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ANTIMICROBIAL EFFICACY OF JUNIPERUS EXCELSA M. BIEL AND HIBISCUS ROSA-SINENSIS L LEAF EXTRACTS AGAINST MULTIDRUG RESISTANT BACTERIAL STRAINS

Arooj Khan[1], Nazia Kanwal[2]*, Aisha Tahir[3], Fareeha Akram[4], Rehana Badar[5], Muzamal Hussain[6], Sayyeda Ayesha Hashmi[7], Muhammad Mushtaq Ahmed[8], Momna Amjad[9], and Zeeshan Haider[10]

[1,2*,4,5,6,7,9]Department of Biological Sciences, The Superior University Lahore
[3]Department of Biochemistry, University of Health Sciences, Lahore
[8]Department of Chemistry, Govt. College University Lahore
[1, 5, 10]Institute of Molecular Biology and Biotechnology, University of Lahore

***Corresponding author:** Nazia Kanwal
* Department of Biological Sciences, The Superior University Lahore, kanwal.n@superior.edu.pk

Abstract
Antibiotic resistance is one of the major problem in developing countries due to either insufficient use or over-use of antibiotics. Current study was designed to evaluate the antimicrobial activity of *Hibiscus rosa-sinensis* L (P1) and *Juniperus excelsa* (P2) against six clinically multidrug-resistant bacterial species. Leaves of experimental plants were collected from local areas of Lahore. Different formulations of both plants were prepared using methanol, ethanol, water, n-hexane, deep-eutectic methanol, deep-eutectic ethanol, and deep-eutectic water designated as F1-F7 respectively. Highest zone of inhibition (38) mm was seen by F5 of P1 against *P. aeruginosa* followed by F1, F6, F2, F7 and F3 (35, 34, 32, 30 and 28 mm) respectively. Likewise, the ZI was also higher (24 mm) by F5 of P2 against *P. aeruginosa* and the lowest ZI (15 mm) by same plant was seen by F3. There was no clear inhibition of growth by F4 against all bacterial species. The second higher ZI was also seen by F5 (35) mm against *S. aureus* followed by F1, F6, F2, F7 and F3 (32, 31, 29, 28 and 25) mm. Furthermore, ZI was also higher by F5 of P1 and P2 (31 and 17 mm) against *E. coli* followed by F1, F6, F2, F7 and F3 for P1 (29, 28, 26, 25 and 22 mm). Growth inhibition of all the formulations (F1, F6, F2, F7 and F3) by P2 was in same pattern against *E. coli*. F5 showed higher ZI against *A. baumannii* for both P1 and P2 (29 and 14) mm and (24 and 10 mm) against *K. pneumonia*. Therefore di-eutectic methanolic extracts of both plant had greater potential to control the bacterial growth so could be used for antibacterial applications against any of the five bacterial strains.

Keywords: Antimicrobial activity, di-eutectic, medicinal and antibacterial

INTRODUCTION

Multidrug resistance in microbes specifically in bacteria develops by the accumulation, of genes, on transposons or resistance (R) plasmids, with each coding for resistance to a drug, or by the function of multidrug efflux pumps, that can pump more than one type of drug (Livermore DM, 2004). Many bacterial pathogens have developed resistance against many antibacterial agents such as *Acinetobacter baumannii, Streptococcus spp., Pseudomonas spp., Escherichia. Coli, Klebsiella pneumonia* and *Proteus spp. etc*. (Weisblum B, 1995).

Acinetobacter species are widespread in environment, soil, and water (Shamsizadeh, 2017). 45% of all the strains of this pathogenic bacteria have been reported as Multidrug Resistant (MDR) (Shamsizadeh et al. 2017). The infections caused by this bacterium are ventilator-associated pneumonia, and central line-associated bloodstream infections (Cheesman, 2017). *Acinetobacter baumannii* has become resistant to many antibiotics including beta-lactams, carbapenems, cephalosporins, and aminoglycosides etc. (Morombaye, 2018). Carbapenems were the only effective class of antibiotics against clinically multi-drug resistant *A. baumannii* (MDRAB) few years ago (Colalto, 2017). *A. baumannii* have developed many resistance mechanisms against carbapenem like antimicrobial inactivating enzymes (beta-lactamase), bacterial targets decreased access (reduced permeability of the outer membrane) and altering targets mutations or alternating cellular functions (Shasank, 2017).

Many bacteria are now resistant to almost all the available antibacterial agents. A notable case is the methicillin-resistant *Staphylococcus aureus* (MRSA), that is not only resistant to methicillin (synthesized against penicillinase-producing *S. aureus*), but also to many other antibiotics such as macrolides, chloramphenicol, lincosamides, aminoglycosides, and tetracycline (Falagas and Rafailidis, 2007). These strains also developed resistance against many disinfectants, and MRSA is a main source of hospital-acquired infections (Blonk, 2017). *P. aeruginosa* infections are life threatening and its treatment is very difficult due to the emergence of antimicrobial resistance. Antibiotic resistance in *P. aeruginosa* is a major problem. It is resistant to many antibiotics such as macrolides, sulfonamides, tetracyclines, aminoglycosides, fluoroquinolones, and many beta-lactams (Patel et al. 2010).

E. coli shows resistance to a broad-spectrum of antibiotics (Oteo and Lázaro, 2005). It produces beta-lactamase enzymes which inactivate beta-lactam drugs making it highly resistant to many beta-lactam antibiotics such as penicillin, cephalosporins, amoxicillin etc. It is also resistant to chloramphenicol, ciprofloxacin, levofloxacin, cefuroxime, trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, and ceftriaxone. Multidrug resistant *Klebsiella pneumoniae* is one of the major bacterial pathogen which have developed resistance against various antibiotics such as beta-lactam drugs (Drag and Nicola, 2010). It shows high resistance to ampicillin/sulbactam. It developed resistance to the broad spectrum of antibiotics, including many beta-lactam antibiotics due to the production of beta-lactamase enzymes. Beta-lactamase-producing *K. pneumoniae* is resistant to penicillins, carbapenems, cephalosporins, and aztreonam (monobactam). *Proteus mirabilis* causes blood-stream infections. It is resistant to some antibiotics, mainly beta-lactam drugs (Magiorakos and Srinivasan, 2011). *P. mirabilis* produce extended-spectrum beta-lactamases (ESBL), carbapenemases and cephalosporinase.

Medicinal plants can provide new antibiotics in future. Medicinal plants contain many bioactive antimicrobial compounds in leaves, barks, stems, and roots (Cock, 2017). These bioactive compounds are active against many bacteria, fungi and viruses (Miyasaki, 2013). These bioactive compounds including flavonoids, tannins, and phenolic compounds are active and efficient against *Acinetobacter*, *Staphylococcus aureus*, *Streptococcus* spp., *Pseudomonas* spp., *Escherichia coli*, *Klebsiella pneumonia* and *Proteus* spp. Etc. (Rhea, 2013). Medicinal plants provide new approaches to infectious diseases (Bandh, 2011). Plant based drugs are becoming popular nowadays because of their minimal side effects, better patient tolerance, cost effectiveness than synthetic drugs (Morombaye, 2018). plants have been utilized in traditional medicines in many countries. They have many ethnobotanical effects vulnerary, antispasmodic, tonic, diuretic, antitussive, febrifuge, asthmatic and carminative activities (Kimwele, 2016).

MATERIALS AND METHODS

Experimental plant

Leaves of two identified plant species such as *Hibiscus rosa-sinensis L.* (GC. Herb. Bot. 3002) (Roselle), and *Juniperus excelsa M. Biel.* (GC. Herb. Bot. 3001) were used as an experimental plant.

Preparation of plant extracts

The leaves of these plants were dried under shade at room temperature for 30 days. Dried leaves were ground to fine powder and crude organic extracts were prepared according to already established methods of experts (Morombaye and Agaie, 2018).

Preparation of deep eutectic solvent extracts

deep eutectic solvent extracts were further prepared by following a protocol of Trusheva (2007).

Culturing of bacterial strains

Bacterial strains were isolated from blood, respiratory and urine samples of patients. The clinical specimens were cultured using standard microbiology techniques and incubated aerobically at 37℃ for 24 hours (Chaudhary, 2012). Bacteria were identified based on colony morphology, Gram staining and biochemical tests (Khan, 2011). These bacteria were sub-cultured on Nutrient agar and incubated for 24 hours (Sushila, 2010).

Antibacterial Assay of plant extracts

Fresh and pure colonies of bacterial strains were utilized for susceptibility testing on the Mueller Hinton agar (Onyeyili, 2011). Bacteria were sub-cultured on Nutrient agar overnight at 37℃ (Bamidele, 2010). The fresh overnight cultures were diluted in distilled water to obtain the test organism and inoculated on Mueller Hinton agar plates to demonstrate the antibacterial activity of the plant extracts (Akinnuga, 2011). Different concentrations of plant extracts were weighed and stored in centrifuge tubes (Ismail, 2014). To prepare the working solution, the organic and crude (aqueous) extracts were diluted with distilled water (Mohammad, 2014). The antimicrobial activity of the plant extracts was demonstrated by well diffusion method (Idris, 2013). Utilizing sterile swabs, the bacterial suspensions were spread on the agar plates (Ahmed, 2016). Six-millimeter wells were made in the agar plates with the sterile cork borer. Utilizing pipette, 100 µl of the solutions made were added into each well at different concentrations (Sarah, 2017). The plates were incubated overnight at 37℃. The antimicrobial activity of the extracts was recorded by zone of inhibition measurement around the wells in millimeter (Morombaye, 2018).

RESULTS

Physical Analysis of crude and organic extracts

Plants extracts were physically analyzed based on their density, viscosity, and color. Aqueous extracts were denser than organic extracts (ethanolic, methanolic, n-hexane) and had brown color. Organic extracts had a greenish appearance (Figure 1) higher yield was seen in aqueous extracts.

Figure 1. Physical analysis of plant extracts. (A) Dried aqueous and (B) organic extracts.

Physical Analysis of Deep eutectic solvent extracts

Deep eutectic solvent extracts were denser than normal aqueous and organic extracts. The extracts were not evaporated completely as they contained deep eutectic solvent. The percentage yield of deep eutectic solvent extracts was seen higher than normal crude and organic extracts as they were not evaporated properly and had some extent of the solvent (Figure 2).

Figure 2. (A) Crude and (B) Organic extract of deep eutectic solvent respectively.

Evaluation of Antibacterial potential of formulations

Highest zone of inhibition (38) mm was seen by F5 of P1 against *P. aeruginosa* followed by F1, F6, F2, F7 and F3 (35, 34, 32, 30 and 28 mm) respectively. Likewise, the ZI was also higher (24 mm) by F5 of P2 against *P. aeruginosa* and the lowest ZI by same plant was seen 15 mm by F3. There was no clear inhibition of growth by F4 against all extracts and formulations against all bacterial species (Figure 3). The second higher ZI was also seen by F5 (35 mm) against *S. aureus* followed by F1, F6, F2, F7 and F3 (32, 31, 29, 28 and 25 mm). Furthermore, ZI was also higher by F5 of P1 and P2 (31 and 17 mm) against *E. coli* followed by F1, F6, F2, F7 and F3 for P1 (29, 28, 26, 25 and 22 mm). Growth inhibition of all the formulations (F1, F6, F2, F7 and F3) by P2 was in same pattern against *E. coli.* F5 showed higher ZI against A. *baumannii* for both P1 and P2 (29 and 14 mm), and lowest ZI was seen F3 of P1 and P2 (19 and 7 mm) for same bacterial species (Figure 3). Formulation 5 prepared by both plants (P1 and P2) were found more effective in controlling growth of P. *mirabilis* (27 and 12 mm), while there was no inhibition in growth by F4 and the lowest ZI was seen in F3 of both plants (16 and 5 mm) respectively (Figure 3). A similar pattern of growth inhibition was observed against *K. pneumonia* as highest ZI was shown by F5 of both plants 24 and 10 mm.

Bacterial species	Plants	F1	F2	F3	F4	F5	F6	F7	Highest
		Zone of Inhibition (mm)							
SP1	P1	35	32	28	0	38	34	30	
	P2	20	17	15	0	24	20	18	
SP2	P1	32	29	25	0	35	31	28	
	P2	17	15	12	0	20	17	14	
SP3	P1	29	26	22	0	31	28	25	
	P2	14	12	10	0	17	15	12	
SP4	P1	26	22	19	0	29	25	21	
	P2	11	9	7	0	14	12	9	
SP5	P1	24	20	16	0	27	23	18	
	P2	9	7	5	0	12	10	7	
SP6	P1	21	17	13	0	24	20	15	
	P2	7	5	3	0	10	7	5	Lowest

Figure 3 Antibacterial potential of plant-based formulations against five bacterial strains *P. aeruginosa, S. aureus, E. coli, A. baumannii, P. mirabilis and K. pneumonia*. Bacterial species SP1: *Pseudomonas aeruginosa*, SP2: *Staphylococcus aureus*, SP3: *Escherichia coli*, SP4: *Acinetobacter baumannii*, SP5: *Proteus mirabilis* and SP6: *Klebsiella pneumonia* treated with the formulations of (P1) *Hibiscus rosa-sinensis* L. and (P2) *Juniperus excelsa M. Biel.* While F1, F2, F3, F4, F5, F6 and F7 are polarity-based formulations prepared using methanol, ethanol, water, n-hexane, deep- eutectic methanol, deep-eutectic ethanol and deep- eutectic water.

DISCUSSION

Plant extracts had great potential to be used as anti-microbial, leaf extract of Hibiscus was found effective anti-bacterial treatment during present course of study. Highest anti-bacterial potential of leaf extracts of *H. rosa-sinensis* had numerous antibacterial compounds such as saponins, flavonoids, and tannins. This study was also supported by the study conducted by Sari and Islamulyadin, (2017).

It had been reported by Safitri et al. 2017 that flavonoids are responsible for the inhibition of cell membrane by forming complex compounds against extracellular proteins of bacterial cells that disrupt the integrity of the bacterial cell membrane. This plant is also enriched in saponin content, which damages bacterial cell membranes and degrades protein and nucleic acid of microbes (Safitri et al. 2017). Further it had been reported by Ruban and Gajalakshmi, 2012 that tannins are responsible for the inhibition of production of bacterial enzymes, and further it can bind to cell walls and destroy bacterial cell membranes. Leaf extracts of hibiscus possessed numerous phytochemicals due to which it had great potential to inhibit bacterial growth. Al-snafi, 2018 reported that quantitative analysis of hibiscus showed the highest quantity of flavonoids. These are natural substances having similar structures like phenolics (Panche et al. 2016). A similar study was conducted Xie et al. 2015 they suggested that hibiscus had higher efficacy against bacterial strains due to the presence of flavonoids. Leaf extracts of *J. excelsa* were also found effective in controlling bacterial growth because these plants also possessed higher content of phytochemicals such as flavonoids, phenolics, terpenoids and tannins. Results of present study were in line with the study conducted by Lesjak et al. 2017. It had higher content of coumarins, flavonoids, lignans, sterols, terpenoids and α-pinene (Seca and Silva, 2006). This plant is also blessed with greater concentration of polyphenols, due to which it could be used as anti-microbial material to inhibit the growth of tested microbes. Study conducted by Semerdjieva et al. 2019 suggested that this plant has variety of phytochemicals due to which it is commonly used for various applications. Outcomes of another study highlighted the presence of important phytochemicals in *J. excelsa* such as phenols, gallic acid, cinnamic acid, vanillic acid, hydroxybenzoic acid, sinapic acid, ellagic acid, myrcetin, and hesperidin (El-Achi et al. 2014). Number studies had been conducted on this plant and marked it as an anti-microbial plant (Topcu et al. 2005; Weli et al. 2014; Angioni et al. 2003).

CONCLUSION

Experimental outcomes of the present study suggested that both plants had a greater efficacy against all the five tested bacterial strains *P. aeruginosa, S. aureus, E. coli, A. baumannii, P. mirabilis and K. pneumonia*. But di-eutectic methanolic extracts of both plants were found more effective in contrast to all other used extracts.

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