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Comparative antimicrobial activities of wild and cultivated varieties of *Olea europaea* different leaves extracts in Pakistan

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ABSTRACT

This research aims to find out the antimicrobial activities of the crude extract of wild and cultivated olive leaves. Leaf extracts of wild and cultivated varieties of Olive plants were screened for their antimicrobial activities against nine bacterial strains (six gram-positive, three gram-negative) and a single fungus. The activity of both varieties was compared to determine which variety is more affective against human pathogens. The obtained results of measurement of ZOI from the disc diffusion method specified that the methanolic extract exhibited strong inhibitory activities against Bacillus atrophus, with the highest inhibition zones (10±0.40mm) that was computable with its antibiotic counterpart, Clarithromycin with inhibition zones (16±0.21mm) for both varieties. The inhibitory activity of these extracts confirmed the antimicrobial activity and its potential use in the treatment of microbial diseases. The lowest ZOI value (2±1.07mm) was observed for n-hexane extract against Pseudomonas aurigonosa and Salmonella typhi that was six times lower than its antibiotic standard, Ciprofloxacin. Antimicrobial activities of different plant extracts obtained were seemed to be too different in the sense of efficiency as some of the bacterial strains are found to extra resistance and others are having more susceptibility towards the plant crude extracts as compared to their respective antibiotics used in this study. All the bacterial strains showed fewer susceptibilities to both plant extracts as compared to standard antibiotics used which shows that both the standard antibiotics and plant crude extracts have higher antibacterial functions to Gram positive bacterial strains as compared to those of Gram-negative bacterial strains. The screening of both wild and cultivated varieties of olive leaves extracts especially wild variety proved that they possess high medicinal values and may be effectively used as potential and valuable drugs reservoirs.

***Key words**: Olea europaea, leaf extract, antimicrobial activity, zone of inhibition*

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INTRODUCTION

The antimicrobial activity of plants has numerous characteristic abilities of disease therapy, so effectively they are used for the treatment of infections causing diseases although synthetically prepared antimicrobial drugs can produce many the side effects [1]. Plant extracts and their essential oils (Eos) are very popular in modern science. From ancient times, numerous flora are useful as drugs, perfumes and food and their extracts even in lesser amounts possess bioactive components proud to efficient antimicrobial agents as compared to artificial medicines[2-3].The antimicrobial activities of medicinal plants have been reported in literature[4-7]. *Olea europaea* possesses durable natural resistivity microbes and show medicinal potentials for the cure of infectious diseases and fever controlling[8-9]. Olive leaf extracts 'useful constituents holding antimicrobial activities[10-14]. These components are certainly in the *Olea europaea* leaf and fruits display antimicrobial activities, free-radical antagonism and antioxidant potentials for healthcare interests. Among the non-polar phenol's components, oleuropein is the high abundance while the tyrosols and hydroxyl tyrosols are the polar phenols of *Olea europaea*. These polar and non-polar phenolic extracts of *Olea europaea* are showing potent against antiviral, antiretroviral, antibacterial, antiyeast, antifungal and additional antiparasitic capabilities [15-16]. Presently, commercially available antimicrobial and antifungal drugs are commonly used for the treatment of serious disease due to which multiple drug resistibility has been given development. Apart from this serious matter, sometime antibiotic drugs cause unfavourable consequences like hypersensitivity reactions, suppression of immunity and allergy on host physiology. So, a need is developed for the alternate antimicrobial and antifungal drugs from plants having medicinal values to treat infection causing diseases. This research aims to find out the antimicrobial activities of the crude extract of wild and cultivated olive leaves. Leaf extracts of wild and cultivated varieties of Olive plants were screened for their antimicrobial activities against some pathogenic bacterial strains like *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Agrobacterium tumefaciens, Erwiniacarrotovora, Staphylococcus aureus, Bacillus subtillus, Bacillus atrophies* and a fungus, *Candida albicans*. The antimicrobial activity was measured by using disk diffusion method. The activity of both varieties was compared to determine which variety is more affective against human pathogens.

MATERIAL AND METHODS

Experimental Assays

Collection of Plant Materials

Olive fruits and the two selected specimens' plant materials containing fresh leaves were collected from the local area of Dir district of Khyber Pakhtunkhwa, Pakistan. A botanical taxonomist at the PCSIR laboratories complex Peshawar, Ministry of Science and Technology, Government of Pakistan done its botanical identification. For reference determination, its voucher samples have been deposited airtight glass containers to be protected from direct sun light in the PCSIR labs complex Peshawar departmental herbarium until needed for further phytochemical analysis and use.

Preparation of leaves extracts

The olive leaves were thoroughly cleaned and washed with tap water and then rinsed with distilled water of analytical grade. After chopping into small pieces, the rinsed olive leaves of collected samples were air shade dried and finely powdered by crushing in electronic grinder. 100 g of finely ground olive leaves powder was extracted with 350 mL of ethyl acetate, ethanol, methanol, distilled water and hexane each **(**Technical grade- Merck**)** and boiled water in 1000 ml conical flasks. Flasks were vigorously shaken in a Labotec model 20.2 shaking machine at high speed overnight. The supernatant was transferred into pre-weighed and labelled flasks after shaking. To fully extract the leaves material, the procedure was repeated for three times. The rotary evaporator at 40°C was used for the removal of solvents under vacuum by and the extraction efficiency was quantified by determining the weight of each of the extracts [17-18].

Antimicrobial activities

Test organisms

The obtained leaves extract was analyzed for antimicrobial activities against nine bacterial strains (six gram positive, three gram-negative) and a single fungus (Table 1). The manual of clinical microbiology recommendations was used for the identifications of microbial strains using of biochemical profiles accordingly [19]. First of all, the microbial strains were cultured in a nutrient's broths and then their 24 hours' incubation was done at 37°C.

Table: 1Microorganisms used for antimicrobial study of olive leaves extracts.

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

Antimicrobial Assay and media preparation

A 24 hours longstanding subculture of every microbial strain was inoculated for the antimicrobial test. The agar medium was used for microorganisms' growth. In vitro antibacterial analysis was performed by disc diffusion technique as represented. The nutrient agar medium was sterilized at the temperature range 40- 45°C and was decanted bacterial suspension containing petri dishes. The experiments were conducted twice. In the first series of experiments, olive leaves crude extracts were verified for the antimicrobial screening against previously stated bacterial strains and a single fungus. 6 µL and 12µL Dimethylsulfoxide **(**DMSO**)** extracts were absorbed by the filter paper discs which were used for the determination of the antimicrobial potent activity. In the second experiment series the commercially obtainable customary reference antibiotic discs that area unit are Clarithromycin (50µg $6\mu^{-1}$) Ciprofloxacin (30 µg $6\mu^{-1}$) and Clotrimazole (50 µg $6\mu^{-1}$) were placed on the highest of the medium within the center of petri dishes by following the disc diffusion technique [20-22]. The aim of this experimental set up was to check the antimicrobial activity of the quality reference antibiotics there with of the solvent extracts of wild and cultivated olive leaves. The plates containing microorganism cultures were incubated at 37°C for 24 hours' time. All of the plates were the studied for the existence of inhibition as an antimicrobial screening property after the incubation time. The antibiotics sensitivities tests were carried out by the use standard antibiotics (Clarithromycin, Ciprofloxacin and Clotrimazole). Solvents like ethanol and methanol showing antimicrobial activities were also investigated against specific strains of bacteria.

RESULTS AND DISCUSSION

Antimicrobial activities

Wild and cultivated olive leaves (both varieties) crude extracts were prepared in different solvents (Hexane, Ethanol, Methanol, Ethyl acetate and distilled Water). The antimicrobial activities of these extracts were analyzed with the help of susceptible disc diffusion technique according to the protocol described[23].

The antimicrobial activity of both varieties leaves extracts and standard antibiotics were observed clearly against the verified microbial strains (bacterial and fungal). The results shown in Tables (2and3) demonstrated the anti-microbial activities of wild and cultivated varieties of olive plants leaves extracts respectively. In this study, nine bacterial (six Gram negative and three Gram positive strains) and 1 fungal strain (Table1) were tested for antimicrobial assay of leaves extracts of olive plant against antibiotics including Clarithromycin, Ciprofloxacin and Clotrimazole. The antimicrobial activities of both varieties leaf extracts (wild and cultivated) was compared with each other and standard antibiotics. The results were based on the measurement of minimum zone of inhibition (ZOI) that was shown in millimeter (mm). It was observed that ethyl acetate and methanol extracts of both varieties (wild and cultivated) of olive

leaves extracts exhibited maximum activity; while minimum antimicrobial activities were observed for hexane extracts (Tables 2 and 3)

Both varieties extracts displayed significant antimicrobial activities against all of the tested strains as shown in Tables (2 and 3). The disc diffusion method resulted from the measurements of minimum zone of inhibition (ZOI), specified that methanollic extract exhibited major inhibition activity contrary to *Bacillus atrophus*, with the highest inhibition zones (10 mm) that was computable with its antibiotic counterpart, Clarithromycin with zone of inhibition (16 mm). The lowest ZOI value (2 mm) was observed for hexane extract against *Pseudomonas aurigonosa* and *Salmonella typhi* that was 6 times lower than its antibiotic standard, Ciprofloxacin. Antimicrobial activities of different plant extracts obtained were seemed to be too much diverse in the sense of efficacy as some of the bacterial strains are found more resistant while some others are found to be highly susceptible to the extracts in comparison with their respective antibiotics used in this study. All the bacterial strains show fewer susceptibilities to both plant extracts as compared to standard antibiotics used which shows that both the standard antibiotics and plant crude extracts have higher antibacterial efficacies to Gram-positive bacterial strains as compared to those of Gram-negative bacterial strains (Table 1). Test results are shown in the attached figure 1.

Table: 2 Antimicrobial activity (mm) of Wild Variety of olive leaves extracts along standard antimicrobial drugs.

Microbial strains	Solvent Extracts					Standard drugs		
	Zone of Inhibition (mm)							
	Hexane	Ethanol	Methanol	Ethyl acetate	Aqueous	Clarithromycin	Ciprofloxacin	Clotrimazole
Bacillus atnophus	7±1.247	9±0.812	10±1.248	9±0.812	7±1.247	16±5.403	-	-
Salmonella typhi	2±0.816	5±0.808	9±0.812	9±0.812	8±1.247	-	13±0.942	-
Escherichia coli	3±0.817	5±0.808	8±1.247	9±0.812	3±0.817	-	14±1.021	-
Agrobacterium tumefaciens	4±1.112	5±0.808	7±1.247	6±1.633	3±0.817	-	13±0.942	-
Pseudomonas aeruginosa	2±0.816	4±1.112	8±1.247	7±1.247	5±0.808	-	15±0.419	-
Staphylococcus aureus	3±0.817	5±0.808	9±0.812	7±1.247	7±1.247	14±1.021	-	-
Klebsiella pneumonia	5±0.808	6±1.633	9±0.812	7±1.247	5±0.808	-	14±1.021	-
Bacillus subtillus	4±1.112	6±1.633	8±1.247	8±1.247	7±1.247	14±1.021	-	-
Erwinia carrotovora	7±1.247	7±1.247	10±1.248	7±1.247	7±1.247	-	15±0.4189	-
Candida albicans	6±1.633	5±0.808	8±1.247	7±1.247	5±0.808	-	-	14±1.021

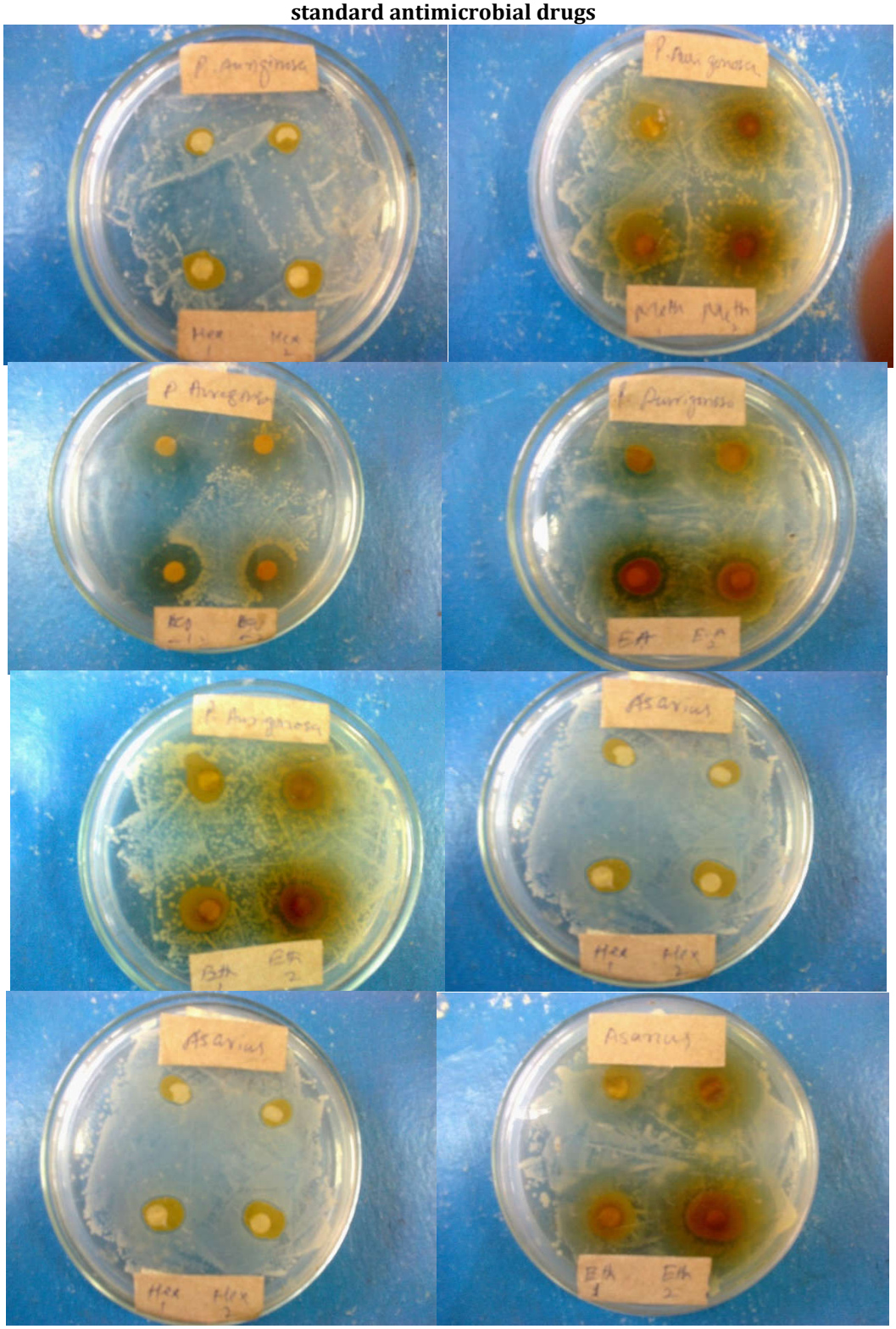
The results are taken in taken in triplicate standard deviation for each quantity.

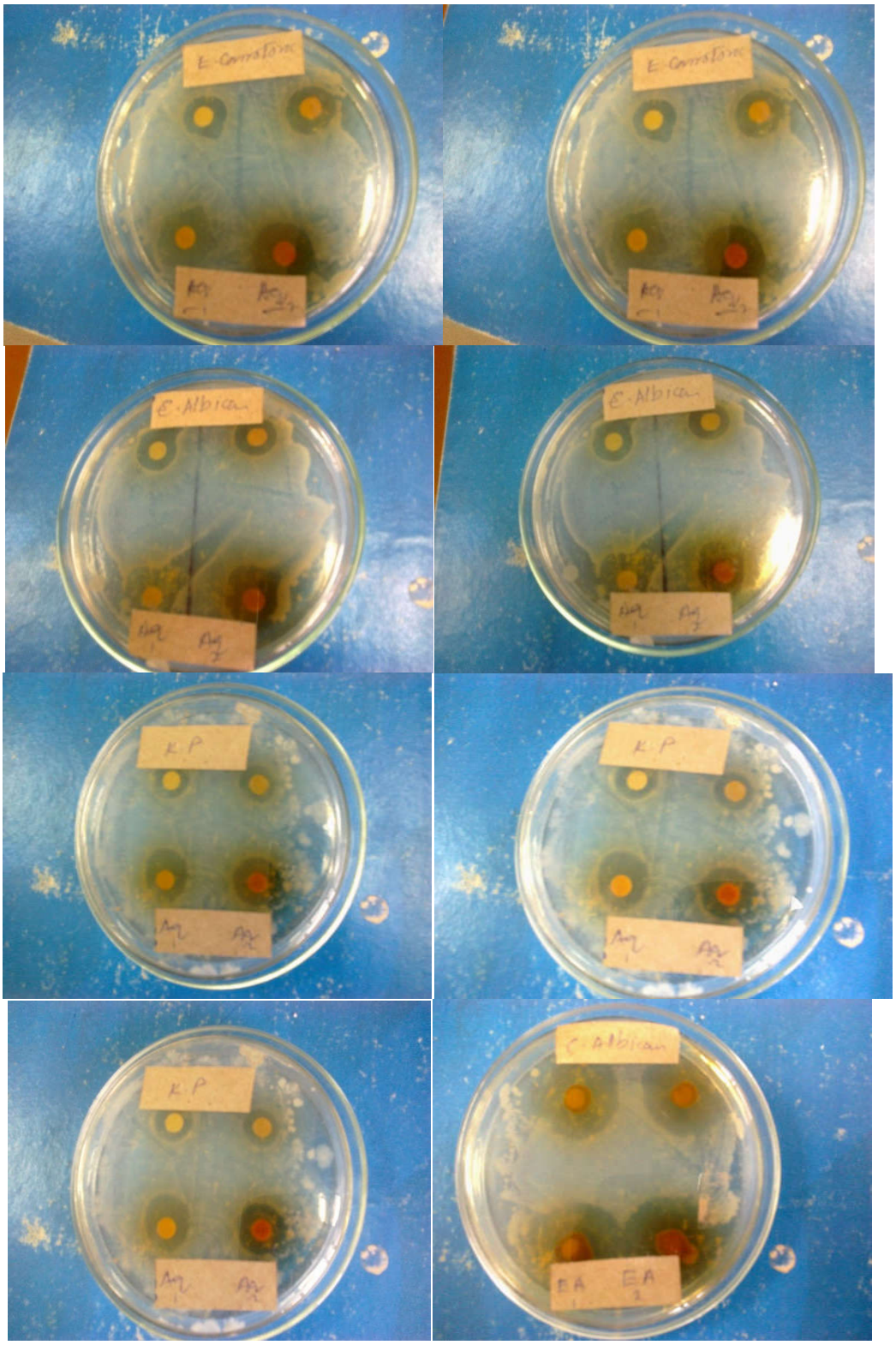
Table: 3 Antimicrobial activity (mm) of cultivated variety of olive leaves extracts along standard antimicrobial drugs.

Microbial strains	Solvent Extracts					Standard drugs		
	Zone of Inhibition (mm)							
	Hexane	Ethanol	Methanol	Ethyl acetate	Aqueous	Clarythromycin	Ciprofloxacin	Clotrimazole
Bacillus atnophus	7±1.247	8±1.372	10±1.248	9±0.812	7±1.247	16±5.403	-	-
Salmonella typhi	2±0.816	5±0.808	9±0.812	9±0.812	7±1.247	-	12±1.247	-
Escherichia coli	4±1.112	7±1.247	9±0.812	9±0.812	4±1.112	-	14±1.021	-
Agrobacterium tumefaciens	3±0.817	5±0.808	7±0.476	8±1.372	7±1.247	-		-
Pseudomonas aeruginosa	2±0.816	4±1.112	9±0.812	7±1.247	5±0.808	-	12±1.247	-
Staphylococcus aureus	3±0.817	5±0.808	6±1.633	7±1.247	7±1.247	15±0.419	-	-
Klebsiella pneumonia	5±0.808	6±1.633	7±1.247	7±1.247	5±1.247	-	13±0.942	-
Bacillus subtillus	4±1.112	6±1.633	8±1.372	8±1.372	7±1.247	16±5.403	-	-
Erwiniacarrotovora	6±1.633	8±1.247	6±1.633	7±1.247	9±0.812	-	13±0.942	-
Candida albicans	6±1.633	5±0.808	8±1.372	7±1.247	5±0.808	-	-	12±1.247

The results are taken in taken in triplicate standard deviation for each quantity.

Fig. 1 Antimicrobial activity of Wild and Cultivated Varieties of olive leaves extracts along standard antimicrobial drugs




E. Coli
E. Coli
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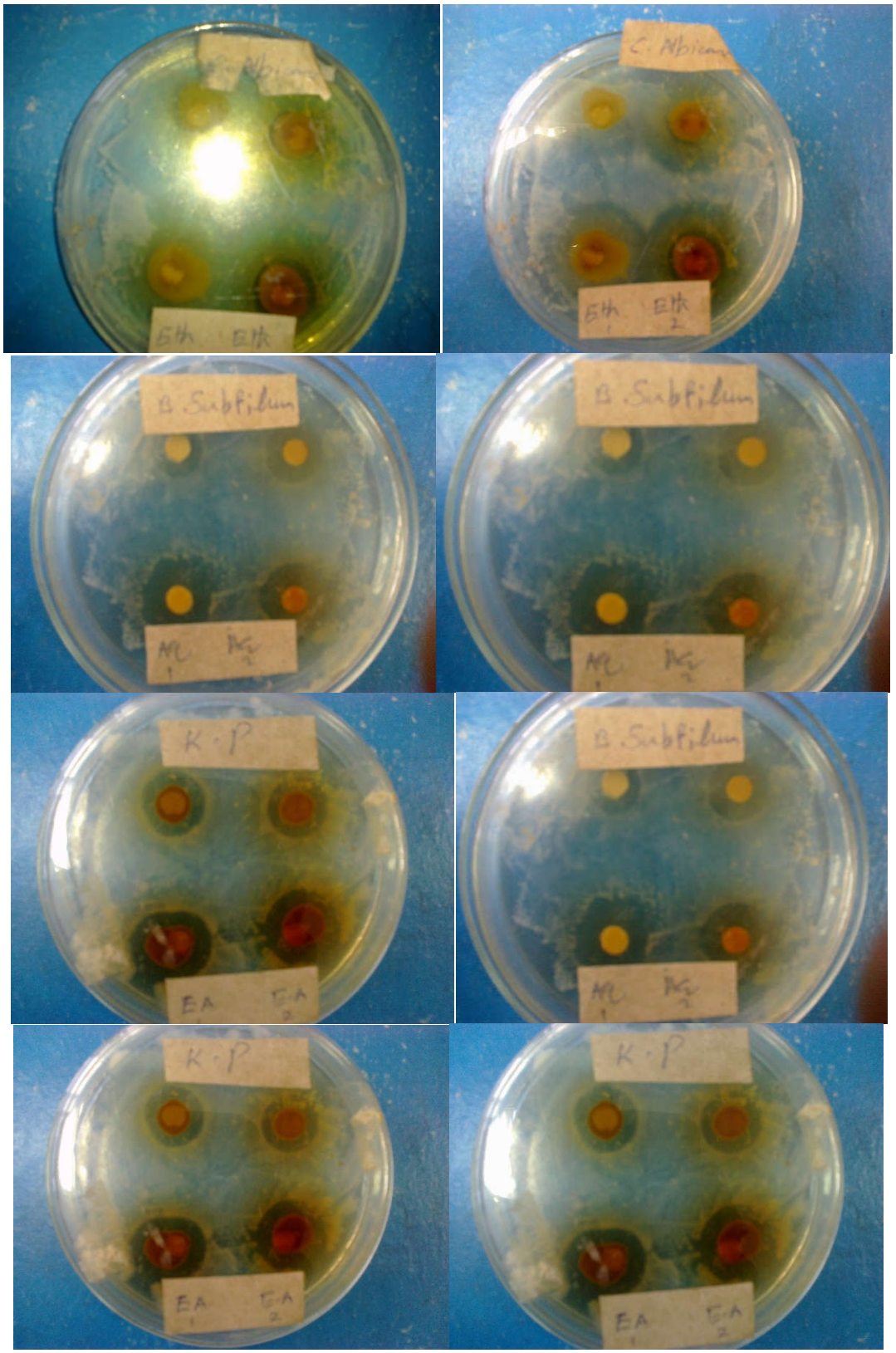
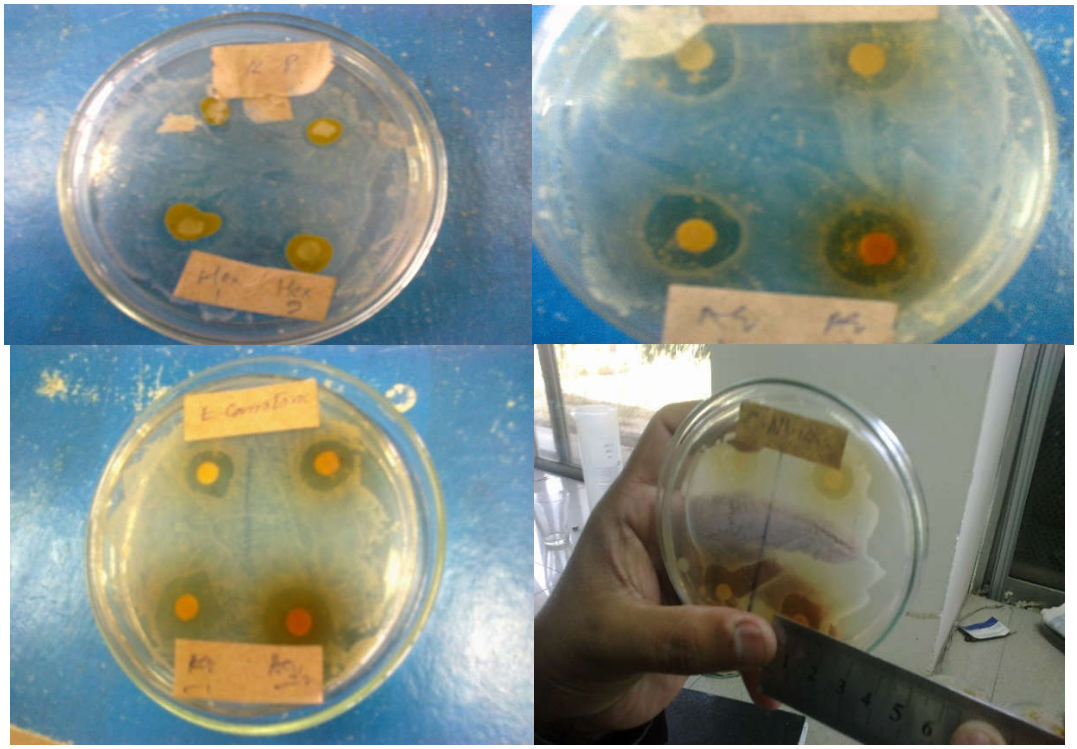
E. Albicans
K. P
K. P
K. P
C. Albicans


C. Albicans
B. Subtilum
B. Subtilum
K. P
B. Subtilum
K. P
K. P



Due to microbial multi-drugs resistivity, herbal plants are chief priority sources to novel medicines against microorganisms. Saponins are also important phytochemical component of plants which exhibit antimicrobial activities[23]. Antimicrobial activities of olive leaves extracts have been reported by several studies. It has studied that antimicrobial activities of olive leaves extracts against *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Klebsiela pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Streptococcus pneumoniae* and *Citrobacter freundii* [24-25].

In this study, the antimicrobial activity of both varieties olive leaves extracts was checked against gram positive, gram negative strains of bacteria and single fungus. The plant showed a broad spectrum of antimicrobial activities against both types of bacterial strains. Antifungal activity of olive leaves extracts was evaluated for the first time in this study and results were much more encouraging as expected. Moreover, different types of organic solvents were used in this study to examine the effects of solvents on antimicrobial activity and to determine which solvents are more effective in showing antimicrobial activity and the results obtained supported methanol and ethyl acetate as the best options for antimicrobial olive leaves extracts. The antimicrobial activities of wild varieties methanollic extracts in comparison with the applied antibiotics. The results of this study were also found novel to the previous studies [23-26].

It has been reported that alkaloids, saponins and tannins are important to be used in antibiotic agents against known pathogens[27-28]. Chemically, flavonoids are hydroxylated phenol rich compounds which are believed that these show antimicrobial activities by the complex formation with extracellular as well as soluble proteins and with cell wall of bacteria[29]. These are also proved useful antioxidant agents and possessing effective anticancer characteristics [30-31]. Saponins are capable of precipitation and coagulation of erythrocytes. Saponins have some major activities like lather formation in aqueous media, hemolysis, and cholesterols binding and acid tastes[32-33].

The antimicrobial activities revealed that methanolic and Ethyl Acetate extracts of both varieties (wild and cultivated) of olive showed maximum activity while minimum antimicrobial activities were observed for hexane extracts. It is because alcohol and ethyl acetate extracts give rise to flavonoids and phenolic phytochemicals as compared to hexane.

The obtained results of measurement of ZOI from the disc diffusion method specified that the methanolic extract exhibited strong inhibitory activities against *Bacillus atrophus*, with the highest inhibition zones (10±0.40mm) that was computable with its antibiotic counterpart, Clarithromycin with inhibition zones (16±0.21mm) for both varieties. The inhibitory activity of these extracts confirmed the antimicrobial activity and its potential use in the treatment of microbial diseases [14] also studied antimicrobial activity of Olive leaf extracts against different bacteria. The lowest ZOI value (2±1.07mm) was observed for n-hexane extract against *Pseudomonas aeruginosa* and *Salmonella typhi* that was six times lower than its

antibiotic standard, Ciprofloxacin. Antimicrobial activities of different plant extracts obtained were seemed to be too different in the sense of efficiency as some of the bacterial strains are found to extra resistance and others are having more susceptibility towards the plant crude extracts as compared to their respective antibiotics used in this study. All the bacterial strains showed fewer susceptibilities to both plant extracts as compared to standard antibiotics used which shows that both the standard antibiotics and plant crude extracts have higher antibacterial functions to Gram positive bacterial strains as compared to those of Gram-negative bacterial strains. The screening of both wild and cultivated varieties of olive leaves extracts especially wild variety proved that they possess high medicinal values and may be effectively used as potential and valuable drugs reservoirs.

CONCLUSION

Disc diffused technique was employed for determination of the antimicrobial activities of wild and cultivated varieties extracts against diverse bacteriological and fungal strains in different solvents. Strong antibacterial and antifungal activities were exhibited by all investigated extracts clearly. Amongst all, Methanol extracts have highest biocidal activities whereas n-hexane extracts had the least activities in case of both varieties leaves extracts in five different solvents (Water, Methanol, Ethanol, Ethyl acetate and n-hexane).

Antimicrobial activities of both varieties in different solvent extracts concluded that the Methanol extracts of wild variety had highest biocidal activities than the cultivated variety leaves while n-hexane had the least antimicrobial activities. From the antioxidant activity of various extracts, it is concluded that wild variety n-hexane and ethanol extracts showed highest while aqueous extracts showed lowest free radical scavenging activities than the cultivated variety in different concentrations taken in µg/mL. From the conclusion of obtained results of this study, it is indicated that the olive fruits and leaves extracts possess pain killer, antiinflammatory, antioxidant and antimicrobial efficacies which fully supports the prescription of this useful and famous plant species as traditional medication to treat serious inflammatory and infectious diseases in the world.

FUTURE PERSPECTIVE

This whole study reveals that the investigated plant is valuable to be explored furthermore in much better manner using different microbial strains, advance instrumentations and either some or all the available conventional as well as traditional techniques.

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