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ANTIBACTERIAL ACTIVITY OF *LAURUS NOBILUS* EXTRACT AGAINST *Pseudomonas aeruginosa* ISOLATED FROM WOUNDS IN SHEEP AFTER FALSE WOOL SHEARIN

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ABSTRACT

Although sheep shearing is considered an important and a widely used process to cut off the sheep's wool, false wool shearing can cause serious problems by giving a chance to grow bacteria. This study is aims to identify *Pseudomonas aeruginosa* isolated from the inflamed wounds after false wool shearing process and to evaluate the antibacterial activity of *Laurus nobilus* extract against this bacteria. The results of bacterial growth showed that *P. aeruginosa* produced characteristic colonies on nutrient agar with pigment pyocin and β- hemolysis on blood agar and grew on MacConkey agar but did not ferment lactose sugar. In addition, the isolates were positive for biofilm formation using polystyrene 96 well plate. Among 6 antibiotic agents, the highest resistance was found with novobiocin, chloramphenicol and tetracycline, respectively. *Laurus nobilis* extract had an antimicrobial activity against *P. aeruginosa*. The results of this study revealed that hot and cold alcoholic extracts of *Laurus nobilis* with MICs 6.5 mg/ml, 12.5 mg/ml and 50mg/ml, respectively, were more effective than hot water extract.

INTRODUCTION

Pseudomonas aeruginosa (family *Pseudomonadaceae*) is an aerobic, motile, Gram-negative rod, widely present in the environment, e.g. in water and in humid places (1), it is also an important opportunistic pathogen for humans, plants and animals. *P. aeruginosa* can cause acute and chronic infections in different mammalian hosts and organs due to the production of a wide arsenal of virulence factors. Virulence factors associated with *P. aeruginosa* include flagella, adhesion proteins and extracellular proteins, or secondary metabolites, with proteolytic and/or cytotoxic activity (e.g. exotoxin A, elastase, proteases, pyocyanin, hemolysins) (2). Inflammation of wounds is a defensive immune response that is coffered by the host against foreign body (3). The innate immune system on encountering pathogen elicits the acute inflammatory response that is accompanied by vascular leakage and leukocyte emigration. The redness, swelling, heat and pain are natural signs of healing process during inflammation period (4). The wound healing and tissue repair are complex processes that involve a series of biochemical and cellular reaction (5). Most of the vascular changes observed in acute inflammation are due to inflammatory mediators that are released by inflammatory cells at site of wounds such as histamine and other mediators (3).

Laurus nobilis L, is one of the most well-known plants from the *Lauraceae* family, which is also known as Bay or laurel leaves, it has an antimicrobial activity. Phytochemical studies on Bay leaves and its fruits have indicated various secondary metabolites including alkaloids, flavonols, glycosylated flavonols sesquiterpene lactones, monoterpene and germacrane alcohols (6,7). Interestingly there is a worldwide concern around that use of antibiotic to treat bacterial and fungal infection. Which can lead to rise and spread of organisms resistant to broad spectrum antibiotic . That concern open ways to use plants as natural sources for novel antimicrobial agents with a similar activity (8,9). Natural medicinal plants, as *L. nobilis,* are rich sources of bioactive compounds. Thus, the biological properties of Bay extracts and its essential oil are documented, specifically their antimicrobial activity.

MATERIALS AND METHODS

Sample collection for isolation and identification of *Pseudomonas aeruginosa*: A total of 50 samples were collected from sheep wounds after wool shearing by using sterilized swabs. The swabs were incubated in brain heart infusion broth for activation. Following incubation for 24 hrs. at 37°C, the inoculums were streaked on nutrient agar for isolation, then the isolates were cultured on blood agar and MacConkey agar and the plates were incubated overnight at 37°C. *P. aeruginosa* was identified by its colony characteristic, pigment production, grape like odor formation. Suspected colonies were identified using motility test, Gram stain (10) and biochemical test like catalase, oxidase, citrate utilization, methyl red test, Indole production and growth at 42°C (11).

Biofilm formation: Biofilm formation by *P. aeruginosa* was studied on 96 microtiter plate (12). All the isolates were grown in trypticase soy broth with 0.25% sucrose and incubated overnight at 37°C. Two hundred microliters were diluted overnight and the culture was transferred into 96-well microtiter plate and incubated at 37^0C for 24 hrs., and the broth without culture was used as a control. After incubation, the content of each well was gently removed by slightly tapping the plates. The wells were then washed three times with 300 µl of sterile distilled water. Bacterial adhering to the wells were fixed with 250 µl of methanol per well for 15 mins. Then each well of plates was stained with 250 µl of 0.1% (w/v) crystal violet solution for 5 mins. Excess stain was removed by washing with sterile distilled water and air dried.

Antibiotics susceptibility test: *P. aeruginosa* isolates that were inoculated on Mueller– Hinton agar plates were incubated into nutrient broth overnight until the turbidity was equivalent to 0.5 Mcfarland standards, and then left for few minutes at room temperature. Antimicrobial susceptibility was performed on Mueller-Hinton agar by the standard disk diffusion method. This was done by dipping a sterile swab stick overnight in nutrient broth and the entire surface of Mueller– Hinton agar plates was carefully swabbed. The antibiotics used against the tested bacteria were: Tetracycline (10 µg), Ciprofloxacin (10 µg), Chloramphenicol (10 µg), ceftriaxone (10 µg), Gentamicin (10 µg) and Novobiocin (10 µg). The antibiotic multi disc (Oxoid) was then placed on the surface of the inoculated plates and gently pressed. The plates were incubated at 37°C for 18–24 hrs. The diameter of the inhibition zone was measured in millimeters and the

isolates were scored as sensitive or resistant by comparing with the recommended values on standard charts (13).

Preparation of *Laurus nobilis* extracts

Cold alcohol extract: Three hundred milliliter of 70 % ethyl alcohol were added to 50 gm of *L. nobilis* leaves The mixture was stirred for 3 days at room temperature then filtered through Whatmann No. 3. The filtrate was evaporated at 80°C by rotary evaporator, then poured in Petri dish and left at room temperature to dry (14).

Hot water extract: Three hundred milliliters of distilled water were added to 50 gm of *L. nobilis* leaves powder to obtain hot water extraction. The solution was left in reflux for 3 days then filtered, and evaporated by rotary evaporator at 60°C (15).

Hot alcohol extract: Three hundred milliliter of 70 % ethanol were added to 50 gm of *L. nobilis* powder to obtain hot alcohol extract. The solution was left in reflux for 3 days then filtered by Whatmann No. 3 and evaporated by rotary evaporator at 60°C (15).

Antimicrobial activity: The antimicrobial activity was evaluated by the disk diffusion method (16), which consists of using absorbent sterilized paper discs (9 mm in diameter) wetted with extracts. The discs were placed on the surface of the agar. The bacteria were spread all over the agar. The micro-organisms grew all over the surface of the agar except where the product that would inhibit their growth. Following incubation around the discs, a clear circular inhibition zone was observed. The effect of the extracts on *P. aeruginosa* was estimated by the appearance of clear zones around the discs. The diameter of the halo of growth inhibition was measured by vernier calipers (Mauser) and expressed in mm (including the diameter of the disc of 9 mm) (17). All assays were performed in triplicate.

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) of *L. nobilis* extracts was determined by using disc diffusion method according to (18) with a little modification. Briefly, sterilized filter paper discs (9 mm in diameter) were impregnated with different concentrations of *L. nobilis* extracts (50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml), which were prepared before 24 hrs and kept at 4°C. Muller Hinton agar media were seeded with 0.1 ml of bacterial culture and adjusted to 0.5 McFarland

turbidity standard. After 15 min, the prepared discs were placed on the surface of the seeded agar. MIC was defined as the lowest concentration that inhibit the visible bacterial growth.

RESULTS

The gross sections in Fig. 1 and 2 showed the contaminated wounds after three and five days of infection, respectively, following false wool shearing process. Ten isolates of *P. aeruginosa* were isolated by using nutrient agar which were confirmed primarily based on characteristic colony morphology in nutrient agar, blood agar and MacConkey agar media and Gram's staining technique. *P. aeruginosa* produced mucoid colonies with emitted sweat grape odor and produced characteristic pigment pyocins in nutrient agar (Fig. 3). The isolates produced β-hemolysis on blood agar (Fig. 4) and on MacConkey agar, but did not ferment lactose sugar (Fig. 5). Thin smears were prepared on glass slides from a single colony for Gram's staining. Following staining, the morphology of the isolated *P. aeruginosa* showed Gram-negative, rod shaped appearance. The motility test revealed that the isolates were found to be motile.The purified isolates of *P. aeruginosa* were examined by different biochemical reactions (Table 1).

Fig. 1: Contaminated wound after three days from infection period

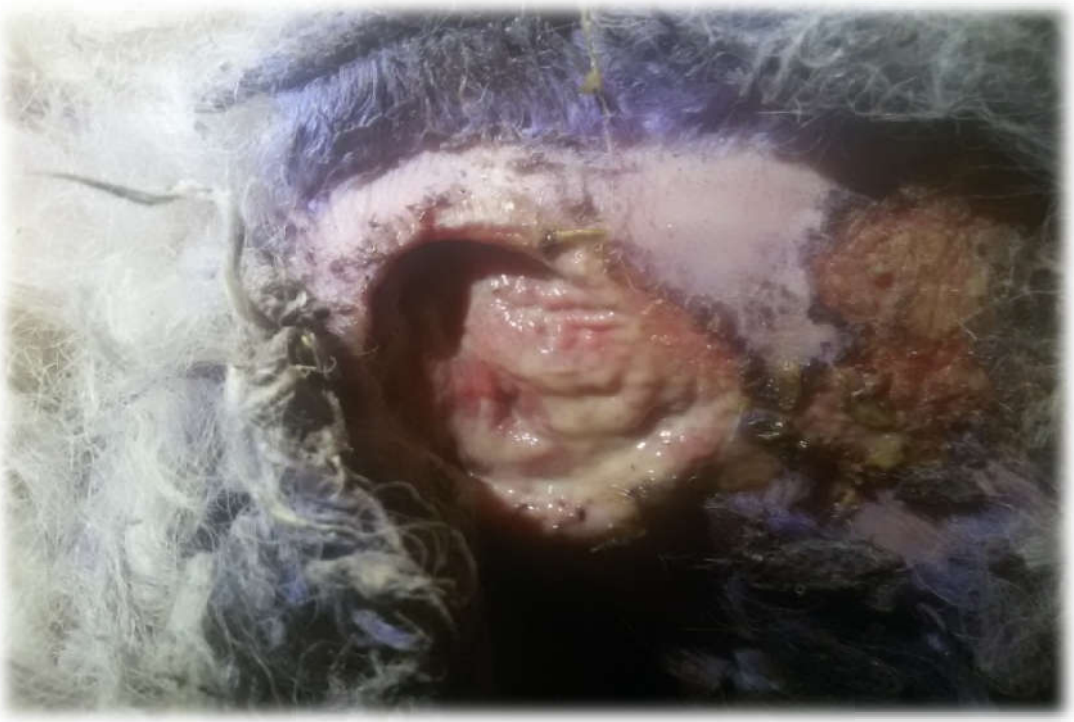

Fig. 2: Contaminated wound after five days from infection period with abscess appearance.

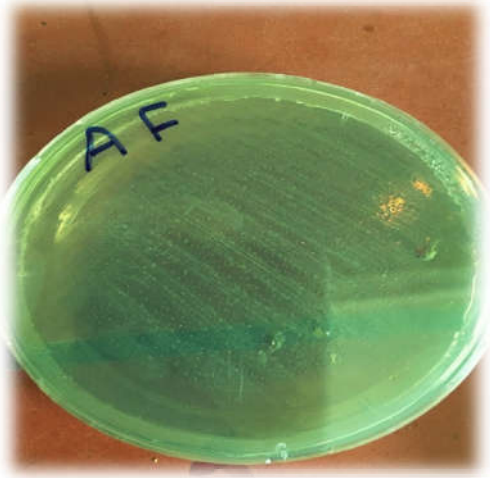

Fig. 3: Pyocin production on nutrient agar by *P. aeruginosa*

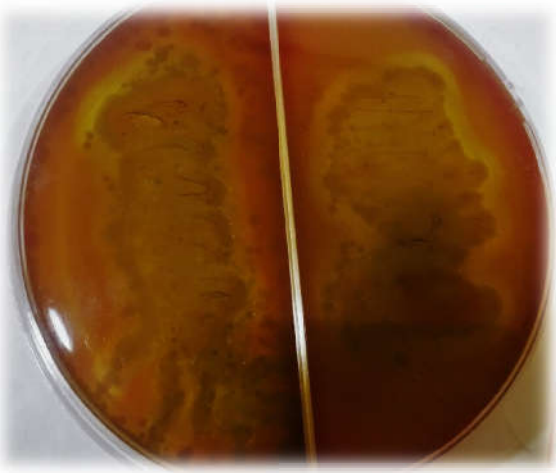

Fig. 4: β- hemolysis on blood agar by *P. aeruginosa*

Fig. 5: Bacterial growth on MacConkey agar

Table 1: Biochemical test of *P. aeruginosa*

biochemical reactions	Result
Oxidase	+
Catalase	+
Indol production	-
Citrate utilization	+
Methyl red test	+
growth at 42C	+

Biofilm formation

Biofilm formation by *P. aeruginosa* was also studied on polystyrene, 96 well-flat bottom tissue culture plates at 37°C for 24 hrs. It was found that all the isolates were positive for biofilm formation (Fig. 6).

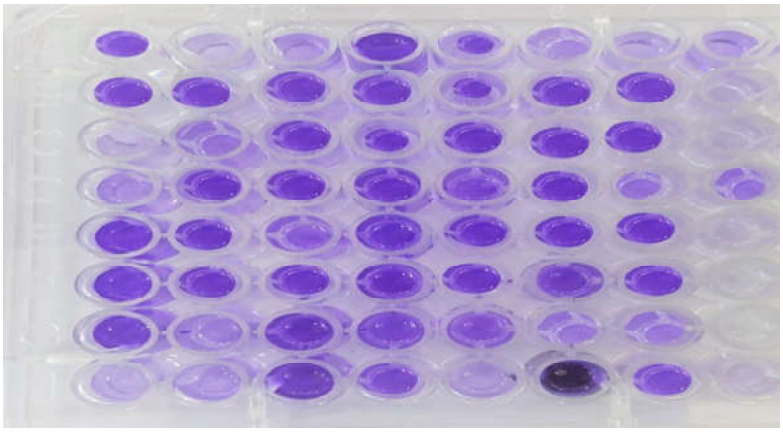

Fig. 6: Biofilm production *by P. aeruginosa* in microtiter plate

Antibiotics sensitivity test

Antimicrobial sensitivity test was done on 6 antimicrobial agents using: Tetracycline (10 µg), Ciprofloxacin (10 µg), Chloramphenicol (10 µg), ceftriaxone (10 µg) , Gentamicin (10 µg) and Novobiocin (10 µg), (Table 2) .

Table 2: Antibiotics sensitivity test results

Antibiotics	**Symbol**	**Disc content (mcg)**	**Diameter of inhibition (mm)**		
			Sensitive	**intermediate**	**Resistance**
Tetracycline	TE	10	-	-	± 1.3 0≤ 14
Ciprofloxacin	CIP	10	±30.3 ≥21	-	
Chloramphenicol	C	10	-	-	± 2.33≤ 12
Ceftriaxone	CRO	10	-	-	0 ≤
Gentamicin	CN	10	±24.33 ≥ 15	-	-
Novobiocin	NV	10	-	-	± 1.66 ≤ 18

Antimicrobial activity of *Laurus nobilis* extracts

Following the measure of the antibacterial activity of *L. nobilis* extracts, the hot alcohol extract and cold alcohol extract were more effective than hot water extract (Fig. 7) and table 3.

Table 3: Antimicrobial activity of *L. nobilis* extracts

Mean and standard deviation of Inhibition zone diameter		
Hot alcohol extract (100mg/ml)	Cold alcohol extract(100mg/ml)	water extract(100mg/ml)
33±0.75	23±0.23	19±0.23

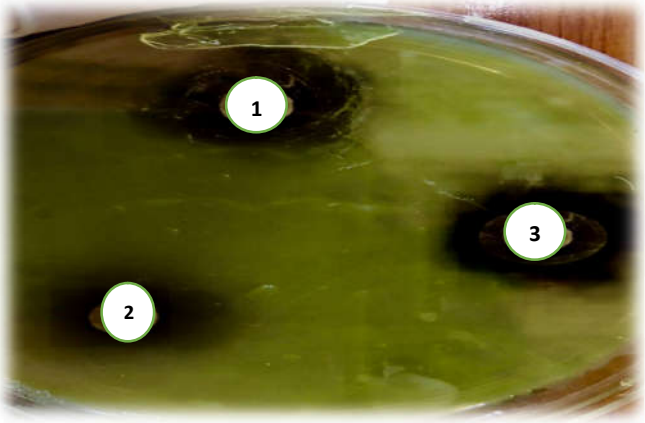

Fig. 7: Antimicrobial activity of *L. nobilis* against *P. aeruginosa*

Determination of minimum inhibitory concentration (MIC)

Hot ethanolic extract of *L. nobilis* had the highest bacterial activity with an MICs of 6.5 mg/ml against *P. aeruginosa*. Cold ethanolic extract had an MICs of 12.5 mg/ml, while the water extract showed less activity against *P. aeruginosa* with an MICs of 50 mg/ml (Fig. 8)

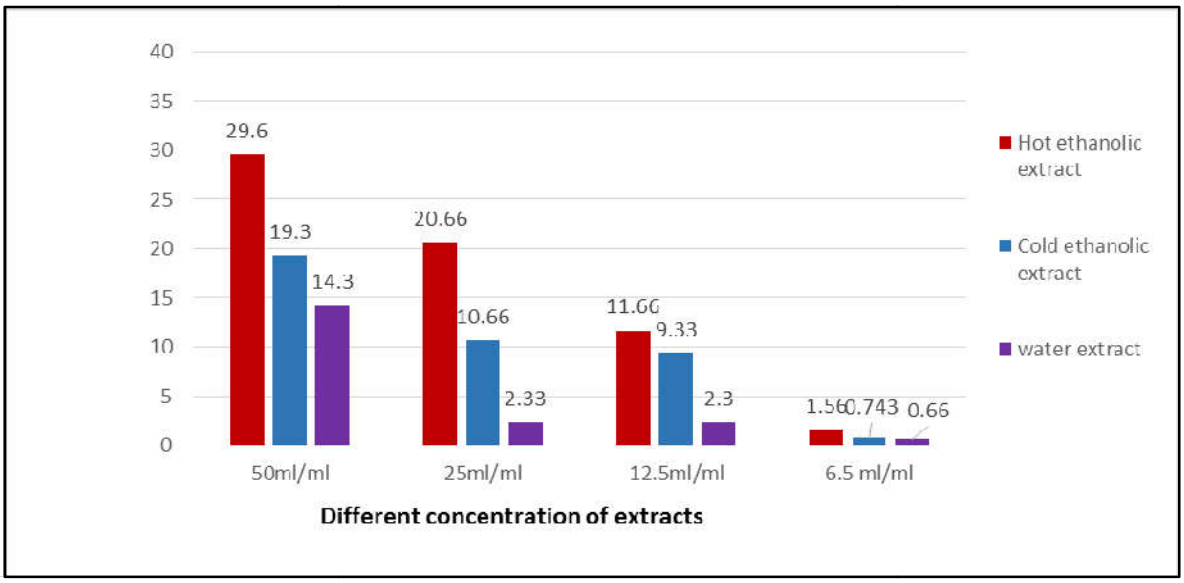

Fig. 8: Minimum inhibitory concentration (MIC)

DISCUSSION

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacterium that belongs to the *Pseudomonadaceae* family. It is a free-living organism, commonly found in soil, vegetation and water, marshes, and coastal marine habitats (19) and occasionally animals and humans.

P. aeruginosa has been known to cause opportunistic and chronic infections in wounds where it produces series of virulence factors such as enzymes and toxins which breakdown the host's tissues (20). Mucus and extracellular polymeric substances including alginate are often produced by the organism to prevent the action of phagocytes and other immune mechanisms (21). The ability to multiply quickly and produce biofilm within a short period (22) has enhanced its pathogenicity. *P. aeruginosa* is one of the organisms that are predominant in wound infections (23). The ability to form biofilm enhances the resistance to the body's immune system and antimicrobials, which further increases the virulence capability this phenomenon has been linked to the impediment of wound healing and causes of chronic wounds (24).

The present study exhibited the medical importance of the plant through the existence of antimicrobial activity of cold and hot alcoholic extracts and water extract against *P. aeruginosa*

isolated from the inflammatory wound after false wool shearing in sheep .The results showed that cold and hot alcoholic extracts were more effective than hot water extract. The average diameter of inhibition zone ranges from 33 ± 0.75 , 23 ± 0.23 and 19 ± 0.23 respectively. The antimicrobial activity depends on the antimicrobial compound in the extracts according to the solvent used in the process (25). The hot extract showed the lowest MICs comparing to the other types of extracts and this may be due to the large quantity of active substance that were precipitated during the extraction process due to the solvent.

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