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Full Length Research Paper

Antibacterial screening of leaves of wild and cultivated olive of Azad Kashmir

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Wild and cultivated olive has been used in the treatment of many diseases ethnopharmacologically in Pakistan. In the present manuscript we have demonstrated the antimicrobial effects of various fractions of leaves of *Olea cuspidata* and *Olea europaea.* The leaves of *O. cuspidata* Wall and *O. europaea* L. (Family: Oleaceae) were extracted successively with four different organic solvents. These crude extracts were assessed for antibacterial activities against eight different bacterial human pathogens that is, *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Pseudomonas aeruginosa, Klebsiela pneumoniae, Proteus vulgaris, Citrobacter freundii and Streptococcus pneumoniae* by using disc diffusion method. The chloroform, ethanol, and methanol crude extracts of leaves of wild and cultivated olive had significant antimicrobial activities on all the bacterial strains tested. The standard reference antibiotic discs, Ciprofloxacin (5 µg) and Erythrocin (30 µg) were used as positive control. The capacity of the extracts and antibiotics were evaluated on the basis of their capacity to inhibit the growth of pathogenic bacteria measured as zone of inhibition. Almost all bacteria showed to be sensible against the antibiotics with the value of zone of inhibition ranging from 25 to 35 mm, while the effectiveness of olive leaves extracts varied from one species to other with zone of inhibition values of 10 to 30 mm. The ethanol and methanol crude extracts of leaves of wild and cultivated olive exhibited prominent activities against all bacteria used in comparison to chloroform extract which had moderate activity against the tested bacteria. Petroleum ether extract have no effect on any of the bacteria tested. Extract obtained with ethanol appeared to be the most effective against all pathogenic bacteria compared to those obtained with other solvents.

Key words: Antimicrobial screening, antibiotic discs, disc diffusion, wild olive.

INTRODUCTION

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of antibiotics and their uses as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. Over use of antibiotics has become the major factor for the emergence and dissemination of multidrug resistant strains of several groups of microorganisms. The worldwide emergence of multi drug resistant *s. aureus* and many other ß-lactamase producers has become a major therapeutic problem (Khan et al., 2004). Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of supreme importance. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy. For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against MDR microbe strains (Kafaru, 1994).

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to

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transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality. Montelli and Levy (1980-1990) documented a high incidence of resistant microorganisms in clinical microbiology in Brazil. This fact has also been verified in other clinics around all over world.

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies.

The use of plant compounds for pharmaceutical purposes has gradually increased in Brazil. According to World Health Organization (Santos et al., 1995) medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developing countries use traditional medicine to cure different ailments. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Ellof, 1998). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Almagboul et al., 1985). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Saxena et al., 1994).

The antimicrobial properties of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatments (Anesini and Perez, 1993). It was documented that among the compounds extracted from these plants, twelve inhibited the growth of *S. aureus*, ten inhibited *E. coli*, and four inhibited *A. niger* and also reported that the most potent compound was one extracted from *Tabebuia impetiginosa*. The antimicrobial properties of compounds obtained from *Parthenum argentatum* against *C. albicans, Torulopsis, Hansemula, K. pneumoniae* and *P. aeruginosa* was detected (Martinez et al., 1994, 1996). Results showed that the substances extracted from nine known plants in Uruguai have activity against *C*. *albicans* and *S. cerevisiae*, but inhibited the growth of *B. subtilis*, *E*. *coli* and *P. aeruginosa* (Alonso-Paz et al., 1995).

Effects of phytochemical were conducted (Izzo et al., 1995; Jansen et al., 1987) and was observed the antimicrobial activity of anacardic acid on *S. aureus, B. ammoniagenes, Streptococcus mutans* and *Propionibacterium acnes*. Later, it tested the bactericidal activity of anacardic acid and totarol on methicillin resistant strains of *S. aureus* (MRSA) and the synergistic effect of these compounds associated with methicillin (Muroi and Kubo, 1996).

Hence, more studies pertaining to the use of plants as therapeutic agents should be emphasized, especially those related to the control of antibiotic resistant microbes.

The objective of this research was to evaluate the potential of leaves of wild and cultivated olive extracts that grow in Azad Kashmir on gram positive and gram negative bacterial strains as well as multi-drug resistant bacteria, which were isolated from hospitals.

MATERIALS AND METHODS

The plant material was collected from different parts of Azad Jammu and Kashmir. The leaves of wild and cultivated olive were dried carefully under shade and then homogenized to fine powder and stored in airtight bottles for further studies.

Organic solvent extraction

The 25 g dried powdered leaves of wild and cultivated olive was soaked separately in 250 ml petroleum ether, chloroform, ethanol and methanol. The extraction was carried out by maceration for 7 days in each solvent at room temperature (25 ± 2°C). The solvents extracted material was filtered in separate flaks (Rawlins, 1977). All extracts were then dried in a vacuum rotary evaporator, weighed and stored at 4°C until further analysis.

Preparation of dilution

The dried petroleum ether, chloroform, ethanol and methanol extracts were then dissolved in their respective solvents in a proportion of 10 mg/ml. The concentration of reference antibiotic discs that is, ciprofloxacin was 5 µg and Erythrocin 30 µg.

Microorganisms used

In the present study bacterial strains that is, *S. aureus, B. subtilis, S. typhi, P. aeruginosa, K. pneumoniae, P. vulgaris, C. freundii and S. pneumoniae* were used to evaluate the antibacterial potential of different extracts of the leaves of wild and cultivated olive.

Antibacterial assay

A 24 h old culture of each bacterium was used as an inoculum for the test. The slants were prepared in test tube. The nutrient agar medium was used for bacterial growth. *In vitro* antibacterial screening was performed by disc diffusion method as described by Scorzoni et al. (2007). The sterilized nutrient agar medium when temperature reached between 40 and 45°C was poured in the petri dishes containing bacterial suspension. Two series of experiments were conducted. In first crude extracts were tested for their antimicrobial activity against already mentioned bacteria. The filter paper discs used to determine the antimicrobial potential were absorbing 10 µl extract. In the second series of experiment, the commercially available standard reference antibiotic discs that is, ciprofloxacin 5 µg and erythrocin 30 µg were placed on the top of the medium in the centre of petri dishes by following the disc diffusion method (Scorzoni et al., 2007). The purpose of this experimental set was to compare the antimicrobial activity of the standard reference antibiotics with that of the solvent extracts of leaves of wild and cultivated olive. The plates containing bacterial culture were incubated at 37°C for 24 h. After the incubation time, all the plates were examined for the presence of inhibition as a property of antimicrobial activity.

Statistical analysis

All values were expressed as means ± SEM. The data for each microorganism were analysed by using one way analysis of variance (ANOVA) technique and means were compared by using least significance difference (LSD) at 5% (0.05) probability level (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The antimicrobial activities of the leaves of wild and cultivated olive and antibiotics were clearly observed against the tested bacterial species. In this study, the antimicrobial influence of petroleum ether, chloroform, ethanol and methanol crude extracts of leaves of wild and cultivated olive were determined by using disc diffusion method. These plants are known to have healing properties and are used for the treatment of various diseases in people all over the world. The results of the antimicrobial screening of different solvents crude extracts of leaves of wild and cultivated olive against 8 bacterial strains were presented in Tables 1 and 3. The statistical analyses of results have been tabulated in Tables 2 and 4. Almost all antibiotics were found to be effective against the tested bacterial species in different level. On the other hand, antimicrobial activities of leaves of wild and cultivated olive extracts obtained by using different solvents were appeared to be very different in terms of effectiveness since some bacterial species are more resistant and some other more susceptible to the extracts. In this study petroleum ether extract of the leaves of cultivated olive showed no activity against all the microorganisms tested but petroleum ether extract of wild olive showed low activity against *S. typhi* and *P. aeruginosa* (Tables 1 and 3). The chloroform extract of leaves of *O. europaea* showed activity against *P. vulgaris* (11.50 ± 0.50 mm), *C. freundii* (13.50 ± 0.50 mm) but all other bacterial strains used were not affected by the extract (Table 1). The chloroform extract of leaves of *O. cuspidata* also exhibited moderate activity against all the microorganisms used except *B. subtilis, K. pneumoniae, S. pneumoniae* and *S. aureus.* The mean diameter of zones of inhibition of the extract against these bacterial strains were 08.16 ± 0.28 mm, 10.50 ± 0.50 mm, 10.50 ± 0.50 mm and 12.33 ± 0.57 mm respectively (Table 3).

In most of the previous studies the alcoholic extracts were found to be more effective against gram positive than gram negative bacteria. In present study it was observed that the ethanol extracts of leaves of cultivated olive showed high zones of inhibition against *B. subtilis, S. aureus S. typhi*, *P. aeruginosa, K. pneumoniae*, *P. vulgaris*, *C. freundii* and *S. pneumoniae* that is, 26.33 ± 0.57 mm, 21.50 ± 0.86 mm, 26.33 ± 0.57 mm, 22.50 ± 0.50 mm, 16.50 ± 0.50 mm, 25.50 ± 0.50 mm, 24.16 ± 0.28 mm and 23.83 ± 0.76 mm, respectively (Table 1) and leaves of *O. cuspidata* exhibited appreciable activity against all bacteria tested with zones of inhibitions, 22.50 ± 0.50 mm, 21.33 ± 0.57 mm, 24.16 ± 0.28 mm, 15.33 ± 0.57 mm, 15.50 ± 0.50 mm, 24.16 ± 0.28 mm, 24.50 ± 0.50 mm and 24.50 ± 0.50 mm, respectively (Table 3).

The methanol extracts of leaves of *O. europaea* and *O. cuspidata* also exhibited substantial activity against all the bacteria used. The largest zones of inhibition produced by the *O. europaea* extract against *B. subtilis, S. aureus S. typhi*, *P. aeruginosa, K. pneumoniae*, *P. vulgaris*, *C. freundii* and *S. pneumoniae* were 20.33 ± 0.57 mm, 26.33 ± 0.57 mm, 26.33 ± 0.57 mm, 24.33 ± 0.57 mm, 15.16 ± 0.28 mm, 21.50 ± 0.50 mm, 20.16 ± 0.28 mm and 23.33 ± 0.57 mm, respectively (Table 1), while the largest zones of inhibition produced by methanol extract of leaves of *O. cuspidata* were 20.33 ± 0.57 mm, 19.16 ± 0.28 mm, 19.33 ± 0.57 mm, 10.33 ± 0.57 mm, 16.33 ± 0.57 mm, 21.33 ± 0.57 mm, 20.33 ± 0.57 mm and 20.16 ± 0.28 mm respectively (Table 3). The antibiotics ciprofloxacin and erythrocin which were used as positive control showed high activity against all the microorganisms used. The mean diameters of zones of inhibition against all bacteria tested were mentioned in Tables 1 and 3.

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another big concern is the

Table 1. Antimicrobial activity of *O. europaea* leaves. Concentration of crud extracts 10 mg/ml, erythrocin 30 µg and ciprofloxacin 5 µg

		Mean diameter of zones of inhibition ± Standard error mean (SEM)							
S/N	**Solvent used**	***B. subtilis***	***S. aureus***	***S. typhi***	***P. aeruginosa***	***K. pneumoniae***	***P. vulgaris***	***C. freundii***	***S. pneumoniae***
1	P.ether	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	Chloroform	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	11.50 ± 0.50	13.50 ± 0.50	0.00 ± 0.00
3	Ethanol	26.33 ± 0.57	21.50 ± 0.86	26.33 ± 0.57	22.50 ± 0.50	16.50 ± 0.50	25.50 ± 0.50	24.16 ± 0.28	23.83 ± 0.76
4	Methanol	20.33 ± 0.57	26.33 ± 0.57	26.33 ± 0.57	24.33 ± 0.57	15.16 ± 0.28	21.50 ± 0.50	20.50 ± 0.50	23.33 ± 0.57
5	Erythrocin	30.33 ± 0.57	28.17 ± 0.28	30.16 ± 0.28	25.16 ± 0.28	28.33 ± 0.57	32.16 ± 0.28	35.33 ± 0.57	30.50 ± 0.50
6	Ciprofloxacin	24.33 ± 0.57	24.33 ± 0.57	25.33 ± 0.57	23.16 ± 0.28	24.16 ± 0.28	22.33 ± 0.57	25.33 ± 0.57	24.33 ± 0.57

Table 2. Antimicrobial activity of *O. europaea* leaves. Least significance difference (LSD).

		Concentration of crud extracts 10 mg/ml, erythrocin 30 µg and Ciprofloxacin 5 µg							
S/N	**Solvent used**	***B. subtilis***	***S. aureus***	***S. typhi***	***P. aeruginosa***	***K. pneumoniae***	***P. vulgaris***	***C. freundii***	***S. pneumoniae***
1	P.ether	0.00^{e}	0.00^{e}	0.00^{d}	0.00^{e}	0.00^{e}	0.00^{f}	0.00^{f}	0.00^{d}
2	Chloroform	0.00^{e}	0.00^{e}	0.00^{d}	0.00^{e}	0.00^{e}	11.50^{e}	13.50^{e}	0.00^{d}
3	Ethanol	26.33^{b}	21.50^{d}	26.33^{b}	22.50^{d}	16.50^{c}	25.50^{b}	24.16^{c}	23.83^{bc}
4	Methanol	20.33^{d}	26.33^{b}	26.33^{b}	24.33^{b}	15.16^{d}	21.50^{d}	20.50^{d}	23.33^{c}
5	Erythrocin	30.33^{a}	28.17^{a}	30.16^{a}	25.16^{a}	28.33^{a}	32.16^{a}	35.33^{a}	30.50^{a}
6	Ciprofloxacin	24.33^{c}	24.33^{c}	25.33^{c}	23.16^{c}	24.16^{b}	22.33^{c}	25.33^{d}	24.33^{b}

Table 3. Antimicrobial activity of *O. cuspidata* leaves. Concentration of crud extracts 10 mg/ml, Erythrocin 30 µg and Ciprofloxacin 5 µg.

		Mean diameter of zones of inhibition ± Standard error mean (SEM)							
S/N	**Solvent used**	***B. subtilis***	***S. aureus***	***S. typhi***	***P. aeruginosa***	***K. pneumoniae***	***P. vulgaris***	***C. freundii***	***S. pneumoniae***
1	P.ether	0.00 ± 0.00	0.00 ± 0.00	13.33 ± 0.57	9.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	Chloroform	0.00 ± 0.00	0.00 ± 0.00	8.16 ± 0.28	10.50 ± 0.50	0.00 ± 0.00	10.50 ± 0.50	12.33 ± 0.57	0.00 ± 0.00
3	Ethanol	22.50 ± 0.50	21.33 ± 0.57	24.16 ± 0.28	15.33 ± 0.57	15.50 ± 0.50	24.16 ± 0.28	24.50 ± 0.50	24.50 ± 0.50
4	Methanol	20.33 ± 0.57	19.16 ± 0.28	19.33 ± 0.57	10.33 ± 0.57	16.33 ± 0.57	21.33 ± 0.57	20.33 ± 0.57	20.16 ± 0.28
5	Erythrocin	30.33 ± 0.57	28.17 ± 0.28	30.16 ± 28	25.16 ± 0.28	28.33 ± 0.57	32.16 ± 0.28	35.33 ± 0.57	30.50 ± 0.50
6	Ciprofloxacin	24.33 ± 0.57	24.33 ± 0.57	25.33 ± 0.57	23.16 ± 0.28	24.16 ± 0.28	22.33 ± 0.57	25.33 ± 0.57	24.33 ± 0.57

development of resistance to the antibiotics in current clinical use. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants. Higher plants produce hundreds

Table 4. Antimicrobial activity of *O. cuspidata* leaves. Least significance difference (LSD).

Concentration of crud extracts 10 mg/ml, Erythrocin 30 µg and Ciprofloxacin 5 µg									
S/N	**Solvent used**	***B. subtilis***	***S. aureus***	***S. typhi***	***P. aeruginosa***	***K. pneumoniae***	***P. vulgaris***	***C. freundii***	***S. pneumoniae***
1	P.ether	0.00^{e}	0.00^{e}	13.33^{e}	9.33^{e}	0.00^{e}	0.00^{f}	0.00^{e}	0.00^{d}
2	Chloroform	0.0^{0e}	0.00^{e}	8.16^{f}	10.50^{d}	0.00^{e}	10.50^{e}	12.33^{d}	0.00^{d}
3	Ethanol	22.50^{c}	21.33^{c}	24.16^{c}	15.33^{c}	15.50^{d}	24.16^{b}	24.50^{b}	24.50^{b}
4	Methanol	20.33^{d}	19.16^{d}	19.33^{d}	10.33^{d}	16.33^{c}	21.33^{d}	20.33^{c}	20.16^{c}
5	Erythrocin	30.33^{a}	28.17^{a}	30.16^{a}	25.16^{a}	28.33^{a}	32.16^{a}	35.33^{a}	30.50^{a}
6	Ciprofloxacin	24.33^{b}	24.33^{b}	25.33^{b}	23.16^{b}	24.16^{b}	22.33^{c}	25.33^{b}	24.33^{b}

to thousands of diverse chemical compounds with different biological activities. The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens.

In our study, we found wild olive and cultivated olive leaves having an antibacterial activity against the tested bacteria although their effectiveness is lower compared to that of standard antibiotics.

Most of the wild floras of Azad Kashmir, Pakistan are rich in medicinal and aromatic properties like antibacterial, antiviral, antihelminitic, anticancer, sedative, laxative, cardiotonic, diuretic and others. They are important sources of bio-molecules, with application for the manufacture of pharmaceuticals and cosmetics (Heinrich and Gibbons, 2001). Further studies are necessary to carry out a detail antimicrobial screening also of other pathogens to establish a database which allow managing and conserving these plant species many of which are recognized also in the traditional medicine.

In present studies, extracts obtained with ethanol and methanol showed a good effectiveness against the tested bacteria compare to those obtained with other solvents. This is probably because, ethanol and methanol, compare to other solvents, allows extracting all the phenolic compounds from the olive leaves. Nevertheless, a thorough investigation is needed to compare phenolic compounds of *O. cuspidata* and *O. europaea* fruit and leaves from Azad Kashmir, Pakistan with those from other countries since environmental condition found to be widely influential on the percentage of acid and phenolic compounds. Study carried out in Kenya on *O. cuspidata* showed that oil extracted from olive fruits has lower content of oleic acid which is found to be associated to the high average temperature of the summer and lack of the winter cold temperature of the country (Hannachi et al., 2009). In addition, further study is to be hoped also to perform a thorough analysis among the phenolic compounds of *O. cuspidata* and those of *O. europaea*.

Conclusion

Based on the present results, it is concluded that organic solvent extracts of leaves of *O. cuspidata* wall and *O. europaea* L. had different levels of antibacterial activity and that they can be used in the treatment of infectious diseases caused by common human bacterial pathogens. The obtained results might be well-thought-out sufficient to further studies for the isolation and identification of the active principles and to evaluate of possible extract components for their antibacterial activity. This study showed that besides fruits of *O. europaea* already tested; also the leaves of *O. cuspidata* and *O. europaea* have antibacterial utilities which can be attributed to the phenolic compound.

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