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Research Article

ANTIBACTERIAL ACTIVITY OF TRADITIONAL HERBS AND STANDARD ANTIBIOTICS AGAINST POULTRY ASSOCIATED *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Present study aims to access the antibacterial activity of medicinal plants and antibiotics against poultry associated *Pseudomonas aeruginosa*. *P. aeruginosa* is the most widespread avian pathogen and it produces a range of toxins and enzymes that may contribute to pathogenicity. *P. aeruginosa* was isolated from the chicken liver and identified through biochemical methods. The antibacterial activity of extracts of medicinal herbs and various antibiotics were analyzed against *P. aeruginosa* through agar disc diffusion method. *P. aeruginosa* was susceptible against Norfloxacin, Chloramphenicol, Streptomycin, Gentamicin, Tobramycin, and Ciprofloxacin. Whereas, moderately susceptible in case of Oxytetracycline, Neomycin, Lincomycin, and Sulfomethoxyzol. It was also analyzed that Ampicillin, Tetracycline, Penicillin G and Trimethoprim had no effect. Among the plants tested *C. zylanicum, C. cyminum, T. ammi*, *S. aromaticum* and green part of *M. charantia* were most active. The maximum antibacterial activity was calculated by the extracts of isoamylalcohol of *C. zylanicum, C. cyminum, T. ammi, S. aromaticum,* and ethanolic and methanol extract of green part of *M. charantia* against *P. aeruginosa*. This study indicated that these medicinal plants could be the potential source for antimicrobial agents. Hence, these medicinal plants can be further subjected to isolation of the therapeutic antimicrobials and further pharmacological evaluation.

Keywords: Antibiotics, Agar disc diffusion method, Medicinal herbs, *Momordica charantia*, Pathogenic bacteria

INTRODUCTION

Food poisoning in developed countries as well as in Pakistan is caused mainly by food-borne pathogens and these pathogens are often associated with poultry meat products. The utilization of contaminated food products lead to the food-borne illness and it has been a critical problem to public health. Garcia et al.[1] demonstrated that food-borne diseases are mainly transmitted through a variety of sources *viz*., dairy products, salad, fruits, vegetables, fish, and processed meat including poultry, egg, and seafood, respectively. Reportedly, 30 to 40 % mortalities have been associated with food-borne diseases[2].

Sanitary measures should be instituted during storage, processing and marketing of sampled chicken products, can reduce health risks to the consumers. Rindhe et al. [3] isolated *E. coli, Streptococcus sp., Clostridium sp., Klebsiella sp., Shigella sp., Pseudomonas sp., Lactobacillus sp., Salmonella, Proteus*, and *S. aureus* from the cooked chicken. *Pseudomonas aeruginosa* is a highly relevant opportunistic human pathogen and also can cause localized or systemic diseases in young and growing poultry. *P. aeruginosa* invades fertile eggs causing death of embryos whereas virulent strains can cause diarrhea, dehydration, dyspnea, and septicemia and also lead to death to newly hatched chicks[4] (Figure 1). This suggested a possible egg borne infection[4].

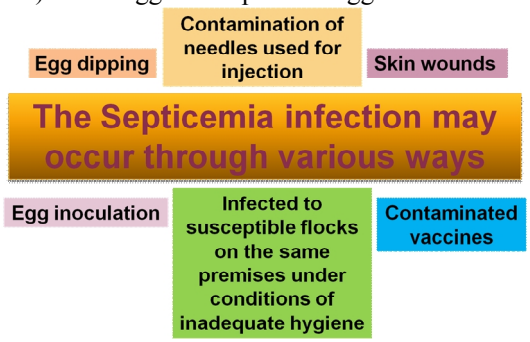

Figure 1: Various ways of septicemia infections caused by pathogens.

It has been trying to promote the growth of chicken broilers by using the doses of antibiotics in feed since 1950s but this contribution has been shown to have negative effect not only to human being, but also to the animal production. In fact it is well documented that the wide use of antibiotics contributes to the development of antibiotic-resistant pathogens both in humans as well as domesticated animals including fish and poultry. This goes a long way to increasing the mortality and decreasing poultry productivity in developing countries. Similarly, the adverse effects of antibiotics *viz*., hypersensitivity, immune-suppression and allergy reactions were also studied on the host[5].

The use of antibiotics in animal feeds has been banned in Europe because of the maturity of antibiotic resistance to human pathogens. For this reason, alternative methods are needed to control poultry associated pathogens and medicinal herbs are being used as antimicrobial agents for the treatment of infectious diseases in different parts of the world[6]. Recently, some scientists have been widely using herbal plants and their essential oils for antimicrobial activity against a range of pathogenic bacteria[7,8].

The current study aims to identify and evaluate the bacterial pathogens associated with chicken products at retail outlets in order to reduce the microbial infection risks to the consumers. Some medicinal plants and antibiotics were screened through agar disc diffusion method which possessed antimicrobial activity against poultry pathogens. It is an interesting way to use the natural products as antibacterial agents to reduce health hazards due to food-borne bacteria[9, 10].

MATERIALS AND METHODS

Preparation of medicinal plants extracts

The medicinal plants *Syzygium aromaticum* (Clove; Lavang; Sample A), *Cinnnamomum zylanicum* (Cinnamon; Dalchini; Sample B), *Cuminum cyminum* (Cumin; Zeera; Sample C), *Trachiyspirum ammi* (Carom seeds; Ajwain; Sample D),

Curcuma longa Linn (Turmeric powder; Sample E/T) and *Momordica charantia* were purchased from the super market of Muzafarrabad, Azad Jammu and Kashmir, Pakistan, and then crushed with the help of a pistil and mortar. The powdered materials of all plants were soaked in different solvents up to 250 ml for 48 h at room temperature except *M. charantia*. After soaking, Whatman 41 filter paper was used for filtration, collected and stored in refrigerator at 4°C. Whereas the Soxhlet extractor was used to collect the extracts of *M. charantia* (both seeds & green parts) successively with chloroform, methanol, ethyl acetate, n-hexane and ethanol after 48 h. Rotary flash evaporator was used to concentrate the extracts in airtight bottle and preserved at 4°C until further use. All the extracts were subjected to antibacterial activity assay.

Isolation and preparation of test pathogens

The test pathogen *P. aeruginosa* was isolated from the chicken liver sample. Chicken liver was brought in the Biotechnology laboratory, cut into small pieces with sterilized aseptic blades, washed with d_3H_2O (double distilled deionized water) for 5 min and later with 70% absolute ethanol. Washed pieces were placed on Nutrient agar (NA) medium supplemented with methyl red and crystal violet. After the incubation of 24 h at 37°C, the small portion of growth area were picked with sterilized loop and again streaked on the different selected medium such as Nutrient gar (NA) with crystal violet and methyl red, MacConky agar (MA), XLD, and TCBS, respectively for the isolation of single pathogen (Figure 2). Glycerol stock cultures were stored at -20°C before used.

Identification of test pathogen through gram staining and other biochemical methods

The bacteria was identified and confirmed by conventional microbiology and biochemical procedures from microbiology lab of Combined Military Hospital (CMH), Muzaffarabad, AJ&K, and Pakistan. The gram staining was aimed at differentiating gram reactions, sizes, shapes and arrangement of cells of the isolates. For the gram-staining of the isolate, glass slides were washed and air dried. A drop of normal saline was placed on the slide. Using a flame inoculation wire loop, a small amount of inoculums was taken and smeared on the drop of normal saline on the slide. The air dried smear was fixed by passing over flame three times, swamped for a min with crystal violet and then rinsed with clean water. Lugols iodine was added for another one minute and this served as a mordant. This was later rinsed and cleaned with distilled water. Acetone-alcohol was added as decolorizer and rinsed immediately with clean water. A counter stain, sefranin, was added and allowed to stand for a minute before being rinsed with clean water. This was allowed to dry before observing under microscope. The other biochemical test such as oxidase, catalase, coagaulase etc were also used for the confirmation of test pathogen.

Antibiotic sensitivity test

Antibiotic sensitivity of test pathogens was determined by the standard agar disc diffusion method of Baur et al.[11] against a number of antibiotics. The potency antibiotics of per disc are as follows; Norfloxacin (10 µg), Chloramphenicol (30 µg), Streptomycin (10 µg), Tobramycin (10 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Oxytetracycline (30 µg), Lincomycin (2 µg), Sulfomethoxyzol (25 µg), Neomycin (30 µg), Tetracycline (10 µg), Penicillin G (10 µg), Trimethoprim (5 µg), Ampicillin (10 µg).

Determination of antibacterial activity

The agar diffusion method was followed for antibacterial susceptibility test[11, 12]. The Nutrient agar (NA) was prepared, allowed to cool up to 40°C, mixed with freshly prepared overnight culture, poured on autoclaved petri plates and allowed to solidify under aseptic conditions. After solidification, the discs (5 mm) with all the extracts of medicinal plants were placed on the surface of the plates with the help of sterilized pincers and gently pressed to ensure contact with the agar surface. Chloroamphenicol used as the positive control. The methanol, ethanol, chloroform, ethyl acetate, n-Hexane and isoamylalcohol were used as blind controls. Finally the inoculated plates were incubated at 37°C for 24 h to allow the maximum growth of the microorganisms[11] and the zone of inhibition was observed and measured in millimeters. Experiments were repeated three times in each case and the mean values of zone of inhibition have been recorded and made known in figures and tables.

RESULTS AND DISCUSSION

P. aeruginosa was isolated from chicken liver (Figure 2). The morphological and biochemical tests were performed according to Bergey's manual of systematic bacteriology[13]. *P. aeruginosa* is Gram (-) rod shaped bacteria and it showed positive characteristics in case of various tests like citrate utilized, urease, nitrate reduction and oxidase test while negative results were obtained in case of methyl red, indole, voges prosteur and catalase tests, respectively[14] as indicated in Table 1.

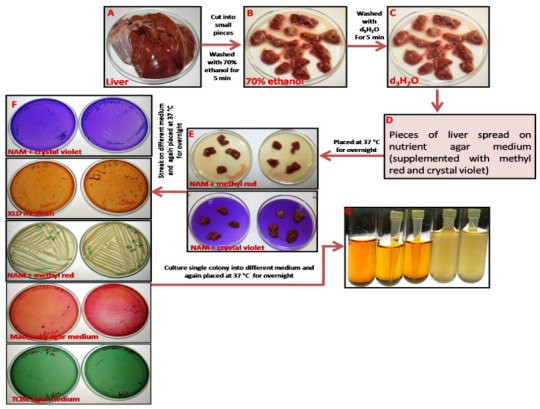

Figure 2: Isolation and screening of *P. aeruginosa* from chicken liver.

Table 1: The morphological and biochemical tests of bacterial strain *P. aeruginosa* isolated from chicken liver.

Characteristics and morphology		*P. aeruginosa*
1.	Gram staining	-
2	Shape	Rod
3	Motility	M
4	Indole test	-
5	Methyl red test	-
6	Voges proskeur test	-
7	Citrate utilization test	+
8	Urease test	+
9	H_2S	-
10	Gas	-
11	Nitrate reduction test	+
12	Catalase test	-
13	Oxidase test	+
	Carbohydrate test	
14	Glucose	+
15	Maltose	+
16	Sucrose	-

Seul et al.[15] pointed out *P. aeruginosa* is a common environmental bacterium and this is an opportunistic pathogen under certain conditions. Therefore, it is concluded that the *P. aeruginosa* is pathogenic for chicken embryos and baby chicks, but has less effect on broilers. In present study the antibacterial activity of chloroform and isoamylalcohol extracts of *C. zylanicum* (Cinnamon; Dalchini), *C. cyminum* (Cumin; Zeera), *S. aromaticum* (Clove; Loang), *C. long Linn* (Turmeric powder), *T. ammi* (Carom seeds; Ajwain), various antibiotics and the n-Hexane, chloroform, ethanolic, methanol and ethyl acetate extracts of *M. charantia* (both seeds and green parts Bitter gourd) were screened against this opportunistic pathogen using agar disc diffusion assay to control or reduce its pathogenecity. Negative control discs containing n-Hexane, chloroform, ethanolic, methanol, isoamylalcohol and ethyl acetate showed no inhibition zone. Growth inhibition (zone of inhibition) was recorded as very high (++++), medium (+++), low (++), which indicated zones of inhibition between 36-49, 21-35 & 12-20 respectively.

Sensitivity test against antibiotics

Sensitivity test revealed that the *P. aeruginosa* was highly sensitive to Norfloxacin, chloramphenicol, Streptomycin, Tobramycin, Gentamicin and Ciprofloxacin (45, 42, 35, 29 45, 49 mm) (Table 2). Our result elucidated that *P. aeruginosa* is sensitive to Chloramphenicol, Streptomycin and Gentamicin (Table 2). These antibiotics involved in the inhibition of peptidoglycan, protein synthesis, DNA replication, folic acid metabolism and murien assembly. Our study is consistent with Walker et al. (4) who found that Gentamycin was most effective for the organism including *P. aeruginosa*. Our results also showed that Oxytetracyclin, Lincomycin, Sulfomethoxyzol and Neomycin had moderately effect (25, 25, 24, & 23 mm) whereas Tetracycline, Penicillin G, Trimethoprim and Ampicillin had no effect because of low permeability membrane barriers to many antibiotics (Table 2). This situation is supported by many evidences like (1) may be an enzyme was produced by *P. aeruginosa* that is capable of inactivating the antibiotic (2) may *P. aeruginosa* altered the receptor target site to block its binding for the antibiotic (3) may the access of the antibiotic was prevented into the bacterium and an efflux pump was used to transport the antibiotic out of the bacterium (4) may more bacterial enzymes were produced through changing the gene expression that is altered by the antibiotic.

Antibacterial activity of medicinal plants

In previous literature the antibacterial, antimycobacterial, antidiarrhoeal and antifungal[9, 16, 17] activities of the plants have been reported. Among the plants screened, *C. zylanicum* (Cinnamon; Dalchini), *C. cyminum* (Cumin; Zeera), *T. ammi* (Carom seeds; Ajwain), *S. aromaticum* (Clove; Loang), and green part of *M. charantia* were most active (Figure 3 & 4). The maximum antibacterial activity was observed by isoamylalcohol extract of *C. zylanicum*, *C. cyminum,* and *T. ammi* (19, 21, & 21 mm) while the chloroform extracts of *C. cyminum* and *T. ammi* showed moderate results (15 & 10 mm) (Figure 3). Our results are closely related to the previous literature that the growth of pathogens was inhibited by the isolated extracts of clove, jambolan, pomegranate and thyme[18]. These results are interesting, because *P. aeruginosa* was opportunistic pathogen and difficult to control by use of therapeutic means. Studies regarding the mode of action should be performed for these compounds in the bacterial cell. Cinnamon and clove are used by the several people due to antibacterial and antifungal properties[19].

Table 2: Antibacterial activity of selected antibiotics against chicken liver associated *P. aeruginosa*.

Sr #	Antibiotic used	Concentration of antibiotic	Sensitivity of *P. aeruginosa*	Zone of inhibition (mm)
1	Norfloxacin	10µg	++++	45±1
2	Chloramphenicol	30µg	++++	42±3
3	Streptomycin	10µg	+++	35±5
4	Tobramycin	10µg	+++	29±2
5	Gentamycin	10µg	++++	49±8
6	Ciproflaxin	5µg	++++	45±2
7	Oxytetracycline	30µg	+++	25±4
8	Lincomycin	2µg	+++	24±0.5
9	Sulfomethoxyzol	25µg	+++	25±4
10	Neomycin	30µg	+++	23±2.5
11	Tetracycline	10µg	-	00
12	Pencillin G	10µg	-	00
13	Trimethobrim	5µg	-	00
14	Ampicillin	10µg	-	00

Singh et al.[20] indicated that the volatile oil of ajwain and cinnamon was found to be highly effective against *P. aeruginosa, E. coli, B. subtilis* and *S. aureus*. On the other hand the turmeric oil was found to be inactive against *Salmonella* Typhimurium and *P. aeruginosa*[20]. A drug based on cinnamon was used against infections caused by *Candida* in AIDS patients. Similarly, it was illustrated that essential oil of *C. zeylanicum* demonstrated strong antifungal activity on *Aspergillus*.*In vitro* antimicrobial activity of *C. zelyanicum* (bark) was used against human pathogenic fungi and commensally bacteria were studied by Matan et al.[21].

P. aeruginosa indicated that it was resistant microbial strains in the presence of both isoamylalcohol and chloroform extracts of turmeric (Figure 3). Resistance exhibited by *P. aeruginosa* may be attributed to the differences in the structural integrity of the cell wall that is, the lack of "binding material" and hence interaction between the cellular lipoproteins (present in the peptidoglycan dense cell walls of Gram-positive microorganisms providing a greater target surface for the active components to attach and initiate its antimicrobial action) and the active compounds present in turmeric extracts.

Antibacterial activity of *M. charantia*

Cucurbits are among the most essential plants supplying humans with edible fruits and useful seeds. Plants have high genetic diversity for fruit, shape and other characteristics,

resulting in a variety of uses. The most important cultivated genera bitter gourd (*Momordica charantia* L.) is the summer vegetable grown extensively throughout the country and covers an area of about 5697 ha with an annual production of about 52099 tons in the country[22] which serve as the main source of nutrition, energy, valuable vitamins and minerals. From literature survey it was found that *M. charantia* possessed antiviral, antiobesity, antidiabetic, antifeedent and antioviposition, anti fertility, anti-genotoxic activities and used as alternative source of medicine to treat various diseases[6, 7, 23-25]. On the basis of various activities, antibacterial activity was conducted and it was observed that the extracts of green part of *M. charantia* (N6, N7, N8, N9 & N10) have antibacterial activity as compared to the seeds (N1, N2, N3, N4, & N5). The ethanolic (N9) and methanol (N10) extracts of green part of *M. charantia* showed antibacterial activity (40 & 32 mm) while the n-Hexane (N1), chloroform (N2), ethanolic (N4), methanol (N5) and ethyl acetate (N3) seed extracts of *M. charantia*, had no effect on the growth of *P. aeruginosa*. On the other hand the chloroform (N7) and ethyl acetate (N8) extract of green part indicated slightly susceptible results (11 & 10 mm) (Figure 4).

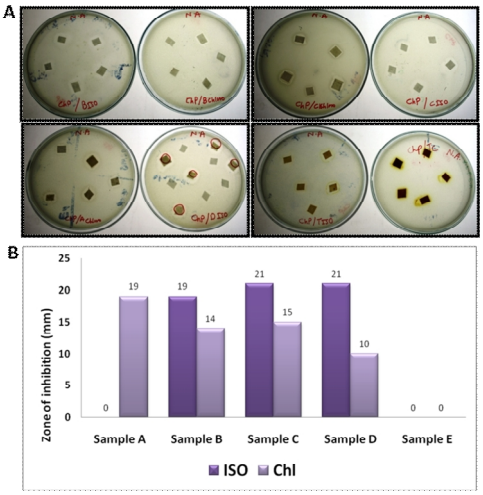

Figure 3: (A) Antibacterial activity of choloform and isoamylalcohol extracts of medicinal plants against *P. aeruginosa.* (B) Mean values of Zone of inhibition of extracts of medicinal plants was measeured in mm.

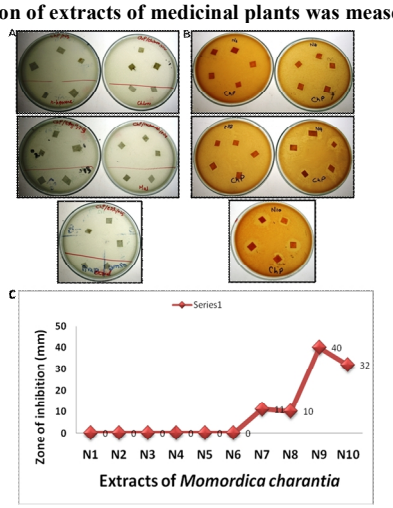

Figure 4: Antibacterial activity of extracts of *Momordica charantia* against *P. aeruginosa.* (A) Indicates the antibacterial activity of seed extracts of *Momordica charantia* through agar disc diffusion method. (B) Shows the antibacterial activity of green part extracts of *Momordica charantia* through agar disc diffusion method. (C) Mean values of Zone of inhibition of both seed and green parts extracts of *Momordica charantia* was measeured in mm.

CONCLUSION

It is concluded from the present study that, the extracts of medicinal plants can effectively be used as a potential antimicrobial agents to overcome the problem of bacterial infection. It was observed that individual compounds such as thyme from ajwain, ar-turmerone from turmeric, eugenol, taninns and flavonoids from clove, menthol and palmitic acid from *M. charantia* have antimicrobial activities and could be used as a new source of therapeutic drugs against bacterial pathogen[26, 27].

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