Inhibitory effects of *Olea ferruginea* crude leaves extract against some bacterial and fungal pathogen

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Abstract: This work aimed to evaluate the inhibitory effects of *Olea ferruginea* crude leaves extract that are commonly used as remedy to cure infections in the tribal (Khyber Agency) areas of Pakistan against some of bacterial and fungal pathogens. The crude n-hexane fraction was appreciably active against both gram positive and negative microorganisms (MIC ranged from 7.5 to 15 mg/ml) followed by butanol fraction (MIC 15 to 30 mg/ml). Conversely least biological activity was shown by chloroform (30mg/ml) and methanol (15 to 30mg/ml) crude fractions. The MBC observed for all crude fractions was same or 2 times higher when compared with MIC for all crude extract fractions. Likewise all the fractions showed activity against *Aspergillus niger* and maximum zones of inhibition were shown by the n-hexane fraction (14±(0.02), butanol (13±(0.02) followed by methanol (9±(0.05) and chloroform fractions (7±(0.02). These results clearly imitate the antibacterial and antifungal potential of *Olea ferruginea* and hence we recommend the whole plant for further futuristic studies.

Keywords: *Olea ferruginea*, susceptibility, traditional medicine, minimum inhibitory concentration.

INTRODUCTION

Medicinal plants not only possess a history of regular use as medicine throughout the subcontinent (Baquar, 1995) but the use of herbal medicine for therapeutic purpose is attaining a worldwide popularity and acceptance especially in developed nations during the last two decades (Lanfranco, 1999). The world health organization's statistics has revealed that more than 85% of people around globe keep believes on traditional herbal medicine for treatment of various health concerns (Augustans and Hoch, 2004).

The *Olea ferruginea* Royle (Oleaceae) is commonly used as traditional medicine (leaves and bark) for treatments of skin ailments, as astringent, during treatment of mouth ulcers in tribal areas of Pakistan (Rehman, 1986) and is an integral component of local cultural, religious beliefs and attitudes (Ibrar *et al.*, 2007, Abebe, 1996). Phytochemical investigations reveal presence of stearnin, cholostrin, olein, limolin, palmatinin leaves while the fruit contain fixed oils and small quantities of linoleic, palmitic acid, stearic and myrstic acid (Siddiqui *et al*., 2011).

The *Olea ferruginea* Royale, a native broad leaved medicinal plant is distributed from 500 to 2000 m in sub tropical, dry temperate and moist temperate regions of Pakistan and India. In Pakistan, this has been reported near salt range, Waziristan agency, Khyber agency, Azad Kashmir, Swat Dir, Chitral, Murree hills and Western hills of Baluchistan (Baquar, 1995; Sheikh, 1993). This frost and drought resistant species has adapted 250 mm to 1000 mm per year precipitation and –10°C to 40°C temperature. Despite the environmental, economical, ecological and medicinal importance of *Olea ferruginea* no detailed literature is available (Siddiqui *et al.*, 2009) covering its antimicrobial and antifungal potential. The scope of this research includes to disclose the antibacterial and antifungal properties of crude leaves extracts of this plant.

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MATERIAL AND METHODS

Plant material

The plant material (Leaves) utilized in present research work was collected from Khyber Agency based on information of local inhabitants regarding ethno medical and traditional uses of plant against infections. Plant samples were authenticated in Department of Plant Sciences, Quaid I Azam University, Islamabad, Pakistan by Dr. Mushtaq Ahmad based on morphological and anatomical features.

Chemicals and reagents

The purified chemical used during research work were commercially purchased from Oxoid, Aldrich & Sigma (Pvt.) Ltd. and Merck (Germany)

Microorganisms

Six bacterial species Viz *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter*, *Staphylococcus areus*, *Micrococcus luteus* for antibacterial and *Aspergillus niger* for antifungal assay

were employed. All strains were provided by microbiology research lab (MRL) Microbiology department, Quaid-i-Azam University Islamabad Pakistan where these were identified and characterized. These strains were maintained on agar slants at 4°C in Gomal Center of Biochemistry and Biotechnology (GCBB) for antimicrobial and antifungal assays. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.4. The reference antibiotics used were ofloxacin (10µg) and itraconzole (Ferozsons (Pvt.) Ltd., Pakistan).

Preparation of crude extracts

The plant material (leaves) were collected during and dried under shade (15-20 days) to avoid the loss of active components. The properly dried leaves were powdered by milling (Wiley Mill, 300 mm mesh). Nearly 200 grams of completely dried and powdered leaves were extracted with 300 ml ethanol (95%) using rotary shaker (195-200 rpm) overnight, followed by filtration, and concentrating it to one-fifth of the total volume. Later the dried crude extract was prepared by subjecting plant material (10g) to slow heat (oven) for 6-8 hours (Vanden Berge and Vlietinck, 1991; Stavri and Gibbons, 2005).

Preparation of fractions

About 100g of dried plant material was extracted with n-hexane, chloroform, methanol and butanol by soxhlet extraction apparatus. Under low pressure, solvent evaporation was performed that yielded a semisolid mass which was stored in silica gel beads (Stavri and Gibbons, 2005).

Antibacterial Screening

Agar well diffusion assay

The antibacterial susceptibility pattern was determined by employing agar well diffusion assay (Ahmad and Beg, 2001). The bacterial cultures were activated through inoculation in nutrient broth followed by incubation at 37°C for 24 hours. The turbidity of inoculum was matched after 24 hours with McFarland's turbidity standard (0.5×10^8 cfu/ml). Later the inocula of the bacterial culture were applied separately on to the surface of sterilized Muller Hinton agar (Oxoid) plates with a sterilized cotton swab to ensure uniform thickness of bacterial lawn after incubation. Afterwards a sterilized cork borer (6mm diameter) was employed to form well on agar plates. About 100 µl of each plant extract fraction was applied in respective well for diffusion for 1–3 hours in a septic environment. The inoculated plates were then incubated for 24-48 hours at 37°C and observed for zone of inhibitions (mm). The Ofloxacine (10µg) was used as standard.

Table 1: Zone of Inhibitions (mm) of *Olea ferruginae* crude leaf fractions

Plant specie	Microorganism/Zone of inhibition						
	Fraction	Ec	Ps	Kp	Ent	Sa	Ml
Olea ferruginae	Methanol	2±(0.05)	0	4±(0.02)	10±(0.02)	6±(0.02)	6±(0.02)
	Butannol	2±(0.02)	3±(0.02)	5±(0.05)	11±(0.05)	5±(0.02)	5±(0.05)
	n-Hexane	2±(0.05)	8±(0.01)	4±(0.02)	14±(0.05)	5±(0.02)	6±(0.02)
	Chloroform	2±(0.02)	3±(0.05)	4±(0.05)	8±(0.02)	4±(0.02)	3±(0.05)
	Ofloxacine	15±(0.02)	16±(0.05)	13±(0.05)	14±(0.02)	13±(0.05)	13±(0.05)

Ec: *E. coli*, Kp: *Klebsiella pneumoniae*, Ent: *Enterobacter*, Ps: *Pseudomonas aeroginosa*, Ml: *Micrococos Luteus*, Sta: *Staphylococcus areus* (methicilline resistant)

Table 2: Minimum Inhibitory concentrations (MIC) of *Olea ferruginae* crude leaf fractions.

Plant specie	Microorganism/MIC* (mg/ml)						
	Fraction	Ec	Ps	Kp	Ent	Sa	Ml
Olea ferruginae	Methanol	15	0	15	30	15	15
	Butannol	15	30	30	15	15	15
	n-Hexane	15	15	15	7.5	15	15
	Chloroform	30	30	30	30	30	30
	Ofloxacine	1.87	0.93	1.87	0.93	0.93	1.87

Ec: *E. coli*, Kp: *Klebsiella pneumoniae*, Ent: *Enterobacter*, Ps *Pseudomonas aeroginosa*, Ml: *Micrococos Luteus*, Sta: *Staphylococcus areus* (methicilline resistant)
*MIC minimum inhibitory concentration

Determination of minimum inhibitory concentration (MIC)

The MIC was considered as the lowest concentration of the crude extract that inhibited the visible growth (90%) of bacteria (Mukherjee, 2002) The MIC of *Olea ferruginea* crude leaves extract was determined by agar dilution method (EUCAST Definitive Document, 2000). Briefly sterilized Muller Hinton agar (oxoid) was cooled to 50°C followed by pouring of 19 ml of this into sterilized test tubes containing 1ml of various concentration of crude leave extract. This mixture was thoroughly mixed and poured into sterilized petri plates. The concentrations of the leaves extracts used during assay ranged from 30 mg to 0.007 mg/ml. The turbidity of microbial suspension (test strains) was adjusted to 0.5 McFarland standard and then inoculated (0.05µl) onto the series of agar plates containing various concentrations of crude leaves extract. The loaded agar plates were incubated for 24-48 hours at 37°C.

Screening for antifungal activity

A disc of fungal strain (*Aspergillus niger*) was suspended in 2ml of inoculation broth (sabaraued dextrose). The fungal suspension was equally spread on agar petriplates by using sterilized cotton swabs. Wells were prepared using sterilized cork borer (6 mm).The crude plant extracts (100ul) were applied into the wells using micropipette and incubated for 3-5 days at 25°C and later examined for zones of inhibition around each well. Itraconazole (30mg/ml) was used as a positive control.

RESULTS

Almost all fractions of *Olea ferruginea* presented encouraging antibacterial activity against both gram positive and negative bacterial strains. The widest zone of inhibition [14±(0.05)] was presented by n-hexane fraction against *Enterobacter*. This fraction proposed significant biological activity against all gram positive and negative bacterial strains. Never the less, both butanol and methanol fractions were noticed as equally active against all pathogens but it was not considered as significant as a result of reduced zone of inhibitions. Only the chloroform fraction was reported as least active (table 1). The similar pattern of bacterial inhibition was proposed by MIC results. The MIC of the fractions ranged from 7.5-30 mg/ml for all fractions (table 2).

The antifungal activity of all crude fractions was investigated against the *Aspergillus niger* by agar diffusion assay. Both the n-hexane and butanol fractions presented maximum zones [(14±(0.02) and 13±(0.02)mm respectively] whereas o f inhibitions comparably lesser zone of inhibitions were presented by methanol and chloroform fractions (table 3).

Table 3: Antifungal activities of *Olea ferrugoniae* by agar well diffusion method

Plant specie	Fraction	Antifungal activities (Zones of Inhibition mm*)
Olea ferruginae	Methanol	9±(0.05)
	Butannol	13±(0.02)
	n-Hexane	14±(0.02)
	Chloroform	7±(0.02)
	Ofloxacine	20±(0.02)

*mm millimeter

DISCUSSION

Despite the availability of a condensed information covering antimicrobial and antifungal activities of *Olea ferruginea* after detailed literature review (Siddiqui *et al*., 2009, Wahab *et al*., 2008), numerous ethanobotanical surveys include the traditional, common use of *Olea ferrugoniae* decoctions as effective remedy for fungal and bacterial infections in various parts especially tribal areas (Khyber, Malakand, North/South Waziristan agencies, Dir, Chitral districts) of Pakistan (Hussain *et al*., 1998; Ahmad *et al*., 2008; Khan *et al*., 2010).

This study aimed to investigate the traditional antibacterial and antifungal claims linked with *Olea ferrugoniae* (leaves). Almost all crude leaf fractions presented encouraging results and were reported as active against all bacterial pathogens. The susceptibility pattern observed for various crude leaf fractions was not highly encouraging against *Micrococcus luteus*, and *Staphylococcus aureus* and were reported as least susceptible. This tends to imply least effectiveness of leaves extract towards gram positive microorganisms. In this context least activity of the crude leaves fraction towards the gram positive strains is an indicator of innate reduced activity, as the gram positive strains are generally employed for detection of sensitivity of newly discovered crude extracts or antimicrobial peptides (AMPc) (Awais *et al*., 2010; Bushra *et al*., 2008, Muhammad *et al*., 2009).

Likewise no significantly variable trend was noticed by viewing the susceptibility pattern of gram negative strains against all crude fractions. Almost all crude fractions presented least effectiveness against *E-coli, Pseudomonas aeroginosa* and *Klebsiella pneumoniae*. In general the gram negative strains are supported by modified resistance mechanisms, additional envelops and high potency of peptidoglycan (Khan *et al*., 2010; Siddiqui *et al*., 2009; Lal Badshah *et al*., 2009; Augustans and Hoch 2004) which makes them resistant or non responsive to anti-infective agents.

Surprisingly, irrespective of antibacterial potential the results of present study are a clear indication of potential antifungal activity of *Olea ferrugoniae* crude extract leaves. *Nearly all* crude fractions exhibited significant activity against *Aspergillus niger.* These encouraging results are in support of classical claims supposed with to *Olea ferrugoniae* (Siddiqui *et al*., 2009; Wahab *et al*., 2008; Hussain *et al.,* 1998; Ahmad *et al*., 2008).

CONCLUSION

Based on present findings this research work therefore ascertains the medicinal value of *Olea ferrugoniae leaves* towards human bacterial and fungal pathogens and supports this medicinal plant for futuristic studies.

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