

The effect of Alcoholic plant (*tribulus terrestris*) extract on the bacteria isolated from genital tract of infertile cows in vitro

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Abstract

Out of Two hundred swabs collected from genital tract of infertile cows during the period between 7/1/2012 to 30/6-2012. Cultured on Blood agar, Nutrient agar, MacConkey agar and Mannitol salt agar. 195 swab represented (97.5%) give positive result and 5 swab (2.5%) give negative result. from the positive culture 78 isolated (39%) mixed bacterial infection. All bacteria isolates were identified by using different biochemical tests and API-20E, the single bacterial isolation in this study were *Esherichia coli* 41.8%, *Staphylococcus aureus* 36.8%, *Pseudomonas aeruginosa* 17%, *Citrobacter braakii* 1.7%, *Providencia rettergi* 1.7% and *Shewanella putrefaciens* 0.9%. In mixed bacterial isolation, the highest percentage was 53.8% *Staphylococcus + Esherishia coli aureus* and the lowest percentage was 1.3% *Esherishia coli + Citrobacter braakii + Pseudomonas aeruginosa*. Antibiotic susceptibility test were carried out on bacterial isolates using 10 single antibiotic discs of commonly used drug, the test showed that most bacterial isolated resist for most antibiotics (Amikacin, Amoxicillin, Nalidix acid, (Amoxicillin and Clavulanic acid), Cefixime, Ceftriaxone, Tetracycline, Nitrofurantoin, Norfloxacin and Gentamycin) on the other hand observed sensitive for alcoholic plant *tribulus terrestris* extract (fruit) in different concentrations

Introduction

Most cattle producers probably do not realise the extent of economic loss that can occur through reproductive failure in their cattle. Low calving rates may be due to infertility (failure of cows to conceive) or to early embryonic losses, abortion and stillbirth (1). Uterine function is often compromised in cattle by bacterial contamination of the uterine lumen, and pathogenic bacteria often persist, causing uterine disease, a key cause of infertility in cattle (2). Non-specific and specific bacteria are important causes of infertility in the cow. Uterine infections reduce the reproductive efficiency of cows and the profit potential of dairy Farms, uterine infections are difficult to prevent especially during calving (3). Herbal remedies used in folk medicine

provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help overcomes the growing problem of resistance and also the toxicity of the currently available commercial antibiotics (4). *Tribulus terrestris* is an annual plant belonging to the family zygophllaceae, its summer growing medicinal plant now has worldwide distributions which include warm regions of Asia, Africa, Europe, America and Australia. It used in folk medicine as tonic, aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic, lithon-triptic and urinary anti-infective Also used for the treatment of cardiovascular disease (5; 6).

Material and methods

1- Sample Collection: 200 swabs collected from the genital tract (cervix and uterus) of infertile cows, in the Abu-Ghraib and its adjacent area. After restraining the animal and securing its tail, the perineal region

cleared by washing and disinfectant. After that preparation tool for swabs obtained using a sterile double guarded swabs device which inserted through the vagina into the lumen of the cervical canal, guided by

palpation per rectum. The inner rod of the catheter was then pushed forward to expose the swab and rotated four times against the mucosa. While moving the swab backward and forward, and then withdraw within the catheter under rectal guidance. All swabs transported to the laboratory in the appropriated media for bacteriology at 4°C, within 3-18 hours and according to (7).

2- Bacterial isolation: According to (8) Specimens for bacterial cultures were transferred from genital tract of cows into the transport media by aseptic swabbing. Each cervical and uterus swabs cultivated on Nutrient agar and Blood agar media aerobically and Microaerophilic at 37°C to promote growth of bacteria. Then transferred on Eosin methylene blue agar, MacConkey agar and Mannitol salt agar, isolation and identification of bacteria based upon Gram's staining colonial morphology biochemical tests and EPI 20 E for diagnosis of gram-negative bacteria.

3- Antibiotics susceptibility test: According to (9) plates were prepared with Muller – Hinton Agar, 3-4- similar colonies were selected to prepare pure culture, these colonies were transferred into about 5 ml of nutrient broth incubated at 37°C for 2-8 hrs until light to moderate turbidity develops, turbidity adjusted by using MacFarland tube to (1.5×10^8) cfu/ml Dipasterile non toxic cotton swab on a wooden applicator into the standardized inoculums and rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid, streak the surface of the plate with the swab three times, allow the inoculums to dry for 5-15 minutes with lid in place, antibiotics discs which include (Amikacin, Amoxicillin, Amoxicillin and , Clavulanic acid,

Cefotaxime, Ceftriaxone, Gentamycin, Nalidix acid, Nitrofurantoin, Norfloxacin and Tetracyclin) these disc were fixed by using aseptic technique and then incubated immediately at 37°C and examine after 24 C°, the complete inhibition zone was recorded and compared with standard tables.

4- Preparation of alcoholic extract of Tribulus terrestris: It was done according to (10), Fifty grams of the fruit of tribulus terrestris powder were extracted with 500 mL of 70% ethanol under continuous stirring for eight hours at room temperature, the suspension was filtered by Whatman no. 0.22 filter paper and the filtrate was concentrated using vacuum rotary evaporator. The crud extract was stored in dark sterile screw bottle at 4 C° until use to prepare the required concentration.

5- Detection of Active compounds of tribulus terrestris L: Which including A- Tannins B- Flavonoids C- Saponins D- Alkaloids E- Terepenes and steroids according to (11)

6- Antibacterial activity of alcoholic extract of tribulus terrestris in vitro: Used agar – well diffusion method according to (12), by inoculation of nutrient agar with (0.1) ml bacterial suspension which was spread by the sterile spreader, wells with diameter (6Mm) made on the surface of the nutrient agar plate using a sterile cork borer, (0.1) ml of different concentrations(100, 50, 25, 12.5, 6.25, 3, 1.5, 0.75 mg/ml)., were poured in the wells , one well used as a control and filled with (0.1)ml of sterile distilled water, the plates were incubated up down at 37°C for (24) hours, the activity of the extract limited by measurement of inhibition zone in (Mm) around each well.

Result and discussion

Biochemical tests result: The isolates can be biochemically distinguished from the unknown species and confirm the specific characteristics of each bacterial isolate. Furthermore, tables (1) and (2) showed the results of some biochemical tests diagnostic

and differential to isolated bacteria. It has been possible to develop a practical laboratory test based on the characterized biochemical activities and can be devised a diagnostic strategy for the specified species of a bacteria.

Table (1) Biochemical tests result for gram negative isolated bacteria.

Types of isolate bacteria	Lactose fermentation macConkey	Motility	Indol	Citrate	urease	Methyl red	Voges	TSI			Oxidase
								Gas	H ₂ S	reaction	
<i>Escherichia coli</i>	LF	+	+	-	-	+	-	+	-	A/A	-
<i>Pseudomonas aeruginosa</i>	NLF	+	-	+	V	-	-	-	-	ALK/ALK	+
<i>Providencia rettgeri</i>	NLF	+	+	+	+	+	-	+	-	A/ALK	-
<i>Citrobacter braakii</i>	NLF	+	-	+	+	+	-	+	+	A/A	-
<i>Shewanella putrefaciens</i>	NLF	-	-	-	-	+	-	+	+	A/A	-

V = variable reaction between species, NLF = no lactose fermentation, - = negative, + = positive, a = acid, Alk= Alkaine reaction.

Table (2) The result of biochemical tests for gram positive isolate bacteria.

Isolate bacteria	Mannitol salt agar	Motility	Catalase	Coagulase	Gelatin liquification
<i>Staphylococcus aureus</i>	+	-	+	+	+

The results of biochemical tests by using Api-20E system: The Api-20E system is one of the most important diagnostic tests

and more accurate diagnosis of most of the gram negative bacteria as show in table (3).

Table (3): Api-20E system for diagnosis of gram- negative bacteria.

Api-20E	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>providencia rettgeri</i>	<i>citrobacter braakii</i>	<i>Shewanella putrefaciens</i>
ONPC	-	-	-	-	-
ADH	+	-	-	+	-
LDC	-	+	-	+	-
ODC	-	+	-	-	+
CIT	+	-	+	+	-
H2S	-	-	-	+	+
URE	-	-	+	+	-
TDA	-	-	+	+	-
IND	-	+	+	-	-
VP	-	-	-	+	-
GEL	+	-	-	+	+
GLU	-	+	+	+	-
MAN	-	+	+	+	-
NO	-	-	+	-	-
SOR	-	+	-	+	-
RHA	-	+	+	+	-
SAC	-	+	-	-	-
MEL	-	+	-	-	-
AMY	-	-	-	+	-
ARA	-	+	-	+	-
OX	-	-	-	-	-

The results of bacterial isolation: The results appear isolation of different bacteria from genital tract of infertile cows 195 swabs origin of 200 swabs (97.5%) and 5 swab (2.5%) have no-growth. The samples give positive results for isolation include 117 single isolates (58.5%), 78 mixed isolates (39%). The bacterial isolates including five genera of gram negative bacteria

which are *Escherichia coli* represent the highest percentage of bacterial isolate (41.8%), *Pseudomonas aeruginosa* (17%), while *Citrobacter braakii* and *Providencia rettgeri* have the same ratio (1.7%) also *Shewanella putrefaciens* (0.9%) and one gram positive bacteria which is *Staphylococcus aureus* in a percentage of (36.8%) as shown in the table (4).

Table (4) Species numbers and percentage of single bacterial isolates from genital tract of infertile cows.

Types of bacteria isolates	number of isolated bacteria 200	percent%
<i>Escherichia coli</i>	49	41.8
<i>Staphylococcus aureus</i>	43	36.8
<i>pseudomonas aeruginosa</i>	20	17
<i>Citrobacter braakii</i>	2	1.7
<i>Providencia rettgeri</i>	2	1.7
<i>Shewanella putrefaciens</i>	1	0.9
Total	117	99.9%

In mixed isolation, (*Staphylococcus aureus* + *Escherichia coli*) isolated in highest percentage 53.8 %, followed by (*E. coli* + *Pseudomonas aeruginosa*) 11.5% , (*Escherichia coli* + *Pseudomonas aeruginosa* & *Staphylococcus aureus*) 10%. (*Staphylococcus aureus* + *Pseudomonas aeruginosa*) 6.4%, (*Escherichia coli* + *Citrobacter braakii*) 5.1%, (*Escherichia coli* + *Providencia rettgeri*) 3.8 %, while the lowest

percentage were 1.3 % for each of (*Citrobacter braakii* + *Pseudomonas aeruginosa*), (*Providencia rettgeri* + *Staphylococcus aureus*), (*Citrobacter braakii* + *Staphylococcus aureus* + *Pseudomonas aeruginosa*), (*Escherichia coli* + *Staphylococcus aureus* + *Citrobacter braakii*) and (*Escherichia coli* + *Citrobacter braakii* + *Pseudomonas aeruginosa*); as shown in the tables (5).

Table (5) Species Numbers and percentage of mixed bacterial isolates from genital tract of infertile cows.

Types of bacteria isolation	number of isolated bacteria 200	percent %
<i>Staphylococcus aureus</i> + <i>Escherichia coli</i>	42	53.8
<i>Escherichia coli</i> + <i>Pseudomonas aeruginosa</i>	9	11.5
<i>Escherichia coli</i> + <i>Pseudomonas aeruginosa</i> + <i>Staphylococcus aureus</i>	8	10
<i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i>	5	6.4
<i>Escherichia coli</i> + <i>Citrobacter braakii</i>	4	5.1
<i>Escherichia coli</i> + <i>Providencia rettgeri</i>	3	3.8
<i>Citrobacter braakii</i> + <i>Pseudomonas aeruginosa</i>	2	2.7
<i>Providencia rettgeri</i> + <i>Staphylococcus aureus</i>	2	2.7
<i>Citrobacter braakii</i> + <i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i>	1	1.3
<i>Escherichia coli</i> + <i>Staphylococcus aureus</i> + <i>Citrobacter braakii</i>	1	1.3
<i>Escherichia coli</i> + <i>Citrobacter braakii</i> + <i>Pseudomonas aeruginosa</i>	1	1.3
Total	78	99.9%

Escherichia coli are the most common bacteria isolated from the uterine cavity of healthy cows and cows that develop disease of the uterus. (13; 14) This result agreed to our study, also (15) in Iran (16) isolate *E.coli* in same percentage. The other studies refer to low percentage of isolation (8; 17); In Iraq (18) isolated *E.coli* from cows suffering from inflammation of the endometrium of the uterus (27.4 %) and from vaginal secretions (19.2%). Recently, it was observed that a specific *E. coli* causes uterine disease, which is different from known diarrhoeic or extra-intestinal pathogenic *E. coli*. This specific *E. coli* was named endometrial pathogenic *E. coli* (EnPEC). EnPEC was found to be more adherent and invasive to endometrial cells (13). The second bacteria isolated from genital tract of infertile cows were *Staphylococcus aureus* in a percentage of (36.8%) and this result agreed with (8; 16). And disagreed with (19) because they isolated these bacteria in lower percentage. *Staphylococcus aureus* is pathogens that are part of the normal microflora inhabitants of the skin and mucous membranes, including the genital tract of cow or present in wide range of the environment that bacteria are able to spread hematogenously to the fetoplacental unit and cause abortion with other bacterial pathogens such as *Escherichia coli*, *Bacillus* spp. and *Pseudomonas* spp. (19). The third isolate in percent study was *Pseudomonas aeruginosa* in a percentage of (17%) which were the same percentage same percentage isolated from cases of repeat breeding and uterine infections in dairy cows by (8). While (20) and (16) isolated these bacteria in a higher percentage. Also (20) Isolated *Pseudomonas aeruginosa* from the oviduct and uterus of Iraqi cows. In Iraq (17) isolated the same bacteria from cows suffering from inflammation of the endometrium of the uterus and from vaginal secretions. *Citrobacter braakii* and *Providencia rettgeri* represent the low percentage of isolates

(1.7%) for each one. (21) Able to isolate *Citrobacter* from the uterus of cows and foetal membrane In Iraq *Citrobacter* was isolated from uterus of cows and buffalo by (22) in percentage agreed to the percentage of isolation of this study. Whereas (23) mention *Providencia rettgeri* is consider high potential pathogenicity of uterine infection (endometritis) in bovine followed *Staphylococcus aureus* (Coagulase +) than *Escherichia coli*. *Shewanella putrefaciens* isolated in this study in very low percentage (0.9%) and the pathogenic role of this agent remains largely undefined (24). Non-specific bacteria consider the most common cause of uterine infection and are the pathogenic microorganisms affecting productivity and fertility of cows (25; 26). Pathogenic organisms isolated from an infected uterus are found generally in livestock environments and are capable of infecting other tissues and organs (27). Numerous bacteria in a variety of combinations have been isolated from infected uterus (21). Embryonic mortality has a substantial impact on the fertility of bovine. Most of the embryonic losses occur during the first days after fertilization and during the process of implantation. Non-specific pathogens are mainly bacteria that enter the uterus by ascending infection; uterine pathogens may cause embryonic mortality by changing the uterine environment or by a direct cytolytic effect on the embryo. (28).

The results of antibiotic susceptibility test: Susceptibility test showed that most isolates of bacteria which were isolated from infertile cow resist to most antibiotics used as follows: *Escherichia coli* resistant 100% to Amikacin, Nalidix acid. Amoxicillin. (Amoxicillin and Clavulanic acid), Cefixime, Ceftriaxone and Tetracycline 80%. Nitrofurantoin and Norfloxacin 60%. Gentamycin 50%. *Staphylococcus aureus* resist 100% for each of Amikacin and Nalidix acid. Amoxicillin, Cefixime and Tetracycline

90%.Gentamycin and Norfloxacin 80%. (Amoxicillin and Clavulanic acid), Ceftriaxone and Nitrofurantoin 70%.Pseudomonas aeruginosa resistant 100% to all types of antibiotics with exception to Gentamycin and Nitrofurantoin 90%. Ceftriaxone 80%. Citrobacter braakii resist 100% of Amikacin, Amoxicillin, Cefixime, and Tetracycline. While (Amoxicillin and Clavulanic acid), Ceftriaxone, Nalidix acid, Nitrofurantoin and Norfloxacin

50%; but sensitive to Gentamycin. Providencia retergi resist 100% the most antibiotic except (Amoxicillin and Clavulanic acid), Ceftriaxone and Nitrofurantoin 50%. And sensitive to Norfloxacin. Also Shewanella putrefaciens resist 100% the most antibiotic, Amikacin, Amoxicillin, (Amoxicillin and Clavulanic acid), Cefixime, Gentamycin, Nalidix acid, Norfloxacin and Tetracycline but sensitive to Ceftriaxone and Nitrofurantoin, as show in the table (6).

Table (6): The results of susceptibility test to antibiotics for isolated bacteria from cow infertile.

isolated Bacterial Types	No. of isolated	Amikacin		Amoxicillin		+ Amoxicillin Clavulanic acid		Cefixime		Ceftriaxone		Gentamicin		Nalidix acid		Nitrofurantoin		Norfloxacin		Oxytetracycline	
		No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance	No. of Resistance isolated	% of Resistance
<i>E.coli</i>	10	10	100%	10	100%	9	90%	8	80%	8	80%	5	50%	10	100%	6	60%	6	60%	8	80%
<i>Staphylococcus aureus</i>	10	10	100%	9	90%	7	70%	9	90%	7	70%	8	80%	10	100%	7	70%	8	80%	9	90%
<i>pseudomonas aeruginosa</i>	10	10	100%	10	100%	10	100%	10	100%	8	80%	9	90%	10	100%	9	90%	10	100%	10	100%
<i>Citrobacter braakii</i>	2	2	100%	2	100%	1	50%	2	100%	1	50%	0	0%	1	50%	1	50%	1	50%	2	100%
<i>providencia retergi</i>	2	2	100%	2	100%	1	50%	2	100%	1	50%	2	100%	2	100%	1	50%	0	0%	2	100%
<i>Shewanella putrefaciens</i>	1	1	100%	1	100%	1	100%	1	100%	0	0%	1	100%	1	100%	0	0%	1	100%	1	100%

Application of antibiotics in veterinary medicine for more than 50 years had a major impact on microbial communities, resulting in an enrichment of bacterial populations which were resistant to, or developed or acquired resistance to, the antibiotics used; therefore it's essential to know the susceptibility and resistance of antimicrobial agents available for the principal pathogens isolated from the uterus. (29; 30). Gentamicin is now widely used as the treatment of choice for many serious Gram-negative infections, so increase in resistance was specific and nonspecific; nonspecific mean the some of the bacterial enzymes are unaffected due to inactivate gentamicin (31). The recent studies refer that bacterial resistance to aminoglycosides including amikacin are the most resistance to the action of bacterial modifying enzymes such as in *E.coli* (32). These results of the current study agree with (33) who that mention the most antimicrobial multidrug-resistant *E. coli* was isolated from uterine dairy cows. Oxytetracycline was already widely used for the treatment of uterine infection in dairy

cattle and as prophylaxis after caesarian section, whereas Oxytetracycline is widely used for the treatment of uterine infection in dairy cattle, there was evidence of resistance in most of the bacteria (27). Added amoxicillin has been largely used in veterinary medicine. All forms of livestock around the world are treated with it, alone or in combination with clavulanic acid, determined the antibacterial resistance of *E. coli* and *staphylococcus spp.*; also all *Pseudomonas aeruginosa* isolates were resistant to amoxicillin-clavulanic acid the same of most antimicrobial used in current study (34).

The results of antimicrobial activity of *Tribulus terrestris*: The result shows that *tribulus terrestris* extracts of different concentrations were able to inhibit the growth of isolated microorganisms from genital tract of infertile cow which include *Escherichia coli*, *Pseudomonas aeruginosa*, *Citrobacter braakii*, *Providencia rettergi* and *Shewanella putrefaciens* and *Staphylococcus aureus* as show in the table (7).

Table (6) The result of plant susceptibility test

<i>Isolated bacteria</i>	Different concentration of alcoholic extract of the fruit <i>tribulus terrestris</i>							
	Inhibition of zone(mm)							
	100	50	25	12.5	6.25	3	1.5	0.75
<i>Escherichia coli</i>	35	31	27	23	20	16	13	11
<i>Staphylococcus aureus</i>	30	26	23	18.5	15	11	9	6
<i>Pseudomonas aeruginosa</i>	23.5	21	18	15	11	8	5	2
<i>Citrobacter braakii</i>	37	34	31	27	23	22	18	13
<i>Providencia rettergi</i>	32	27	22	19	16	12	8	5
<i>Shewanella putrefaciens</i>	29	24	21	18	16	12	7	4

The activity of the plant against both Gram positive and Gram-negative bacteria may be indicative to the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant, in addition to the plant (fruits) content of pharmacological active metabolites like saponins (35) Saponins have been reported

to possess a wide range of biological activities. The toxicity of Saponins to insects (insecticidal activity), parasite worms (anthelmintic activity), and their antifungal, antiviral, and antibacterial activities are well documented (36). The mode of action of antibacterial effects of saponins seems to involve membranolytic properties, rather

than simply altering the surface tension of the extracellular medium, thus being influenced by microbial population density (37; 38). Flavonoids are known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (39). More

lipophilic flavonoids may also disrupt microbial membranes (40). Alkaloids isolated from plant are commonly found to have antimicrobial properties (41). Berberine and harmane are important representatives of the alkaloid group. The mechanism of action of berberine and harmane is attributed to their ability to intercalate with DNA (42).

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تأثير المستخلص الكحولي لنبات القطب للبكتريا المعزولة من القناة التناسلية للابقار العقيمة

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الخلاصة

جمعت مائتي مسحة من الجهاز التناسلي للابقار العقيمة خلال الفترة بين 2012/1/7 الى 2012/6/30. زرعت البكتريا المعزولة على اكار الدم ، اكار المعدي، اكار الماكونكي واكار المانيتول الملحي. 195 مسحة تمثل (97.5%) أعطت نتيجة إيجابية و 5 مسحات (2.5%) أعطت نتيجة سلبية. من البكتريا الموجبة للزرع 78 عزلة (39%) كانت اصابات بكتيرية مختلطة. كل العزلات البكتيرية المعزولة ميزت باستخدام اختبارات الكيموحيوية المختلفة ونظام API-20E، العزلات البكتيرية المفردة في هذه الدراسة كانت *Esherishia coli* 41.8%، *Staphylococcus aureus* 36.8%، *Pseudomonas aeruginosa* 17%، *Citrobacter braakii* 1.7%، *Providencia rettergi* 1.7% و *Shewanella putrefaciens* 0.9%. بالنسبة الى العزلات البكتيرية المختلطة كانت اعلى نسبة 53.8% *Staphylococcus + Esherishia coli aureus* وأقل نسبة كانت 1.3% *Pseudomonas aeruginosa + Citrobacter braakii* اختبرت الحساسية للجراثيم المعزولة وتم استخدام 10 أنواع من الأقراص المفردة للمضادات الحيوية الأكثر استخداما، أغلب الجراثيم المعزولة كانت مقاومة للمضادات الحيوية (Cefixime, Nitrofurantoin, Ceftriaxone, Nalidix acid, Amoxicillin, Amikacin (Tetracycline, Norfloxacin, (Amoxicillin and Clavulanic acid), Gentamycin). من جهة اخرى نلاحظ حساسيتها للمستخلص الكحولي لنبات القطب (الثمار) للتراكيز المختلفة.