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Isolation and Identification of Proteus bacteria from mouth and nose of human and animals with studying the Sensitivity to ethanol: aqueous extracts of pomegranate peel and Lantana cammara leaves.

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ABSTRACT

The aim of this study is to determine the antibacterial activity of alcohol and aqueous extract of Pomegranate peel, *Lantana. cammara* leaves against *proteus mirabilis* bacteria. Therefore 60 samples from mouth and nose swabs from human and different animal species were dependent for the current study. The swabs were submitted to routine bacterial procedures isolation, through culturing on nutrient broth, then MacConkey and blood agar, followed by gram stain examination, and fixing the macromorphological characters of colony and finally confirmed by API 20 system. A total of Proteus mirabilis was 2 out 60 were isolated in pure form nose and mouth , in addition to 1 case mixed with *Klebsiella pneumoniae* from nose, in addition 1 case mixed with Bacilli, and 1 case with Bacilli and Streptococcus, from mouth. These isolates were examined for their sensitivity to ethanol: aqueous (70:30) extract of Pomegranate peel, *Lantana. cammara* leaves using disc agar diffusion method. The sensitivity was highest for the ethanol: water, extract for the pomegranate peel and *Lantana cammara* leaves were showed an inhibitory effect.

Key words: Proteus , ethanol extracts, pomegranate peel and *Lantana cammara.*

عزل وتمييز بكتريا المتقلبات Proteus من الفم والانف للانسان والحيوان مع دراسة حساسيتها لمستخلص الايثانول :الماء لقشر الرمان وأوراق المينا الشجرية

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الخلاصة :

هدفت هذه الدراسة تحديد الفعالية المضادة للبكتريا للمستخلص المائي والكحولي لقشر الرمان واوراق المينا الشجرية اخذت 60 مسحة من الفم والمنخر من الانسان وانواع مختلفة من الحيوانات في الدراسة الحالية . خضعت المسحات إلى الإجراءات الروتينية للعزل البكتيري من خلال الزرع على مرق المغذي ، واكار الماكونكي والدم ، تبع ذلك التصبيغ بصبغة كرام وتحديد الصفات الشكلية للمستعمرة ، وتم التثبيت النهائي باستخدام نظام API 20.. تم الحصول على عزلتين نقيتين من المتقلبات الرائعة من مسحات الانف وعزلة واحدة نقية من الفم ، بالإضافة إلى حالة واحدة مختلطة مع الكليبسيلا الرئوية من الأنف، وتم الحصول على حالة واحدة ممزوجة بالعصيات ، وحالة واحدة مع بكتيريا العصيات والسبحية من الفم. تم فحص العزلات لمعرفة حساسيتها تجاه لمستخلص الايثانول : الماء 70:30 لقشر الرمان ، المينا الشجرية الاوراق بطريقة الاقراص في الاكار وان الحساسية كانت الاعلى للمستخلص الايثانول : الماء لقشر الرمان. وأظهرت أوراق المينا الشجرية تأثير مثبط.

الكلمات المفتاحية: المتقلبات الرائعة ، لمستخلص الايثانول ، قشر الرمان واوراق المينا الشجرية.

INTRODUCTION

Proteus is Gram negative, facultative anaerobic, rod shaped bacteria. It has swarming motility, urease activity, do not usually ferment lactose. Since it belongs to the family of *Enterobacteriaceae* (1) In this family it's place in the tribe *Proteeae*, together with the genera *Morganella* and *Providencia*. Proteus rods are distinguishable from most other genera by their ability to swarm across agar surface of solid media (2). Proteus is widely distributed in the natural environment. It can be found in polluted water and in soil and manure, (3). Plants are rich source of antibacterial agents because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection. (4). Pomegranate (*Punicagranatum* L.) has been used in the folk medicine of many cultures especially in the Middle East ,edible parts of pomegranate fruit represent 52% of total fruit weight, comprising 78% juice and 22% seed (5). Non edible part called pomegranate peel extract Pomegranate has become more popular because of the attribution of important physiological properties, such as anticancer (6) antiproliferative apoptotic (7) cardio protective (8) anti-hyperlipidemic(9),(10). demonstrated that pomegranate has many potential effects such as immune modulator stomachic, antifungal and antibacterial. (11).

L. camara has several uses, mainly as a

herbal medicine and in some areas as firewood and mulch. It is also used for the treatment of cancers, chicken pox, measles, asthma, ulcers, swellings, eczema, tumors, high blood pressure, bilious fevers, catarrhal infections, tetanus, rheumatism, malaria and atoxy of abdominal viscera (12). Extracts from the lantana leaves exhibit antimicrobial, insecticidal and nematicidal activity and also contain verbascoside, which possesses antimicrobial, immunosuppressive and antitumor activities (13). Lantana oil is sometimes used for the treatment of skin itches, antiseptic for wounds, leprosy and scabies.

Aim of study was to isolate the Proteus from the natural orifice of the body (Mouth and Nose) of human and animals and study its sensitivity for ethanol: aqueous extracts of peel pomegranate and lantana cammara leaves .

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MATERIALS AND METHODS

Methods

Collection of samples and specimens

The study was conducted in department of Microbiology, college of veterinary Medicine, University of Diyala, Iraq from November, 2018 to end of December ,2019. Swabs from Nose, and Mouth were collected from human and different animal species.

Sample culturing

The collected swabs were submitted to culture by inoculation into nutrient broth and incubated at 37 oC for 5 hr. (enrichment step) to increase bacterial level.loop full from the incubated broth was distributed onto surface of MacConkey agar then incubated at 37 °C for 24 hr.

Identification of Isolates:

Morphological Characterization:

After the isolates cultured on MacConkey agar and incubated for 24 hours at 37 °C, they were examined by naked eye for colony characterization.

API20 system (confirmation test):-

This system was used for approving the diagnosis of bacterial isolates and study the rest of the biochemical tests for bacteria P. mirabilis (14).

Sensitivity:

McFarland solution was prepared according to (15). The mixture was shacked well and placed in a screw capped test tube and kept in dark place at 4 °C. The solution is mixed well before use to compare it with bacterial turbidity as it gives a turbidity equal to 0.5 x 108 bacteria/ ml.

Sensitivity to plant extract we used brain heart infusion agar for the sensitivity test .we use ethanol solvent for extract the pomegranate peel, and *Lantana cammara* Leaves.

Crude plants Extract Preparation:

Pomegranate Peel Extraction

The peels were collected from local market in Baqubah then washed and left 5 days to get dry under sun light. After that dried peels are cut into small pieces and grinded using electric grinder until became fine powder. A fifty gm of pomegranate peel dry powder was taken, mixed with 70% ethyl alcohol and heated to (60 -70) °C for two hours by using soxhlet extractor then separated by centrifuge 5000 rpm for 20 minutes. The supernatant solution was collected in sterile container, this process was returned three times then the solution was collected in sterile container (16). After the above process done the ethyl-alcohol was removed from the solution by Rotary Evaporator, then the final result of the extracted material was kept, the above extracted material was ethyl alcoholic extract of pomegranate(17). and then different concentrations (100, 200, 400, 800 and 1600) were prepared from the stock solution by dilution with dimethyl sulfoxide (DMSO) solution . and used as the test extracts for antimicrobial activity assay.

Lantana cammara extraction

The fresh Lantana cammara leaves were collected from gardens college of veterinary medicine of Diyala University, air dried at room temperature, and ground into powder by using electric grinder. A quantity of 50g of Lantana camara L. leaves powder was mixed with 250ml of 70% ethanol by soxhlet apparatus for 6 hrs. At 60-70°C, then the solvent was removed under reduced pressure by a rotary evaporator at 40°C. then the final result of the extracted material was kept (18). and then different concentrations (100, 200, 400, 800 and 1600) were prepared from the stock solution by dilution with dimethyl sulfoxide (DMSO) solution . and used as the test extracts for antimicrobial activity assay

Antimicrobial Activity

Agar disc Diffusion Method

The modified disc agar diffusion method according to (19) was depended to evaluate the antibacterial activities of the extracts. A fresh colony forming units (10^8 CFU/ ml) was aseptically spread onto the surface of brain heart infusion agar and then left to dry for 3o min. disc, 5 mm in diameter were prepared from filter paper. Each disc was saturated with 100 µl of the crude extract (100, 200, 400, 800, 1600 mg /ml). the plates were left at room temperature for 30 min to allow diffusion of material in media. The controls were prepared using the same solvent. plates were incubated at 37°C for 18-24 hours. Inhibition zones in mm (including disc diameter) around disc were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extracts against tested bacteria. All tests were performed in triplicate.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the extract was determined. a 16- hours culture was diluted with sterile physiological saline solution (0.9% w/v sodium chloride) with reference to the 0.5 McFarland turbidometry to achievement the inoculum approximately equal to 10^8 CFU/ ml) (20). In the tube dilution assay, standard bacterial suspension and different concentration of extracts, 100., 200, 400, 800 and 1600 mg/ ml) were added to tubes containing 2 ml broth. These tubes were incubated at 37 °C for 24 hours. The first tube of the series with no sign of visible growth was considered as the MIC. This process has been done three times (21). The values are statistically analyzed according to (22).

RESULTS

Isolation of Bacterial spp.

The results of current study showed that from 60 swabs collected from mucocutaneous origin of mouth and nostrils of human and different animal origin, submitted to the study. A total of Proteus mirabilis was 2 out 60 were isolated in pure form, in addition to 1 case mixed with *Klebsiella pneumoniae* from nose. In mouth were isolated 1 case in pure form and 1 case mixed with Bacilli, and 1 case with *Bacilli* and *Streptococcus*, (Table 1).

Table - 1- distribution of proteus isolates according site of isolation.

origin	Sp.	No.
Mouth	Prot. Bacill	1
	Proteus mirabilis	1
	Strep Prot. Bacilli	1
Nose	Proteus mirabilis	2
	Prot. Kleb	1

Identification of Proteus mirabilis :-

Microscopic examination:-

Microscopic examination of the results showed that the bacterial cells isolate are negative to Gram stain, red color coccobacilli , variable in length , frequently occurred singly or in short chains and non-spores forming.

Biochemical test results :-

Several biochemical tests were achieved to identify the isolates of *Proteus mirabilis* were positive for catalase, urease , Phenylalanine , gelatin and methyl red but gave negative results for citrate utilization, oxidase , vogas- proskauer and indole tests . This bacteria showed its ability for H2S production when it was cultured on Triple sugar iron (TSI) agar while the slant turn to red, and its bottom was yellow this is because of the ability of ferment glucose only .

MacConkey agar was used for growing *Proteus* isolates because it differentiates them from other Gram negative species and it contains all required nutrients for *Proteus* growth. Furthermore, on blood agar *Proteus* isolates showed the characteristic swarming motility with no hemolysis behavior (figure 1).

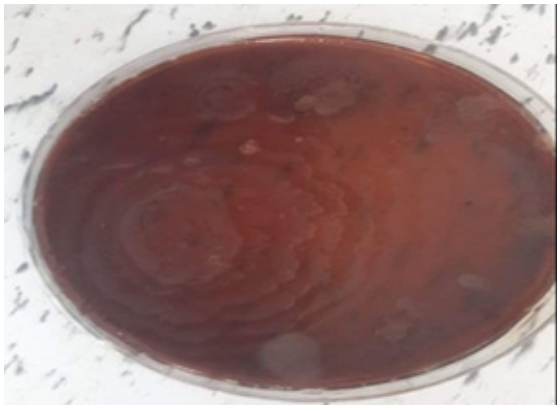

Figure (1):
Swarming of P. mirabilis on blood agar

API 20 test result

Bacterial isolates were tested by API 20 E for confirmation the results are shown as in the figure (2)

Figure (2):API 20 E test results for Proteus mirabilis

Sensitivity to alcohol and aqueous extracts:

The results of sensitivity showed that the pomegranate peel extract exhibit a significant effect as the sensitivity was high and dose dependent. The highest inhibitory zones appear at 1600 mg / ml. *Lantana cammara* leaves extract did not show any significant inhibition and the bacteria was insensitive to the extract fig-(3).

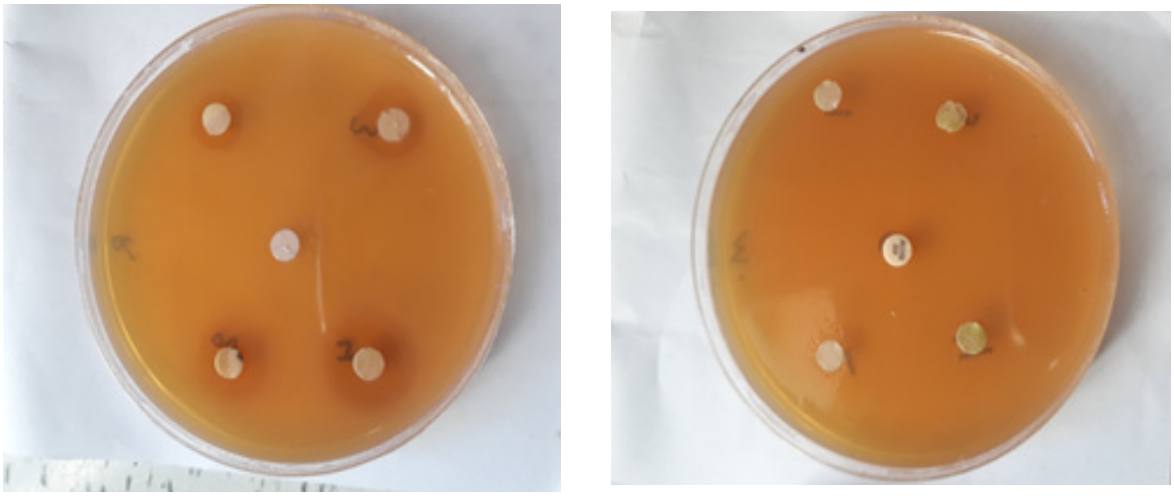

Figure (3) showed the sensitivity of pomegranate peel extract and insensitivity *Lantana cammara* leaves extract by disc agar diffusion method

Table (2): Sensitivity of *Proteus mirabilis* to alcohol and aqueous extract of pomegranate peel and *Lantanas cammara* leaves by disc agar diffusion method

Extract	Conc. mg / ml	Pome. peel	L. leaves
	100	8.0± 0.55Aa	1.0 ± 0.2b
	200	9.0± 0.92Aa	1.5± 0.2b
	400	11.0± 0.43Ba	1.5± 0.4b
	800	11.0± 0.14Ba	2.0± 0.3b
	1600	16.33± 0.34 Ca	2.0 ± 0.5 b
Am	None		
Aug	None		

Value are Mean ± SEM; a, b significant when compared between the two extracts;

A, B , C, when compared between concentrations at P< 0.05 level

DISCUSSION

The results of current study showed that from 60 swabs collected from mucocutaneous origin of mouth and nostrils, only (3; 0.05%) *Proteus mirabilis* were isolated in pure form, with 3 isolates were mixed with other bacteria.one of etiological factors of numerous human and animal diseases are Proteus sp., since in human it is the etiological agents of wound infections, urinary tract, burn, as well to bacterial chronic and acute otitis media (23) and as a causal of pneumonia and nosocomial infections (24).*Proteus vulgaris* (4/98; ratio was 4.1% and *Proteus mirabilis* (2/98; ratio was 2.0%) were isolated from (98) patient suffered from wound infection. while from 173 patients suffered from burn complications Proteus sp. (21/173; 12.1%) were isolated. Urine samples obtained from patients with urinary tract troubles Proteus sp., were isolated from 11/90; 12.2%). From whom suffered from otitis media Proteus sp. (16/50; 32%) were isolated (25).According to (26) diverse Gram negative bacteria are opportunistic infections in immunocompromised patients. The organisms most often occupied members of the genera *Klebsiella, Escherichia, Proteus*, and *Acinetobacter* and *Enterobacter*.

In current study, the ethanol-aqueous infusion extract of pomegranate peel by disc agar diffusion method, showed there were bacterial inhibition, of dose dependent type. The highest inhibitory zone was at 1600 mg / ml. and bacterial inhibitory test showed that 800 mg //ml was the level at which there was no bacterial growth. Meanwhile extract of *Lantana cammara* leaves did not show significant inhibitory effect at all concentration. The isolates were resisted to amoxicillin, and Augmentin as there were no any inhibition. (27) reported that the agar well diffusion method was more important to determine the antibacterial effects of both aqueous and alcoholic extracts of cotoneaster sp., in comparison with agar disc method, the alcoholic extract was more effective and showed higher antibacterial effect against all bacteria sp.in comparison with aqueous extract as well both alcoholic and aqueous extract were concentration dependent (27).All extract (aqueous and methanol extract) of *Urtica dioica display* important antibacterial action against isolated pathogenic bacterial strains of *Staphyloccocus aureus , Bacillus subtilis ,Pseudmonas aeruginosa, E. coli, Klebsiela and Proteus* .In aqueous extract, by well method Proteus was the the majority sensitive at 200mg /ml merely. Although in disc way aqueous extract show

an inhibition to Pseudomonas, followed by Proteus only. ethanol extract via well technique did not showed any inhibition to Proteus, whereas through disc method methanol extract of *urtica dioica* show an inhibition to *E. coli*, *Proteus, staphylococcus and Klebsiela* (28). Results of a study done by (29) confirm that the fruit extracts of *M. azedarach* showed an antibacterial effect. E. coli and Bacillus were the least sensitive bacteria. Pseudomonas, Staphylococcus, Proteus and Klebsiella were sensitive to *M. azedarach* fruit aqueous and ethanol extract. No differences were observed between the well and disc diffusion ethods and between aqueous and ethanol extract.

The susceptibility of 20 *P. mirabilis* to antibiotics was investigated by using Kirby- Bauer method. The results indicated that *P. mirabilis* isolates had variable degrees of resistance toward different categories of antibiotics.it included Clarithromycin, tetracycline, clindamycin and erythromycin 100%, ampicillin, cloxacillin, cefixime and vancomycin 95% in spite of its action is mostly on Gram positive bacteria (30). From the above results, it can be noticed that *P. mirabilis* isolates had moderate to low resistant to antibiotics. That inhibit protein synthesis, such as aminoglycosides (amikacin, streptomycin, gentamycin, tobramycin and kanamycin), Nalidixic acid, and chloramphenicol, so as antibiotics that inhibit nucleic acids synthesis such as rifampicin, while all isolates showed resistance to erythromycin, clarithromycin, clindamycin, and tetracycline(31).

Proteus sp. (*Proteus vulgaris* and *P. mirabilis*) showed a sensitivity to Amp., CTX (otitis media), resistant to CX, AMX, CM, L, E (otitis media) (25). Motamedi et al .,referred to multi resistant to antibiotics showed by local isolated strains of *E. coli* and *Proteus mirabilis*(32).The results indicated that the resistance of *P. mirabilis* to some antibiotics is increased with prescription of years. Because of the wrong and random use of these antibiotics and increasing the rate of Proteus infections. On the other hand, this bacteria had ability to produce beta - lactamases, especially extended spectrum beta- lactamases (ESBLs), as well as, their ability to transfer genetic elements carrying the genes of these enzymes, and number of mutations occur with these type of enzymes leading to increase resistance to antibiotic beta- lactam, in addition to other mechanisms such as alteration the target site or alteration the access to the target site by modification of penicillin binding proteins (OBPs) (33).Inhibitory effect of pomegranate is forever attributed to the antioxidant activity that depends chiefly on the anthocyanin and phenolic content of the fruit (32). the highest antibacterial activity was recorded against Klebsiella pneumoniae and among fungi high activity against Aspergillus was recorded.(34) generally antimicrobial effects can be attributed to the phytochemicals of the plant used in our study. In phenols and polyphenols include phenolic toxicity to microorganisms which include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (35) ; Quinones, they provide a source of a stable free radicals and are also known to complex irreversibly with nucleophilic amino acids in protein (36), often leading to inactivation of the protein and loss of function, they may also render substrates unavailable to the microorganism; Flavones, flavonoids and flavonols, they are known to be

synthesized by plants in response to microbial infection (37) and found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, more lipophilic flavonoids may also disrupt microbial membranes (38). Majority of the *Lantana camara* activity is due to bioactive compounds viz, flavones, isoflavones, flavonoids, isocatechins, alkaloids, tannin, saponins and triterpenoids. (39) have reported that the leaves extract of *Lantana camara* be active against various gram positive and gram negative bacteria. The essential oil of *Lantana camara* exhibited prominent antibacterial activity against all the bacterial strains tested. Gram negative *Klebsiella pneumoniae* and Pseudomonas aeruginosa were not susceptible to the essential oil at lower concentration (40).

CONCLUSIONS:

From results we can concluded that very few cases (3/60;ratio was 0.05%) of *Proteus mirabilis* were isolated in pure form and 3 mixed with other bacteria. Ethanol: aqueous extracts of Pomegranate peel and *Lantana cammara* leaves have an inhibitory effect in sensitivity point of views.

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