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EFFICIENCY ASSESSMENT OF SOME PLANT EXTRACTS ON THE GROWTH OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM BOVINE MASTITIS

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Abstract

Bovine mastitis is the most economically important disease affecting dairy cattle worldwide from an economic, diagnostic and public-health point of view. The study aimed to isolate, identify *Pseudomonas aeruginosa*, investigate the susceptibility of this isolates against some antibiotics and plant extracts with comparison of theirs impact and picking the most efficient. Five pre-diagnosed isolates of *P. aeruginosa* in addition to hundred milk samples from cattle with mastitis harvest by using nutrient agar and MacConkey agar. Disk diffusion method was used to determine the antimicrobial activity of aqueous, alcoholic extracts and standard antibiotics discs against five isolates of *Pseudomonas aeruginosa*. The results showed that the isolates were only sensitive to the amikacin, where the diameter of the non-bacterial growth zone was about (19-21mm). It was found that alcoholic extract of (*Organium majorana* plant) have variable antimicrobial activity (the inhibition zone diameter ranged from (9 to 15 mm) respectively according to the tested concentration). The isolates showed the highest sensitivity when using alcoholic extract of *Achillea falcata*. While no significant effect was observed for the aquatic extracts of both plants.

Key words : Mastitis, *P. aeruginosa* bacteria, Medicinal plant, Antibiotics.

Introduction

Pseudomonas aeruginosa is an adaptable bacteria pathogen related with a wide range of infection in humans and animal. Due to the innate capacity of resistance to antimicrobial agents, this bacterium is greatly difficult to treat. What's more, such resistance is being progressively a problematic issue because of increasingly development of resistance to agents regarded as powerful therapeutic option. (Kerr and Sneeling, 2009).

This pathogen has propensity to initiate sudden clinical or perhaps subclinical cases of most outbreak in number of cows within short period of time (Licia *et al.,* 2015). A group of signs and symptoms accompany the acute cases of bovine mastitis with noticeable udder swelling, fever in addition to abnormal watery as well as clotty milk, which contains flaked or blood. whereas subclinical form does not show any visible symptoms. Clinical mastitis is threatening to a farmer in a dairy herd and treatment is given immediately to control it. But subclinical mastitis, which cannot be identified without a laboratory or field test, mostly remains unnoticed by the farmer. (Park *et al.,* 2014).

Because of Pathogenic germs protect itself from antibiotics and white blood cells in layers of slim and The resistance to antimicrobial drugs has increased, so recently there has been a great deal of attention paid in medical treatments to plant extracts and compounds with biological features. These compounds are not only efficient for the treatment of infectious diseases, but also concurrently diminish existing side effects via their anti-bacterial compounds. (Girish *et al.,* 2015).

Despite the diversity etiological agent of mastitis by bacteria, viruses (Wellenberg *et al.,* 2002), and fungi (Farnsworth, 1977), the most common cause are gram-positive and gram negative bacteria precisely *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* Comes after them. (Hogan and Smith, 2003).

This study guaranty two plants: *Majorana origanium*

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Linn (Laminaceae), and *Achillea falcata* Linn (Asteraceae), which have been tested as alternative source for antimicrobial drugs. The goal of this work to find therapeutic alternatives against *P. aeruginosa* bacteria, especially those resistant to antibiotics and selection of plant extracts that have the strongest influence in these germs. (Monnet *et al.,* 2004) (Corti *et al.,* 2003).

Materials and Methods

Sample collections

Aseptically, hundred milk mastitis samples were gathered from livestock farm in wasit Governorate, by using 10 ml collected vial, delivered to the lab and stored at 4^0C until use. In addition to isolates obtained from the University of Baghdad laboratories as shown in table 1.

Table 1: *Pseudomonas aeruginosa* isolates.

Code of bacterial isolates	Source	Reference
P1 P2 P3 P4	Isolated from udder wounds	Obtained from microbiology laboratory, university of Baghdad
P5	Isolated from milk	

Processing of the samples

The samples were streaked on nutrient agar plates and the plates were incubated at 37C° for 24 hours as described by (Pawel *et al.,* 2008). Then the characteristic suspected single colonies were subjected to Gram's staining then sub-cultured on MacConkey agars and blood agars, The pure isolates of *Pseudomonas aeruginosa* were transferred to 1% nutrient agar slant and stored in the refrigerator. *P. aeruginosa* was identified by biochemical test (sugar fermentation test) depending on methods described in (MacFadden *et al.,* 2000). relying on the method described by (Cheesbrough, 1985) Motility test of the isolated *P. aeruginosa* was performed.

Antibiotic sensitivity test

Disk diffusion method adopted to hold sensitivity test as explained by (bauer and Kirby, 1966), 4-5 pure colonies were taken from the surface of the blood agar with a sterile loop and placed in a tube containing 4 ml of nutrient broth and shacked well until dark resemble to Macfarland tube formed. after 1 to 2 hours cultured petri dish contained Muller- Hinton agar by sterile cotton swab.

After removing the excess quantities of bacterial isolate by pressing the swab strongly on the walls of the test tube inside, then the agar was planned from all sides to ensure equal quantity was distributed in a similar manner and lay the dishes aside for 15 min. by using sterile forceps, four antibiotic discs used in this study explained in table 2 were transferred to the surface of the cultured pseudomonas, with a space between one disc from another to avoid overlapping areas of inhibition, *Pseudomonas aeruginosa* PTcc 1310 was used as quality control strain in susceptibility isolates were defined as these showed resistant to classes of anti-pseudomonas agent.

Table 2: Type of antibiotics disk and source of production.

Antibiotics disk	Content	Company
Gentamycin	10 µg	Bioanilyse(turke)
Tetracycline	30 µg	
Erythromycin	15 µg	
Amikacin	30 µg	

Collection of plant material

The leaves of *Origanum majorana* and the flowers of the *Achillea falcata* were collected from local markets, after washing with cold water and distilled water, dried at 60°C and kept in moisture-free conditions until extraction.

Preparation of aqueous and alcoholic extract

Organium marjorana leaves and *Achilla falcata* flowers were crushed firstly, then based on (AL-Sammaraiae, 2011) for both watery and alcoholic extract as in steps:

1. Mixing 20 g of downy plant with 400 ml of distilled water once and 70% of Ethyl alcohol.
2. Place the mixture in (1L) flask and incubate in a 40 water bath for one day.
3. Exit the extracts from the water bath and have the filtration process by the filter paper Whatman No.1, followed by incubation step at 37C for one day until dried.
4. After the drying of the extracts, the weight and percentage of the extract was recorded. It was taken 5 g of extract in flask complete the size to 100 ml (5% stock solution) from this prepared 10 µg /ml and 25 µg /ml.

By virtue of *(LDD et al. 1978*), Where 10 grams of dry material was soaked in 200 ml of ethanol 70% for 24 hours. after a day of dissolving process, materials were taken and filtered (paper filter of Wattman No.1, 5 mm), therewith the substance was concentrated on using the rotary evaporator at 40-45°C to drain off the solution and to earn effective matter of the alcohol extract, stock solution (2%) prepared by dissolve 2g of concentrated materials of each plant in 5 ml organic solvent and complete the volume to 100 ml by using distill water. finally, Saturated filter paper with 10 µg/ml and 25 µg/ml of

alcoholic extract prepared.

Results and Discussion

A total of 100 milk sample were collected from a cattle. The cattle were suffering from clinical symptoms and did not response to common antibiotics such as Tetracycline and Amoxicillin etc.

The first piece of study was undertaken to isolate the bacterium causing mastitis, out of 100 sample, only 13/100 (13%) diagnostic as *P. aeruginosa* which were confirmed primarily based on characteristics colony morphology in nutrient agar, blood agar and MacConkey agar media and Gram's staining technique. This a consensus with a study conducted in Korea on samples milk was 45/225 (20%), In an Egyptian study done on a group of animals, the ratio was (20.3%). (Nam Hm and kamel, 2011).

P. aeruginosa produces circular mucoid smooth colonies with emits sweat grape odor in nutrient agar (Fig. 1) and cause β-hemolysis on blood agar (Fig. 2) and grew on MacConkey agar, but did not ferment lactose sugar (Fig. 3). These characteristics colonies were similar with finding of Hossain *et al.,* (2013) and Haleem *et al.,* (2011).

Thin smears were prepared on glass slides from a single colony of MacConkey agar for Gram's staining. In Gram's staining, the morphology isolated *P. aeruginosa* showed Gram-negative, pink colored, medium rod shaped appearance (Fig 4). These findings agreed with the findings reported by Saleh ZF *et al.,* (2016) and Tripathi *et al.,* (2011). Isolates of *P. aeruginosa* were found to be motile when examined using hanging drop slide under microscope Quinn *et al.,* (2002) and Tripathi *et al.,* (2011). In sugar fermentation test, the isolates fermented dextrose with the production of both acid and gas but did not ferment lactose and sucrose which strongly supports the observations of Quinn *et al.*, (2002) and Cheesbrough., (1985). Table 1 revealer results of chemical identification:

Antibiogram test means, the test that is done in laboratories to determine the sensitiveness of antibiotics against certain bacteria which is responsible for specific disease. Furthermore, isolates of *P. aeruginosa* were investigated for susceptibility and resistance patterns by Kirby-Bauer test method using four Commercially available antibiotic discs belonging to different groups as detailed in table 2.

Table 1: Results of identification of study isolates.

Gram stain	Negative
MacConkey agar	Pale colonies
Oxidase	Negative
Catalase	Positive
Indole Test	Negative
Methyl Red Test	Negative
Citrate Utilization	Positive
Motility Test	Positive
βHemolysis	Positive

Fig.1: β-hemolysis of *P. aeruginosa* on blood agar.

Fig. 2: Circular mucoid smooth colony of *P. aeruginosa* on nutrient agar.

Fig. 3: *P. aeruginosa* grow on MacConkey agar without sugar fermentation.

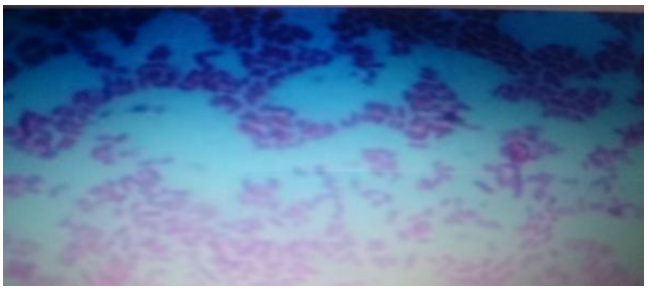

Fig. 4: Gram's staining results of *P. aeruginosa.*

Table 2: Antibiotics sensitivity pattern of isolates of *P. aeruginosa*.

Name of antibiotics	Code No.	Inhibition zone(mm)	Description
Gentamycin	GN10	12	Intermediately sensitive
Tetracycline	TE30	5-7	Resistance
Erythromycin	E15	5-9	Resistance
Amikacin	AK30	19-21	Sensitive

**Zone of inhibition produced by each antibiotics disc on agar plate were measured in millimeter by virtue of national committee on clinical laboratory standards (NCCLS)

Among the variety of antibiotics tested, the highest resistance was found with Tetracycline and erythromycin (table2). These findings were more or less similar to other researchers (Haleem *et al.*, 2011, Ferguson *et al*., (2007) and Ryan and Ray (2004) where the authors concluded that 100% *P. aeruginosa* were resistant to Ampicillin, Amoxicillin and Tetracycline.

On the other hand, both the isolates were intermediately sensitive to Gentamicin. These results were nearly comparable with Tripathi *et al*., 2011 where the authors found that all the clinical isolates of *P. aeruginosa* were sensitive to variety of antibiotics including gentamicin. Both isolates of *P. aeruginosa* were highly sensitive to amikacin (table 2). These results of antibiotic sensitivity test were similar with Heras *et al.,* 1999 and Corona-Nakamura *et al*., 2001 where the authors interpreted that *P. aeruginosa* absolutely sensitive to amikacin.

Recently and significantly, Resistance to aminoglycoside category antibodies (gentamycin and amikacin) has been increasing from *P. aeruginosa*. This resistance is due to the production of an enzyme acting on modulation the antibiotic and therefore loses its properties or come as a result for the loss of some outer membrane proteins which reduces the permeability of the antibody.

With regard to plant extracts, results showed that the watery extract of both plant not appear any effect on bacteria growth, these finding agree with Malu S.P., (2009). A study to determine the effect of ginger plant in a group of germs where it confirmed that the water extract is not have any effectuation Otherwise, alcoholic extract of *O. majorana* (in concentration 10 and 25 µg/ml) had the ability to inhibit growth of tested isolates, noticed inhibition zone was ranged between (8-10) and (14-15) millimeter respectively, also it had been seen that the extract activity increase with increasing extract concentration as shown in table (3, 4) below. These results

Table 3: Detection anti-pseudomonal activity of *Achillea falcata* extract.

Extract	Concentration	Inhibition zone(mm)				
		First isolate (Ps1)	Ps2	Ps3	Ps4	Ps5
Aqueous	5 µg/ml	NE	NE	NE	-	-
	10 µg/ml	-	-	-	-	-
Ethanoic	10 µg/ml	21	21	22	22	21
	25 µg/ml	33	33	33.6	33.6	33.6

*NE=non effect

Table 4: Showed effects of *Origanium majorana* extract on bacteria growth.

Extract	Concentration	Inhibition zone(mm)				
		Ps1	Ps2	Ps3	Ps4	Ps5
Aqueous	5 µg/ml	NE	-	-	-	-
	25 µg/ml	-	-	-	-	-
Ethanoic	10 µg/ml	8	9	10	9	8
	25 µg/ml	14.3	14	14.8	15	15

are consistent with a study conducted by Hogan, 2003.

The powerful influence was for the *Achillea* plant, where the range of diameters of the non-growth zone in 10 µg /ml was 22mm and 33.6 when we use 25 µg/ml, the finding agreed with Frdoos al fadel and shaza al laham., (2013), where the author found the ethanolic extract of the Syrian cesium plant had the strongest effect in the non-growth of the same bacteria.

Conclusion and Recommendation

The alcoholic extracts of the tested plant parts showed an effective impact exceed the antibiotic's effect in curbing the *P.aeruginosa* bacteria. Proceeding from the above, we recommend studying the effect of these plants on other bacterial species, especially those Stubbornness to antibiotics. More research is exigency to know to evaluate the possible toxicity of this extract and its application in the medicinal system.

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