table 1-4** *P.auregenosa* was resistant to augmentin (30µg) used in this study. Since all the extracts were dissolved in DMSO, it was used as control and no inhibition was seen around the wells filled with this solvent alone (**Table1**).

**TABLE1: EFFECT OF DIFFERENT SOLVENTS EXTRACT OF OLD OLIVE LEAVES ON *PSEUDOMONAS AERUGINOSA*.**

| Name of Bacteria | Crude extract | Inhibition zone in millimeter ( mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Extract concentration µg/well | | | | Standard | | Control |
| | | 25 | 50 | 100 | 150 | Amikacine(30µg) | Augmentin(30µg) | DMSO |
| *Pseudomonas aeruginosa* | Ether | 5 | 6.5 | 11 | 14.5 | 14 | R | - |
| | Chloroform | 5 | 8.5 | 8 | 9 | | R | |
| | Alcohol | 4.5 | 5 | 8 | 8 | | R | |

As standard, Amikacin (30µg ) was used against all the extracts for comparative purposes. The inhibition zone of the *P.aeurogenosa* sensitive to ether, chloroform and alcohol were measured and found minimum at 25 µg/well and maximum at 150µg/well with all the extracts (**Table1**). In other words, *P.aeuregenosa* showed concentration dependent behavior in response to all the extracts tested in this study. Extracts of old leaves with ether, chloroform and alcohol at concentration of 25µg/well created the area of inhibition zone as 5mm, 5mm and 4.5mm respectively. A slight increase (as compare to 25µg) in the inhibition zone was observed against all the extracts tested at the concentration of 50µg/well (6.5mm, 8.5mm and 5mm with Ether, Chloroform and Alcohol, respectively). Thereafter, at 100 µg Inhibition zones of *P.aeuregenosa* with old leave extracts were considerably increased (**Table1**). It was interesting to note that at this concentration (100µg) chloroform extract showed the similar area of inhibition zone (8mm) as at the concentration of 50µg (8.5mm). As can be seen on **Table 1**, area of inhibition zone in response to 100µg concentration was considerably increased as compared to the inhibition zone resulted against 50µg/well i.e ether

(11mm) and Alcohol (8mm). Ether extract at concentration of 150µg exhibited maximum zone inhibition i.e. 14.5 mm as compared to chloroform and Alcohol, as both of them generated area of inhibition zone 9mm and 8mm respectively (**Table1**). In conclusion, the results portrayed in **Table 1** shows that Ether extract of old olive leaves was more potent against the *P.aeuregenosa* activities and it exerted the antibacterial effect as the concentration was increased from 25 to 150µg/well.

In the plan of present study, it was anticipated that supplementation of augmentin with olive leaves extract may put a synergistic effect on the inhibition of *P.aerugenosa*.

However, as the results showed in **Table 2**, augmentin supplementation did not alter the inhibition momentum of the ether, chloroform and alcoholic extracts of olive leaves against *P.aerugenosa.* It is to be mentioned here that augmentin alone was completely remained ineffective as *P.aeuregenosa* was stubbornly resistant to this antibiotic. As can be seen in **Table 1**and **2** area of inhibition zone remains unperturbed with the additament of augmentin (30µg).

**TABLE 2: COMBINED EFFECT OF DIFFERENT SOLVENT EXTRACTS OF OLD OLIVE LEAVES AND AUGMENTIN ON *PSEUDOMONAS AERUGINOSA.***

| Name of Bacteria | Crude extract | Inhibition zone in millimeter ( mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Extract concentration µg/well | | | | Standard | | Control |
| | | 25 | 50 | 100 | 150 | Amikacine (30µg) | Augmentin (30µg) | DMSO |
| *Pseudomonas aeruginosa* | Ether | 5 | 6 | 10 | 14 | 14 | R | - |
| | Chloroform | 4 | 5 | 8 | 10 | | R | |
| | Alcohol | 4.5 | 5.5 | 9 | 8.5 | | R | |

We also extracted the young leaves of olive tree with Ether, Chloroform and Alcohol. These crude extracts were tested for antibacterial activity by using the *P.aerugenosa* as was executed with old leaves extracts. As it can be discerned in the **Table 3** and **4** there was no significant difference in antibacterial activity when compared these results with those of the extracts of old leaves. However, results enlightened (**Table 3, 4**) that young leaves showed better activity though not substantial.

**TABLE 3: EFFECT OF DIFFERENT SOLVENT EXTRACTS OF YOUNG OLIVE LEAVES ON *PSEUDOMONAS AERUGINOSA***

| Name of Bacteria | Crude extract | Inhibition zone in millimeter ( mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Extract concentration µg/well | | | | Standard | | Control |
| | | 25 | 50 | 100 | 150 | Amikacine(30µg) | Augmentin(30µg) | DMSO |
| *Pseudomonas aeruginosa* | Ether | 5.5 | 7 | 12 | 15.5 | 14 | R | - |
| | Chloroform | 6 | 9 | 9 | 8 | | R | |
| | Alcohol | 5 | 5 | 8 | 9 | | R | |

**TABLE 4: COMBINED EFFECT OF DIFFERENT SOLVENT EXTRACTS OF YOUNG OLIVE LEAVES AND AUGMENTIN ON *PSEUDOMONAS AERUGINOSA.***

| Name of Bacteria | Crude extract | Inhibition zone in millimeter ( mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Extract concentration µg/well | | | | Standard | | Control |
| | | 25 | 50 | 100 | 150 | Amikacine(30µg) | Augmentin(30µg) | DMSO |
| *Pseudomonas aeruginosa* | Ether | 6 | 9 | 12.5 | 15.5 | 14 | R | - |
| | Chloroform | 7 | 8 | 9 | 8 | | R | |
| | Alcohol | 5.5 | 6 | 8 | 8.5 | | R | |

Total DNA was extracted from $1.5\times10^9$ cells inoculated and grown in the lab. AS it is shown in fig Control and augmentin to which *P.aeurogenosa* is resistant DNA was found 17±1.9µg and 16±2.3µg respectively. The difference between two is non-significant.

DNA content in response to low and high concentration of extracts was also determined. Ether, Chloroform and Alcoholic olive leaves extracts suppressed the DNA of *P.aeuregenosa* from 17±1.9 µg to 6±µg, 7±µg and 8±µg at lower concentration i.e.25µg (**Fig.1**). The maximum inhibition of DNA synthesis was found at 150µg. It was observed that150µg extract of ether (2.5µg) inhibited the DNA more than the inhibition caused by 2.75 µg amikacin (**Fig.1**). At high concentration (150µg) Chloroform and Alcoholic extracts inhibited the DNA but less than Ether extract i.e.4± µg and 5±µg respectively (**Fig.1**). The results regarding young leaves have not been shown here because they were quite similar to the results of old leave regarding total DNA content.

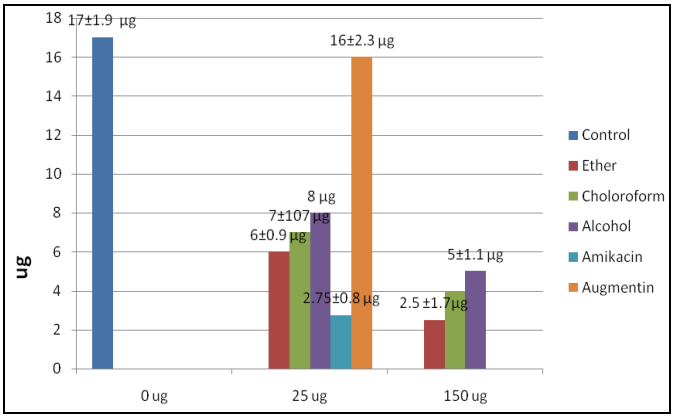


**FIG. 1: EFFECT OF DIFFERENT SOLVENT EXTRACTS OF OLIVE LEAVES ON TOTAL DNA CONTENT OF *PSEUDOMONAS AERUGINOSA***

As it is evident from the results, DNA inhibition coincides and agreed in with the inhibition zone of *P.aeuregenosa* in response to the extracts of olive leaves (**Table 1, 2, 3, 4**).

Regarding inhibition activities of the extracts of olive leaves against *P.aeuregenosa* were noted by N. B. Nora et al, 2012 [28], however, they used aqueous extract and in present study ether, chloroform and alcoholic extracts have been tested for their antibacterial activities. It was noted with interest that aqueous extract inhibition zone which above mentioned authors have noted was 13.5mm which is larger than of our chloroform and Alcoholic extracts but less than the ether extract inhibition zone (15.5). The difference between present study and above referred study is obviously due to different extraction solvent system. In addition the origin of olive trees and the time of leave collection and experimental conditions must be taken into consideration. Similar antibacterial activities of olive leaves were also reported by Markin and Deuk, 2003, [5].

They also reported the inhibition with aqueous extract of olive leaves. To our knowledge it is first time that olive leaves were collected from Al Jouf region Saudi Arabia in the month of March, 2014 and were extracted with Ether, Chloroform and Alcohol to analyze its antibacterial activities against an opportunistic bacteria i.e. *P.aeuregenosa*. Maximum antibacterial activities were found in Ether extract of olive leaves (**Table 1, 2, 3, 4**). As earlier stated by Pereira et al 2007[29] and Nora et al,2012, [28], the antibacterial activity noted in present study might be due to successful inhibition of bacterial respiratory system after the treatment with olive leave extracts.

The wall of the Gram- Negative bacteria has a lipopolysachride components Nikaido and Vaara, 1985 [30], Noora et al, 2012 [28], which may be impermeable to lipophilic solutes. However, in our study, Ether extract is more active which likely suggest some other mechanism to be explored in future.

Minimum Inhibitory Concentration. As depicted in **Fig. 2** extracts of all the three solvents tested in present study inhibited the *P.aeuregenosa* growth but the effective concentration is different for the ether (10µg/ml) than chloroform (20µg/ml) and alcohol (20µg/ml). Minimum inhibitory dose of ether is significantly lower than the other two solvents. It is suspected that constituents of ether extracts more active to break permeability barrier of cell wall and after getting enter it might have depressing impact on respiration of *P.aeuregenosa*. However, to explore the mechanism of action we need further studies and it is plan of our future work.

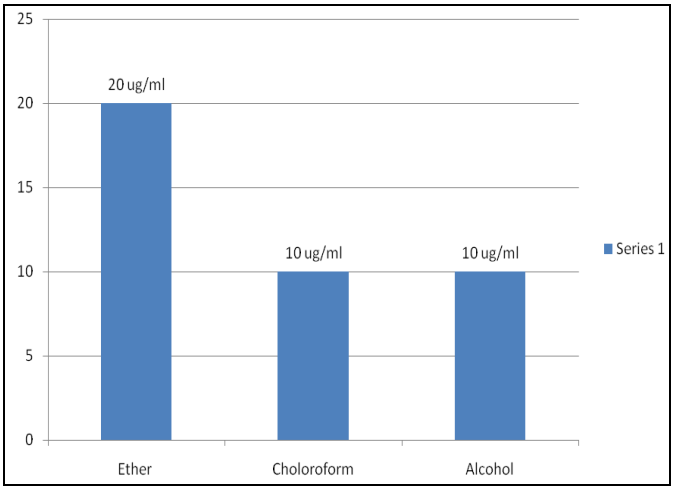


**FIG.2: MINIMUM INHIBITORY CONCENTRATION OF DIFFERENT SOLVENT EXTRACTS OF OLIVE LEAVES FOR *PSEUDOMONAS AEUROGENOSA***

**CONCLUSION:** The narrative of the present work found out that ether, chloroform and alcohol extracts of olive leaves has tremendous inhibitory potential against *P.aerugenosa*. It was further culminated that ether extract was more potent than other two solvent extracts tested in the present work. In the effort to evaluate the difference between the old and young leave extract we found that there is not a significant difference between old and young leaves. There was no additional effect noted when augmentin was supplemented to all the extracts and tested for antibacterial activities. The effect on total DNA content was also concentration dependent and seen more in ether extract than chloroform and alcohol. Ether extract also shown the minimum inhibition concentration as half of the chloroform and the alcohol. From the outcomes of agar well diffusion method, minimum inhibitory concentration and DNA content in response to olive leave extract it can safely be stated that ether extract has a comparable activities to that of amikacin and can be a potential candidate for neutraceutical for eradication of opportunistic bacteria i.e. *Pseuodomonas aeruginosa.*

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