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Antimicrobial potentiality of *Polyalthia longifolia* seed oil against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin

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The antimicrobial potentiality of *Polyalthia longifolia* seed oil and its fatty acid methyl ester (FAME) extract was evaluated against multi drug resistant strains of bacteria and fungi of clinical origin. A sensitivity test was performed to determine the resistance pattern for marketed antimicrobial agents against the selected microorganisms. The results of antimicrobial assay revealed that *P. longifolia* seed oil and its FAME extract were effective against all the microorganisms studied. *Enterococcus faecalis, Streptococcus mutans* and *Streptococcus bovis* were observed to be the most susceptible strains. Among the tested Gram-negative bacteria, *Klebsiella pneumoniae* was found to be the most sensitive, while *Salmonella typhimurium* was the most resistant bacteria. In the case of Gram-positive bacteria, *E. faecalis* was the most sensitive, while *S. aureus* was the most resistant strain. The *Candida albicans* was found to be highly sensitive to the action of FAME extract with the least minimum inhibitory concentration (MIC) of 19.5 µg/ml. The MIC values were lower than MBC/MFC values suggesting that the *P. longifolia* seed oil and its FAME extract inhibit the growth of test organisms while being bactericidal/fungicidal at higher concentrations. In addition, the physiochemical properties and fatty acid composition of *P. longifolia* seed oil were also investigated. Thus, it was found that *P. longifolia* seed oil offers potential antibacterial property against the reference strains and could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms from community as well as hospital settings.

Keywords: *Polyalthia longifolia*, agar diffusion assay, strain sensitivity profile, antimicrobial potentiality.

INTRODUCTION

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents, there has been a belief in the medical group that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant (MDR) strains of several groups of microorganisms (Harbottle et al., 2006). The worldwide emergence of *Escherichia coli, Klebsiella pneumoniae, Haemophilus* and many other β-lactamase producers has become a major therapeutic problem. MDR strains of *E. coli* and *K. pneumoniae* are widely distributed in hospitals and are increasingly being isolated from community acquired infections (Khan and Musharraf, 2004; Akram et al., 2007). Thus, in light of the evidence

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of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. However, the past records of rapid, widespread emergence of resistance to synthetic antimicrobial agents indicate that even new families will have a short life expectancy (Coates et al., 2002). For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against MDR microbial strains (Braga et al., 2005). For thousands of years, natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs. The antimicrobial efficacy attributed to some plants in treating diseases has shown promising results. A number of phytotherapy manuals have mentioned various medicinal plants for treating infectious diseases due to their availability, fewer side effects and reduced toxicity (Lee et al., 2007). *Polyalthia longifolia* (Sonn.) Thwaites (Order: Magnoliales; Family: Annonaceae) is an evergreen plant commonly used as an ornamental street tree due to its effectiveness in combating noise pollution (Kar et al., 2013). The bark is used in skin diseases, fever, hypertension, helmenthiasis and is a febrifuge (Rastogi, 1997). Its aqueous extract also lowers the blood pressure and rate of respiration in experimental animals (Saleem et al., 2005). The hypoglycemic and antihyperglycemic activities of various solvent extracts of *P. longifolia* var. pendula leaf extracts were evaluated in alloxan induced experimental diabetic rats (Nair et al., 2007). The antimicrobial activity from leaves (Annapuma et al., 1983), stem (Faizi et al., 2003) and seed diterpenoids (Murthy et al., 2005) have been reported. However, the antimicrobial potential of *P. longifolia* seed oil still remains unexplored.

Therefore keeping in view the importance of biochemical and pharmacological activities associated with *P. longifolia,* the present study was conducted to access the physiochemical properties, fatty acid composition and antimicrobial potentiality of seed oil obtained from *P. longifolia*. A detailed sensitivity pattern of reference microbial strains was also made against commercially available antibiotics to determine the antibiotic resistance pattern.

MATERIALS AND METHODS

Collection and preparation of seed sample

The *P. longifolia* seeds were obtained from the A.M.U. campus and were identified by a taxonomist in the Department of Botany, A.M.U, Aligarh. The seeds were screened manually to remove bad ones. They were then dried to constant weight in an oven at 70°C, ground using mechanical grinder, put in air-tight containers and stored in desiccators for further analysis.

Extraction of seed oil

The oil from *P. longifolia* seeds was extracted by continuous extraction in Soxhlet apparatus for 12 h using petroleum ether (60 to 80°C boiling range) as a solvent according to the method described by AOCS (1980). At the end of the extraction, the solvent was evaporated. The oil thus extracted was stored in a lightproof, air-tight and moisture proof container at -4°C for further analysis.

Preparation of mixed fatty acids (MFA)

Saponification of seed oil was carried out by refluxing the oily mass with 0.5 N alcoholic KOH for about 3 h. The unsaponified material was removed by diethyl ether extraction. The mixed fatty acids were obtained by subsequently acidifying the aqueous layer with hydrochloric acid (6 N) and then performing diethyl ether extraction. The diethyl ether layer was then dried over anhydrous sodium sulphate and the solvent was evaporated to yield MFA (Christie, 1982).

Preparation of methyl esters of mixed fatty acids

The methyl ester of fatty acids was prepared by refluxing MFA for about 2 h in a large excess of absolute methanol containing 1% sulphuric acid (v/v). The resulting mixture was then extracted with diethyl ether and washed with water. The diethyl ether layer was dried over anhydrous sodium sulphate and the solvent was evaporated. The ester so obtained was chromatographed over a column of silica gel. Elution with petroleum ether-diethyl ether 96:4 (v/v) gave pure methyl esters of mixed fatty acids (Christie, 1982). The ester was subjected to GLC analysis.

GLC analysis of methyl esters of mixed fatty acids

GLC was carried out by using a Varian Vista 6000 instrument equipped with FID (290°C) detector using a stainless steel column (2 M x 2 mm i. d.) packed with 15% of OV-275 on chromosorb-W (80-100 mesh). Separation was carried out at a programmed temperature of 140 to 200°C (10°C min^{-1}).

Microbial test strains

Clinical strains of *E. coli, K. pneumoniae* and *Candida albicans* from nosocomial and community acquired infections were isolated, identified and characterized by conventional biochemical methods (Chakrabarti et al., 1995; NCCLS, 2000). The study includes ESBL producing strains of *E.coli* and *K. pneumoniae* from community acquired infections (Akram et al., 2007). Other microbial strains investigated were *Streptococcus mutans* ATCC-700610, *Staphylococcus aureus* ATCC-29213, *Enterococcus faecalis* ATCC-29212, *Streptococcus bovis* ATCC-9809, *Pseudomonas aeruginosa* ATCC-27853, *Salmonella typhimurium* ATCC-13311, *E. coli* ATCC-25922, *Klebsiella pneumoniae* ATCC-700603 and *C. albicans* ATCC-10231. *S. mutans* were grown in Brain Heart Infusion (BHI) Broth (Himedia Labs, Mumbai, India), rest of the bacteria were grown in Nutrient Broth (Himedia Labs, Mumbai, India) at 37°C. The yeast, *C. albicans* were grown in Yeast Peptone Dextrose (YPD) Broth (Himedia Labs, Mumbai, India) at 35°C.

Determination of the strains sensitivity to antibiotics

The susceptibilities of the microbial strains to different antibiotics were tested using disc diffusion method (Chakrabarti et al., 1995; NCCLS, 2000). Antibacterial agents from different classes of antibiotics were used which included cephalothin, ceftazideme, ceftriaxone, cefepime, amoxyclav, aztreonam, piperacillin, amikacin, gentamycin, tobramycin, fluoroquinones, nalidixic acid, ciprofloxacin, nitrofurantoin, tetracycline and chloramphenicol (Himedia Labs, Mumbai, India). For fungal strains, the antibiotics used were itraconazole, ketoconazole, nystatin, clotrimazole, fluconazole and amphotericin (Himedia Labs, Mumbai, India).

Agar diffusion assay

The extracts were tested for antimicrobial activity using agar diffusion on solid media. Soyabean Casein Digest Agar (TS) was used for *S. mutans*, Nutrient Agar for the rest of the bacterial strains and YPD Agar for *C. albicans*. The solid agar was punched with 7 mm diameter wells. The inoculums (1.5 x 10^8 CFU/ml) were spread onto their respective agar plants using sterile swabs and then filled with 100 µl extracts. The concentrations of the extracts employed were 0.1 g/ml. The plates were then incubated at 37°C for 24 h. After incubation, zone of growth inhibition for each extract was measured.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration

Strains with inhibition zones were considered sensitive to the extract, those without such a zone were considered resistant. For MIC, two-fold serial dilutions of the extracts were performed. Each inoculum was prepared in its respective medium and density was adjusted to 1-2 x 10^8 CFU/mL and diluted to 1:100 for the broth microdilution procedure. Microtiter plates were incubated at 37°C and the MIC was recorded after 24 h. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth. MBC/MFC were determined by subculturing the test dilutions onto a fresh solid medium and incubated further for 18 to 24 h. The highest dilution that yielded no bacterial/ fungal growth on solid medium was taken as MBC/MFC.

RESULTS AND DISCUSSION

The oil content of *P. longifolia* seed was 11.3% and the oil was yellowish in color. The refractive index (1.4853) is in close agreement with the value of most drying oils whose refractive indices were between 1.48 and 1.49. The iodine value of *P. longifolia* seed oil, 92.2 mg iodine g^{-1} is similar to those of unsaturated fatty acid rich oils such as peanut (86.0 to 107.0) (Duel, 1951). The saponification value of 202.1 mg KOH g^{-1} showed that the oil contains high amounts of higher fatty acids.

The fatty acid profile of *P. longifolia* seed oil revealed that the 9(*Z*) oleic (36.7%), 9(*Z*), 12(*Z*) linoleic (31.3%), palmitic (25.4%) and 9(*Z*) palmitoleic (4.2%) are the major fatty acids present. Some minor fatty acids found were capric (0.8%), stearic (0.6%), lauric (0.5%), 9(*Z*), 12(*Z*), 15(*Z*) linolenic (0.3%) and myristic (0.2%).

Further, in this study, the *P. longifolia* seed oil and its fatty acid methyl ester (FAME) were tested for their antimicrobial activity against multi-drug resistant strains. ATCC strains of Gram-negative bacteria, Gram-positive bacteria and yeast species were also used as control sensitive strains. The seed oil and its FAME showed antimicrobial activity against at least four of the types of microorganisms tested, as exhibited by agar diffusion assay (Table 1). *E. faecalis, S. mutans,* and *S. bovis* were the most susceptible strains. Moreover, the extract of *P. longifolia oil* which demonstrated high activity on the basis of inhibition zone diameters (26 to 35 mm) showed low potency at the MIC level (MIC ≥ 78 µg/mL for 6 of the 8 tested ATCC strains). This observation indicated that the relationship between inhibition zone diameters and the MIC values was far from evident. This could be explained by the fact that some constituents may influence the diffusion properties of the active compound as already observed by others (Nair et al., 2006; Walsh et al., 2003). Our data revealed that standard ATCC strains of Gram-positive bacteria were more sensitive than Gram-negative ones and could be explained by the different cell wall structures of these bacteria. Gram-negative bacteria possess an outer phospholipidic membrane with structural lipopolysaccharide components which is not found in Gram-positive bacteria. This composition makes the cell wall impermeable to lipophilic solutes, and the porins in the cell wall do not allow the penetration of high molecular mass hydrophilic solutes, with an exclusion limit of about 600 Da. This data is also supported by previous workers (Nair and Chanda, 2006; Suffredini et al., 2006; Kang et al., 2006). Furthermore, it has been proposed that the mechanism of the antimicrobial effects involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally ion leakage from the cells (Walsh et al., 2003). Amongst the tested Gram-negative bacteria, *K. pneumonia* was found to be the most sensitive, while *S. typhimurium* was the most resistant bacteria.

In the case of Gram-positive bacteria, *E. faecalis* was the most sensitive, while *S. aureus* was the most resistant strain. *C. albicans* was found to be highly sensitive to the action of FAME of *P. longifolia* seed oil (least MIC, 19.5 µg/mL) followed by *P. longifolia* seed oil with the least MIC being 78 µg/mL (Table 2). In contrast to the previous findings that Gram-negative bacteria are hardly susceptible to the plant extracts in doses less than 2 x 10^5 µg/mL, our results showed inhibition at concentrations as low as 19.5 µg/mL (for FAME extract). The variation of susceptibility of the tested microorganisms could be attributed to their intrinsic properties that are related to the permeability of their cell surface to the extracts. Due to the emergence of antibiotic resistant pathogens in hospitals and homes, plants are being looked upon as an excellent alternate to combat the further spread of multidrug resistant microorganisms. In this study, both the *P. longifolia* seed oil and its FAME showed good antimicrobial activity against multidrug resistant strains of *K. pneumoniae, E. coli* and *C. albicans* isolated from nosocomial and community acquired infections (Table 3). The FAME of seed oil was found to be the most active extract against the nosocomial as well as community acquired isolates. The MIC value of the FAME extract of *P. longifolia* oil against different isolates was found to be in the range of 19.5 to 156 µg/mL. Our data show that strains isolated from nosocomial infection were more resistant to the tested extracts than community acquired infection ones. It was also reported earlier that the resistance to antibiotics as well as mortality is almost two times higher in the case of nosocomial infections than in

Table 1. Susceptibility pattern of *P. longifolia* seed oil and its FAME against different microorganisms.

Microbial strain	FAMEextract[#]	Oil[#]
S. mutans ATCC-700610	+++	+++
S.aureus ATCC-29213	++	++
E.faecalis ATCC-29212	+++	++
S.bovis ATCC 9809	+++	+++
P.aeruginosa ATCC-27853	+++	++
S.typhimurium ATCC-13311	++	-
E.coli ATCC-25922	++	++
C.albicans ATCC-10231	+++	++
K.pneumoniae ATCC-700603	++	++
E.coli [11]a)	+++ (11/11)	++ (11/11)
E.coli [17]b)	- (1/17) + (2/17) +++ (14/17)	- (1/17) + (3/17) ++ (13/17)
C.albicans [19]c)	- (1/19) + (5/19) +++ (13/19)	-(2/19) + (3/19) + (14/19)
K.pneumoniae [15]d)	- (1/15) + (2/15) ++ (3/15) +++ (9/15)	–(2/15) + (3/15) ++ (4/15) +++ (6/15)

Diameter of inhibition zone: no inhibition (-); 5-15 mm (+); 16-25 mm (+ +); 26-35 mm (+ + +); > 40 mm (+ + + +); * Values in parentheses indicate number of isolates out of total isolates tested; a) & c) = isolates of nosocomial infection; b) & d) = isolates of community acquired infection

Table 2. MIC and MBC/MFC values for *P. longifolia* seed oil and its FAME extract against Multi-Drug Resistant strains of Nosocomial and Community Acquired Infections and susceptible Standard strains.

Microbial strain	FAME extract*		Oil	
	MIC (µg/mL)	MBC/MFC (µg/mL)	MIC (µg/mL)	MBC/MFC(µg/mL)
S. mutans ATCC-700610	19.5	39	39	156
S. aureus ATCC-29213	19.5	39	78	313
E. faecalis ATCC-29212	39	156	39	313
S. bovis ATCC- 9809	39	313	78	625
P. aeruginosa ATCC-27853	39	78	39	625
S. typhimurium ATCC-13311	78	156	78	625
E. coli ATCC-25922	78	313	78	313
C. albicans ATCC-10231	39	78	78	156
K. pneumoniae ATCC-700603	39	78	78	313
E.coli [11]a)	39	78 (4/11)	39	313 (2/11
(9/11)	78	313 (7/11)	78	625
E.coli [17]b)	39	78 (5/17)	39	156 (4/17)
(13/17)	156	625 (12/17)	156	625
C.albicans [19]c)	19.5	39 (6/19)	78	313 (2/19)
(17/19)	39	78 (13/19)	156	625
K.pneumoniae [15]d)	39	78 (5/15)	78	313 (4/15)
(11/15)	78	156 (10/15)	156	625

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MFC = minimum fungicidal concentration; a) & c) = isolates of nosocomial infection; b) & d) = isolates of community acquired infection; * value in parentheses indicates number of isolates out of total isolates tested; – = No activity at the concentration of the extracts tested.

Table 3. Resistance profile of multi-drug resistant isolates of Nosocomial and community acquired Infections.

Microorganism[a)] Source of Infection	Resistance Pattern of Antibacterial/ Antifungal Agent	Isolates [b]
E. coli (11) Nosocomial	Ch,Ci,Cpm,Ac,Ao,Pc,G, Na,Cf,T	7EC,8EC,9EC
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,G,Cf,T	1EC,6EC	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,G,Na,Cf,T	2EC,5EC	
Ch,Ca,Ci,Cpm,Ac,Pc,G,Na,Cf,T,C	10EC	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,G,Tb,Na,Cf,T,C	3EC,4EC, 11EC	
E. coli (17)Community	Ch,Ci, Ac,Pc,Na,Nf,C	128EC
Acquired	Ch,Ca,Cpm,Ac,Ao,,Ak,Tb,Cf	68EC
Ch,Cpm,Ac,,Tb,Na,Nf,T,C	92EC, 112EC	
Ch,Ca,Ci,Cpm, Pc,Na,Cf,T	137EC	
Ch,Ci,Cpm,Ac,Pc,Ak,Tb,Na,Cf,Nf,T	186EC	
Ch,Ca,Cpm,Ac,Ao,Pc,Ak,Na,Cf,Nf,T,	61EC, 153 EC	
Ch,Ca,Ac,Pc,Ak,G,Tb,Na,Cf,Nf,T,	93EC	
Ch,Ca,Cpm,Ac,Ao,Pc,G,Tb,Na,Cf,Nf,T,	67EC, 144EC	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,G,Tb,Na,Cf,T	158EC	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,G,Tb,Na,Cf,Nf,103EC,133EC	Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,G,Tb,Na,Cf,Nf	59EC,90EC,152EC
K.Pneumoniae (15)Community	Ch,Cpm,Ac,Ak,Tb,Cf,T	173KP,174 KP
Acquired	Ch,Ci,Cpm,Ac,Pc,Ak,G,Cf,T	63KP
Ch,Cpm,Ac,Ak,Tb,Cf,Nf,T	66KP, 155KP	
Ch,Ca,Ci,Cpm,Ac,Pc,G,Tb,T	111KP, 141KP	
Ch,Cpm,Ac,Pc,Ak,G,Na,Cf,Nf,T,C	153KP	
Ch,Ca,Ci,Cpm,Ao,Pc,G,,Na,Cf,Nf,T	159KP	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,Tb,Na,Cf,Nf	150KP	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,G,Tb,Cf,Nf,T	164KP,174KP, 192KP	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,G,Na,T,C	165KP, 194KP	
Ch,Ca,Ci,Cpm,Ac,Ao,Pc,Ak,G,Tb,Na,Nf,T,C		
C. albicans (19) Nosocomial It,Ns,Flu	2CA,10CA,15CA	
It,Flu,Ap	17CA	
It,Ns,Flu,Ap	14CA	
It,Ns,Cc,Ap	11CA	
It,Kt,Cc,Ap,flu	5CA,13CA	
It,Kt,Ns,Cc,Ap	12CA	
It,Kt,Ns,Flu,Ap	9CA,16CA	
It,Ns,Cc,Flu,Ap	3CA,4CA	
It,Kt,Ns,Cc,Ap	1CA,6CA,7CA,8CA,18CA,19CA	

a) = No. of isolates tested in parentheses; b) = Name of the strains studied in our lab. Antibacterial Agent: Cephalosporins; Ch=Cephalothin (30 µg), Ca=Ceftazideme (30 µg), Ci=Ceftriaxone (30 µg), Cpm=Cefepime (30 µg). Other ß-lactam: Ac=Amoxyclav (30 µg), Ao=Aztreonam (30 µg), Pc=Piperacillin (100 µg). Aminoglycosides:Ak=Amikacin (30 µg), G=gentamycin (10 µg), Tb=Tobramycin (10 µg); Fluoroquinones:Na=Nalidixic acid (30 µg), Cf=Ciprofloxacin (5 µg). Others: Nf=Nitrofurantoin (300 µg), T=Tetracycline (30 µg), C=Chloramphenicol (30 µg); Antifungal Agents: It=Itraconazole (10 µg), Kt=Ketoconazole (10 µg), Ns=Nystatin (100 units), Cc=Clotrimazole (10 µg), Fu=Fluconazole (10 µg), Ap=Amphotericin (100 units).

community acquired infections (Kang et al., 2006). The FAME of *P. longifolia* seed oil was found to give the more potent antimicrobial extract than the seed oil (Table 2).

It is interesting to note that the *P. longifolia* seed oil as well as its FAME showed good activity against MDR strains where modern antibiotic therapy has failed. As per our results, the MIC values were lower than their MBC/MFC values, suggesting that the *P. longifolia* oil and its FAME inhibited growth of the test microorganisms, while being bactericidal/fungicidal at higher concentrations.

In conclusion, the present study indicates that *P. longifolia* seed oil has the physiochemical properties within the recommended limits. The iodine value reflects the fact that the oil is rich in unsaturated fatty acids.

Further the results of antimicrobial assay suggest that the *P. longifolia* seed oil offers potential antibacterial property against the reference strains and could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms from community as well as hospital settings. However, it is necessary to determine the toxicity of the active constituents, their side effects and pharmaco-kinetic properties.

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