Pak. J. Bot., 46(4): 1463-1468, 2014.

ANTIMICROBIAL POTENTIAL OF LEAF AND FRUIT EXTRACTS AND OILS OF WILD AND CULTIVATED EDIBLE OLIVE

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Abstracts

Olive tree is the first botanical noted in the Bible. Leaves and fruits of olive are rich sources of Phenols, triterpenes, and flavanoids. Oleuropein obtained from the leaves extract is believed to be important therapeutic compound. Olive leaf and oils are used for the treatment of different diseases as folklore medicines by different ethnic groups in different countries of the world. The present study aims to investigate the potential antimicrobial activities of wild (*Olea ferruginea*) and edible (*Olea europaea*) olive leaf crude extracts, crude oils from ripe and unripe fruits and extra virgin oils against the selected gram positive and gram negative bacterial strains. The results show that olive leaf and oil have potential antibacterial activities against some of the gram positive and gram negative bacterial strains. However, certain strains were resistant to the extracts. It was also found that the activities were higher for the gram negative strains as compared to gram positive strains. The methanolic and ethanolic extracts were found to be more efficient in extraction than the other solvents used. Leaf extracts were more effective than the oil extracted from ripe and unripe fruits. There was no significant difference in the activities of extra virgin oils and crude leaf extracts. From the results it is concluded that the leaf extract is a cheap and effective antibacterial agent that can be used as alternative to purified oil.

Introduction

Resistance of human pathogenic bacteria to drugs is a worldwide problem (Owais *et al.,* 2005). In developing countries like Pakistan low income people mostly in villages use folk medicine for the treatment of various diseases including infections (Walter *et al*., 2011). When people from remote areas get a disease, they go to traditional healers for treatments, because of their expertise in diagnoses, setting bones, treating wounds and making herbal medicines. They claim that traditional medicines are cheaper and more effectives than allopathic medicines (Shinwari & Qaiser, 2011). Ospina (2002) reported that patients from the remote area have a reduced risk to get an infectious disease than people from urban areas when treated with traditional medicines. The resistance of pathogenic bacteria to antibiotics can be prevented by the use of new compounds from plants or plant lead synthetic antimicrobial compounds (Shinwari *et al*., 2012; Shah, 2005). To use folk medicines for the treatment of common infectious diseases, it is necessary to evaluate the potential of folk medicines on scientific basis (Fabricant & Farnsworth, 2001). Antimicrobial properties of both phytochemicals and plant extracts can be of great therapeutic significance in treatments. Mostly the allopathic drugs have been derived from the extracts taken from medicinal plants. Our Holy Prophet (Peace be upon Him) also used certain herbs to cure various diseases (Baqar, 1989). One of them is the olive tree. In western literature, olive is one of the most cited plants. In the Bible, the oil and tree of olive have been mentioned over 30 times. In the Holy Quran, it is praised as a precious fruit and have been mentioned almost seven times (Viktoria, 2010).

Olea europaea L. (Olive) is one of the most important fruit tree. It is native to the Mediterranean region such as Palestine, Syria, Spain, Italy, Greece, France, Turkey, Algeria and Morocco. It accounts for 98% of the world crop and cover about 8 million hectare area (Guinda *et al.,* 2004; Pereira *et al.,* 2007).

Olea ferruginea Royle is found wild in the Himalayas from Kashmir to Nepal up to 2400 m altitude. The olive plant is an important source of nutrition and medicine throughout the history of civilization. They contain many potentially bioactive compounds that may have antioxidant (Aytul, 2010), antihypertensive (Hansen *et al.,* 1996), anti-inflammatory, anti-bacterial (Upadhyay *et al.,* 2010; Aliabadi *et al.,* 2012; Nora *et al.,* 2012), hypoglycemic (Gonzalez *et al.,* 1992), and hypocholesterolemic properties. In the present study, the *in vitro* effect of olive leaf extracts and oils on the survival and growth of certain gram positive and gram negative bacterial strains of American type culture collection (ATTC) was investigated.

Materials and Methods

The present study was conducted in the Iqbal Ahmad Qarshi (IAQ) and Qarshi Research International Labs at Qarshi Industry (Pvt.) Ltd., Hattar, Pakistan. The leaves and fruits of *Olea ferruginea* were collected from the Qarshi Herb Garden and leaves of *Olea cuspidata* were collected from natural plantation of Khanpur hills. The oil from ripe and un-ripe fruit of *Olea europaea* was

extracted in IAQ Lab and virgin olive oil was purchased from a superstore in Islamabad. The shade dried leaves were grinded to fine powder before preparing the extract.

Leaves crude extraction: About 10 gram of fine powder of leaves was dissolved separately using different organic solvents for 48 hours. Rotary flash evaporator was used to concentrate all the extracts. The concentrated extract was weighed and preserved in airtight bottles at 4°C until further use. For antibacterial assay, 15 mg of each extract was dissolved in 1ml of DMSO as a solvent.

Crude oils extraction: Olive oils were extracted from the ripe and unripe fruit by mechanical pressing and using n-hexane as a solvent. The mechanical pressing method involves applying pressure to stacked filter mats, smeared with paste, that alternate with metal disks; a central spike allows the expressed oil and water (olive juice) to exit.

Bacterial strains: Cultures of nine pathogenic bacterial strains of American type culture collection were used in this study. Among these *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC®27853), *Klebsiella pneumoniae,* (ATCC 15380), *Salmonella typhimurium* (ATCC®14028), *Enterococcus faecalis* (ATCC®49452) were gram negative and *Staphylococcus aureus* (ATCC®6538), *Listeria monocytogenes* (ATCC®13932), *Bacillus subtlilis* (ATCC®19659) and *Bacillus cereus* (ATCC 11778) were gram positive. Cultures were maintained in 2% w/v Luria Broth medium at 37ºC. Each bacterial culture was kept overnight and inoculated in 15 ml of media separately for each test and control experiment. For testing the activities, each bacterial culture were stored at 4ºC and sub-cultured after every 8th day in solid agar plates.

Screening of antibacterial activity: Antimicrobial potential of olive oils and leaves crude extracts on bacterial growth was determined at concentration of 15mg/ml. For screening of antimicrobial activity, agar disc diffusion method was used for each essential oil and leaves crude extracts. Equal volume of solvent was added to dilute the essential oils. A known volume (0.5-1 ml) of each extract and oil was coated on 6 mm size sterile filter paper discs (Whatman No. 1) separately. Bacterial inoculums were spread evenly on the surface of each agar plate and extracts coated discs were placed in the centre of inoculated agar plate. Sterile distilled water and solvents were used as negative control. All cultures were incubated at 37ºC for 24 h and zone of inhibition surrounding filter paper disc was measured.

Statistical analysis: Each experiment was repeated twice and each treatment consists of three replicates. Data are presented in Tabulated form and Graphs were prepared using the Excel Spread sheet. Zone of inhibition determined is based on the readings.

Results and Discussion

There is an urgent need to cure new emerging infectious diseases using diverse chemical structures and with novel mechanism of actions (Rojas *et al.,* 2003, Gul *et al*., 2012). Plant based antimicrobial agents play a very important role in the prevention and treatment of various infectious diseases. Therefore, scientists are now turning their attention to folk medicine against microbial infections resistant to synthetic drugs. Biological screening of traditional medicinal plants against pathogenic microorganism makes it logical to develop new drugs. The present study was conducted in order to screen olive plants leaf extracts in different solvents and its fruit oils against different gram positive and gram negative pathogenic bacteria cultures. The results as shown in Tables 1 & 2 indicate that extracts of both the plants have substantial activities against both gram negative and gram positive bacterial strains.

It is also evident from the results that extraction procedure also has substantial effects on the activity of the extracts. The maximum activities were found for the methanolic extracts in both the plants against both types of bacterial strains. This might be due to the good extraction efficacy of methanol compared to other solvents, as it allows the extraction of entire phenolic compounds (Nostro *et al*., 2000; Igbinosa *et al*., 2009; Fazal *et al*., 2011). The second and third effective solvents were ethanol and water, respectively. Other solvent extracts (Ethyl acetate, n-hexane, chloroform and diethyl ether) have no significant difference in activities against the selected bacterial strains.

It was also found that none of the extracts of both the plant species have any effects on certain bacterial strains such as *Bacillus cereus* ATCC® 11778, *Salmonella typhimurium* ATCC®14028 and *Enterococcus faecalis* ATCC®49452. All these strains are resistant to all the extracts tested. The most susceptible bacterial strains were *Staphylococcus aureus* ATCC®6538, *Listeria monocytogenes* ATCC®13932, *Pseudomonas aeruginosa* ATCC®27853, *Escherichia coli* ATCC®25922 and *Klebsiella pneumonia* ATCC® 15380. From the scoring of zone of inhibition, it is also evident that gram negative bacterial strains are more susceptible to both of the plant extracts than gram positive bacterial strains as shown in Figs. 1 & 2 (Tables 1 & 2).

Varying degree of susceptibility in different extracts indicates that some of the solvents are more efficient in extracting the active substances than the others. The use of water, methanol, and ethanol as extracting solvents from the leaves of *Olea europaea* and *Olea ferruginea* proved to be more efficient. Nostro *et al*., (2000) also described that aqueous and alcoholic extracts are more efficient in extraction process. In a study, Igbinosa *et al*., (2009) found that the methanolic extract of some plants are more effective than the ethanol extract. In the present study it was also observed that methanolic extracts of both the plants are more efficient against both gram negative and gram positive bacterial strains and is in consistence to the results of Igbinosa *et al*., (2009).

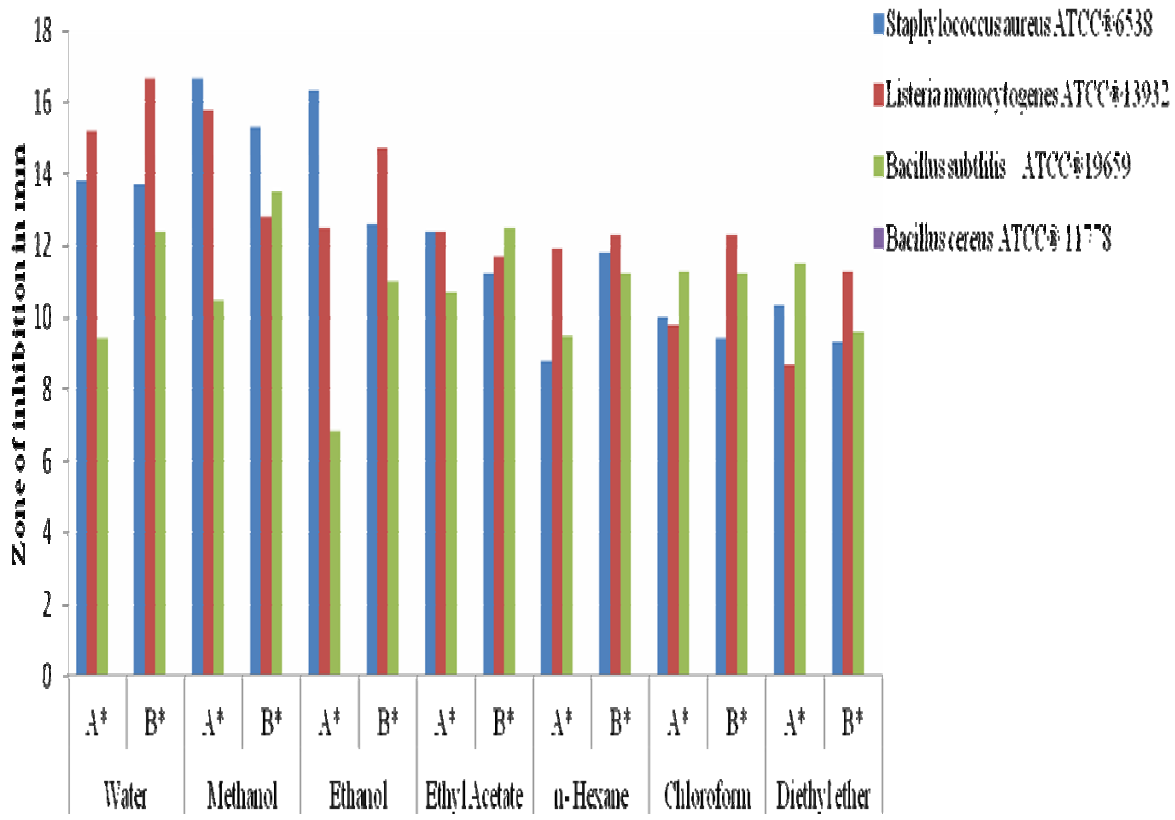

Fig. 1. Activities of olive (*Olea europaea & Olea ferruginea*) leaf crude extract against selected gram positive bacterial strains.

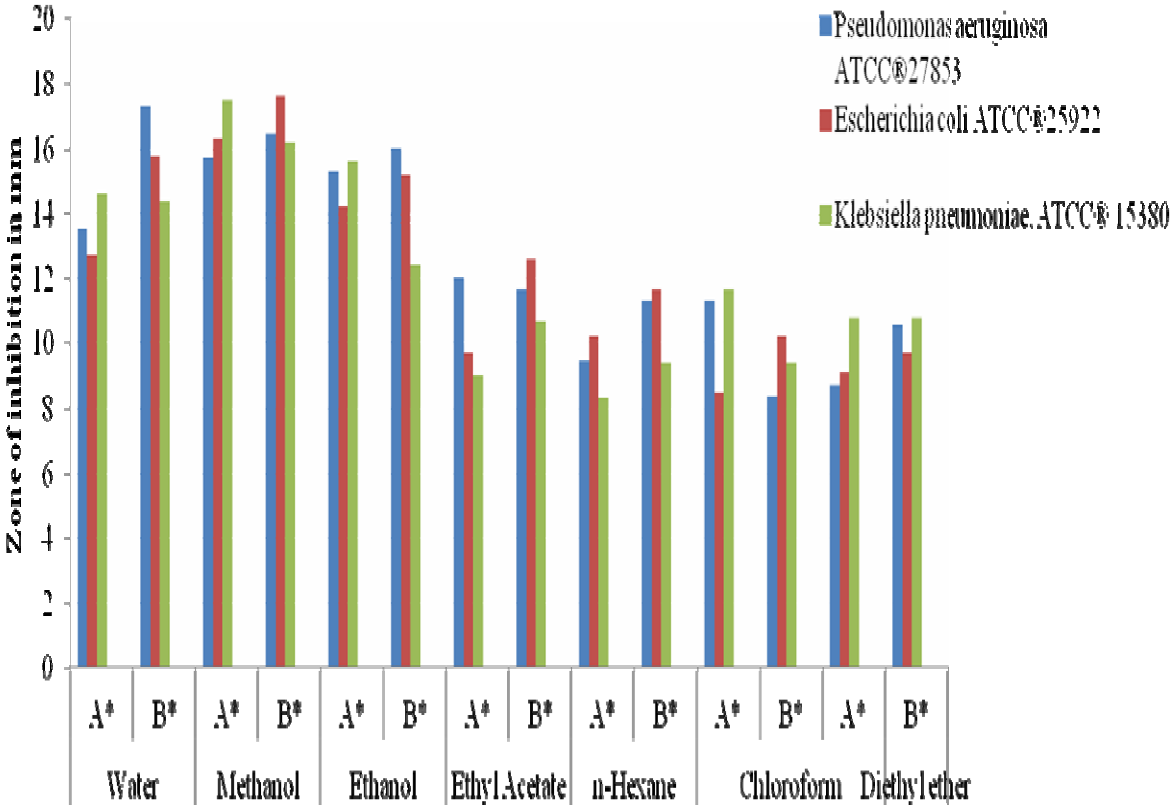

Fig. 2. Activities of olive (*Olea europaea* (A^*) & *Olea ferruginea* (B^*) leaf crude extract against selected gram negative bacterial strains.

Table 1. Antibacterial potential of olive plants (*Olea europaea* and *Olea ferruginea*) leaf crude extract (15mg/ml) against selected gram positive bacterial strains.

Bacterial strains (gram +ve)	Water		Methanol		Ethanol		Ethyl acetate		n-Hexane		Chloroform		Diethyl ether	
	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*
Staphylococcus aureus	13.8	13.7	16.7	15.3	16.3	12.6	12.4	11.2	8.8	11.8	10	9.4	10.3	9.3
Listeria monocytogenes	15.2	16.7	15.8	12.8	12.5	14.7	12.4	11.7	11.9	12.3	9.8	12.3	8.7	11.3
Bacillus subtlilis	9.4	12.4	10.5	13.5	6.8	11	10.7	12.5	9.5	11.2	11.3	11.2	11.5	9.6
Bacillus cereus	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A* = *Olea europaea*, B* = *Olea ferruginea*

Table 2. Antibacterial potential of olive plants (*Olea europaea* and *Olea ferruginea*) leaf crude extract (15mg/ml) against selected gram negative bacterial strains

Bacterial strains (gram +ve)	Water		Methanol		Ethanol		Ethyl acetate		n-Hexane		Chloroform		Diethyl ether	
	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*	A*	B*
Pseudomonas aeruginosa	13.5	17.36	15.7	16.5	15.3	16	12	11.7	9.5	11.3	11.3	8.4	8.7	10.6
Escherichia coli	12.7	15.8	16.3	17.6	14.2	15.2	9.73	12.6	10.2	11.7	8.5	10.2	9.1	9.7
Klebsiella pneumoniae	14.6	14.4	17.5	16.2	15.6	12.4	9	10.7	8.3	9.4	11.7	9.4	10.8	10.8
Salmonella typhimurium	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus faecalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A* = *Olea europaea*, B* = *Olea ferruginea*

It is reported that the oil obtained from wild olive (*Olea ferruginea*) is almost comparable in term of chemical composition to edible oil of *Olea europaea* (Gulfraz *et al.,* 2009). The environmental conditions of most of the Himalayan regions are very similar (Paudel *et al.,* 2011), and oil obtained from the olive fruits found in Pakistan and other countries of the Himalayan regions will have a very analogous properties. The oil tested in the present study was extracted from the edible olive (*Olea europaea*) and extra virgin oil (Borage-Spain) was purchased from the market. Comparing the activities of leaf extracts and different crude oils from ripe and unripe fruits of edible olive and extra virgin oil shows that there is no significant difference in their activities against the gram negative bacterial strains. This indicates that the composition of secondary metabolites both in leaves and fruits have no significant difference. However, the activities of extra virgin oil were slightly higher than the oils obtained through solvent extraction and mechanical pressing of fruits. This might be due to fine processing method of the extra virgin oil that is rich in phenolic compounds (Visioli & Bernardini, 2011). In Kenya, study on the oil extracted from the fruit of *Olea cuspidata* shows lower concentration of oleic acid (Hannachi *et al.,* 2009) which is found to be associated with environmental conditions. In the present study the slight difference in the antibacterial activities of olive oils and extra virgin oil against gram negative bacterial strain might be due to the difference in secondary metabolites concentration imposed by different environmental condition prevailing in Pakistan and Spain.

The leaf extracts and oils have no effect on the growth of *Salmonella typhimurium* ATCC®14028 and *Enterococcus faecalis* ATCC®49452 as shown in Table 4 (Fig. 4). In case of gram positive bacteria the olive oils were effective only against *Staphylococcus aureus* ATCC®6538 and to a lesser extent to *Bacillus cereus* ATCC® 11778, but have no effect on the growth of *Listeria monocytogenes* ATCC®13932 and *Bacillus subtlilis* ATCC®19659 as shown in Fig. 3 (Table 3). The oleuropein is an important constituent in the leaf and fruit extracts and present in higher concentration in the leaves than in the fruits and other parts of the plant. It was also noticed that the activity of leaves crude extracts was much higher than the olive oils on gram positive and gram negative strains that might be due to the higher concentration of oleuropein in the leaf.

Antimicrobial agents with broad spectrum and effective mode of action are priority of each pharmaceutical industry. In order to control the increase resistant to synthetic drugs, medicinal plants should be tested against pathogenic bacteria (Heinrich & Gibbons, 2001). Further studies are needed to compare the phenolic contents of the leaves crude extracts and fruit oils of both *Olea ferruginea* and *Olea europaea* and virgin oils in order to use leaf extracts in preparation of effective natural medicines.

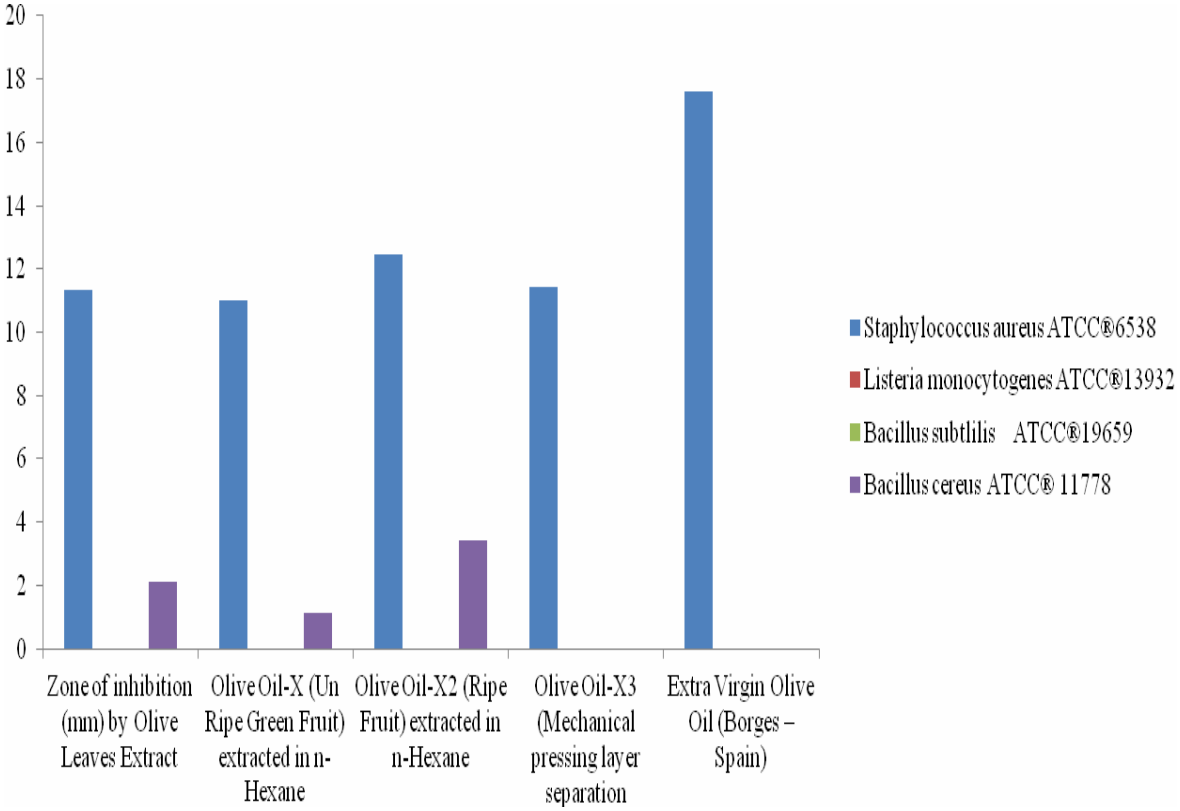

Fig. 3. Activities of olive fruit oils and extra virgin oils against gram positive bacterial strains.

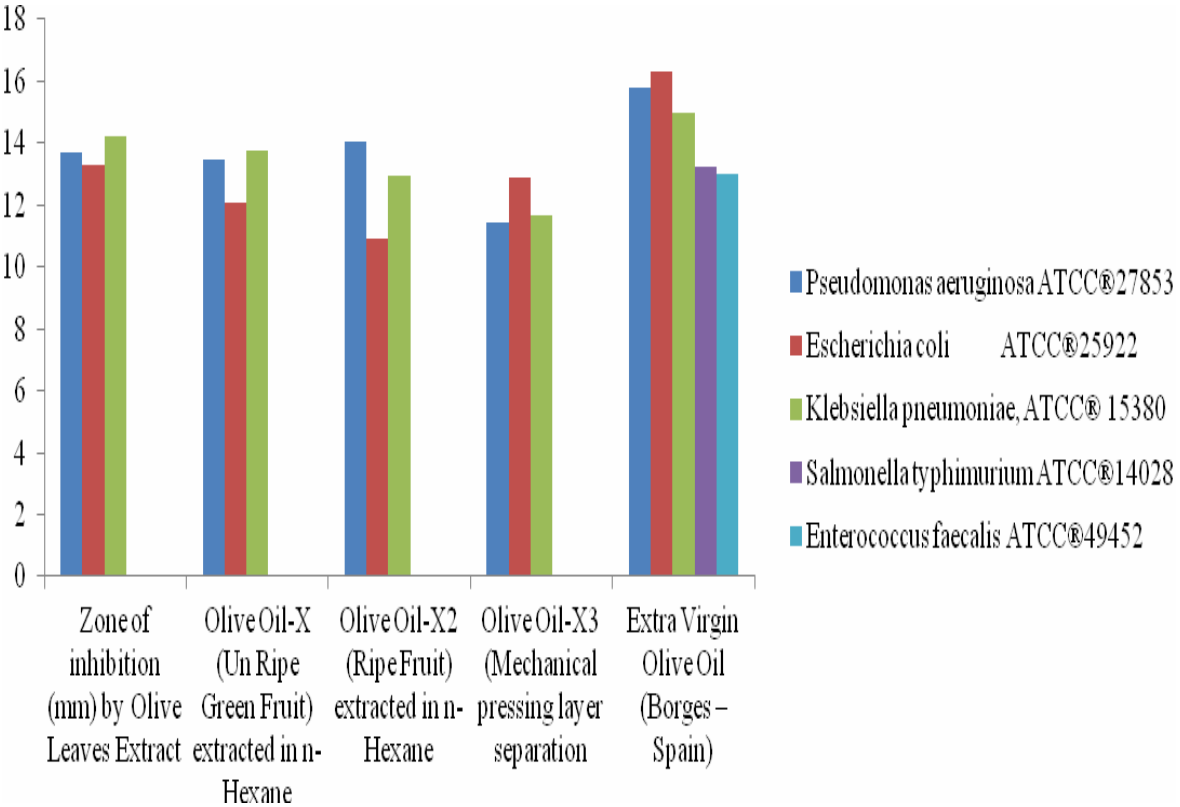

Fig. 4. Activities of olive fruit oils and extra virgin oils against gram negative bacterial strains.

Table 3. Zone of inhibition (mm) of olive oils against gram positive bacterial strains.

Gram positive bacterial strains	Olive leaves extract	Olive oil-X (un ripe green fruit) extracted in n-hexane	Olive oil-X2 (ripe fruit) extracted in n-hexane	Olive oil-X3 (ripe fruit) mechanical pressing layer separation	Extra virgin olive oil (Borges–Spain)
Staphylococcus aureus	11.32	11	12.45	11.44	17.63
Listeria monocytogenes	0	0	0	0	0
Bacillus subtlilis	0	0	0	0	0
Bacillus cereus	2.11	1.14	3.43	0	0

Table 4. Zone of inhibition (mm) of olive leaves extract and fruit oils against gram negative bacterial strains.

Gram negative bacterial strains	Olive leaves extract	Olive oil-X (unripe green fruit) extracted in n-hexane	Olive oil-X2 (ripe fruit) extracted in n-hexane	Olive oil-X3 (ripe fruit) mechanical pressing layer separation	Extra virgin olive oil (Borges–Spain)
Pseudomonas aeruginosa	13.68	13.45	14.02	11.44	15.78
Escherichia coli	13.27	12.05	10.93	12.91	16.32
Klebsiella pneumoniae	14.23	13.75	12.97	11.64	15
Salmonella typhimurium	0	0	0	0	13.25
Enterococcus faecalis	0	0	0	0	13

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(Received for publication 13 February 2013)