ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* FROM DOG'S FECES AND STUDY OF THE CHARACTERISTIC PROPERTIES OF IT'S PRODUCED ANTIMICROBIAL AGENT

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ABSTRACT

Escherichia coli was isolated from 56 dog fecal sample (69.14%) out of 81 collected samples. The antibacterial activity of *E. coli* isolate was primarily and secondarily screened on sold and liquid media. The results of primary screening showed that 56 isolates had antibacterial activity against tested gram positive and negative standard strains. By secondary screening *E. coli* isolate was able to produce antimicrobial agent in fermentation media at different incubation conditions, but larger amount of produced antimicrobial agent was obtained at 37C^{o}, 24h, 180rpm by used seed and fermentation media. Different methods were used to determine the purity and to characterize the produced antimicrobial agent physically, chemically and biologically. The results of purity test revealed that the produced antimicrobial agent appeared as pure compound with only one spot in paper chromatography. The result of physical, chemical and biological characterization showed that the produced antimicrobial agent was peptide material with low toxicity 2350mg\kg body weight.

INTRODUCTION

Escherichia coli is a genetically heterogeneous group of bacteria whose members are typically nonpathogens that are a part of the normal microflora of the intestinal tract of humans and animals [(1)], Although most strains are harmless [(2)], while others can make both animals and people sick[(3)], certain subsets of this bacterial species have acquired genes that enable them to cause intestinal or extraintestinal disease[(1)]. Newborns have a sterile alimentary tract which within two days becomes colonized with E. coli [(4)]. However, *E. coli* is the head of the large bacterial family, Enterobacteriaceae, they are among the most important bacteria medically[(5)], *E. coli* have able to produce many useful biomaterials like vitamin K[(6)] and other affect on microorganisms, like colicins[(7)], microcins[(8)] and methylenomycin[(9)]. On other hand *E. coli* bacteria was used in the study of biological properties in most other organisms. At

least the presumed universality of the central dogma and the flow of genetic information were encapsulated by Jacques Monods famous statement that "what was true for *E. coli* would also be true for the elephant"[10]. More than 700 serotypes of *E. coli* have been identified [4]. We choice the dog in this study, because of the carnivorous containing enteric bacteria more than herbivorous[11].

The aim of the present study is isolate *E. coli* and determined it's ability to produce an antimicrobial agent and to extract the produced antimicrobial agent in vitro and study of it's chemical, physical and biological properties.

MATERIAL AND METHODS

Samples collection: eighty one samples were collected randomly from dog's rectums by sterile cotton swabs[12].

Bacterial isolation: A MacConkey agar were inoculated by the collected swabs, and incubated at 37C^{o} for 24 hours. The representative colonies from the MacConkey agar were transformed to Eosin Methylene Blue agar and incubated at same condition, to observe the cultural characteristics[13 and14].

Biochemical tests: A lope full was taken from growing colony, inoculated into 10 ml nutrient broth then incubated at 37C^{o} for 24 h. to be used in identification of *E. coli* as, gram staining, indol production, methyl red test, voges-proskaur test,citrate utilization, urease activity, catalase production and motility, according to[15;16;17;18 and19].

Primary screening: Antimicrobial agent production in sold medium by *E. coli* isolate, against *Staph. aureus* (NCTC6571), *Bacillus subtlus*, *E. coli* (NCTC5933), *Klebsiella spp* was carried out according to[20].

Secondary screening: *E. coli* isolate in fermentation media was performed in two ways to determine antimicrobial agent production ability of isolated *E. coli* and the best production conditions.

1) **Direct method**. Three 500ml erlenmyer flasks containing 100ml nutrient broth as fermentation medium were inoculated with 2cm^{2} of nutrient agar block containing *E. coli* isolate, one flask was incubated at 37C^{o} for 24 hours in shaker incubator at 180 rpm[12]. Other two flask were incubated at same condition for 36 and 48 h respectively.
2) **Indirect method:** erlenmyer flasks (250ml) containing 50ml nutrient broth were used as seed medium, inoculated with *E. coli* isolate and incubated at 37C^{o} for 24h at 180rpm to obtain a good biomass, then four 500ml erlenmyer flasks containing 100ml nutrient broth inoculated with 3ml seed medium and incubated at 37C^{o} for 24, 36 and 48h at 180 rpm. Standard bacterial strain, *Staph. aureus* (NCTC6571), *Bacillus*

subtilis, *E. coli* (NCTC5933), *Klebsiella spp* were used to determined the ability of isolated *E. coli* to produce antimicrobial agent in fermentation medium according to[21].

Extraction and Purification of produced antimicrobial agent: Method of charcoal adsorption which described by[22] was used to Extract and Purify the produced antimicrobial agent.

Purity test: paper chromatography (PC) technique were used to determine the antimicrobial agent purity[23].

Identification: Methods described by[24 and25] were used to determine the active groups (color tests), which include, ninhydrin reaction, biuret's reaction, molish test and determination of double bond. While the milting point, infra red spectrum, ultra violet spectrum and the solubility in different solvents (distal water, ethanol, petroleum ether, butyl acetate and benzene) were carried out according to[26 and27].

Biological properties:

1) Determination of antimicrobial agent activity: Paper disk agar diffusion method described by[28] were used to determine the antimicrobial agent activity against standard bacteria strain.

2) Determination of lethal dose $_{50}$: The LD_{50} of extracted antimicrobial agent was studied with male mice type (Albino), which were divided into 5 groups (8 mice fore each), one group of them used as control. The control group of mice were injected intraperitoneally with 0.25 ml distal water while other groups of mice were injected intraperitoneally with 0.25 ml antimicrobial agent solution in different concentration (1000, 2000, 3000 and 4000) mg/kg, all mice were examined for 3 days to observe any behavior exchange or death. The results, were analyzed according to probate analysis method described by[29].

RESULTS AND DISCUSSION

Bacterial isolation and identification: *E. coli* was isolated from 56 fecal samples out of a total 81 collected samples, after 24h. incubation, the growing bacterial colonies appeared as round small, bright pink on MacConkey agar. While on Eosin Methylene Blue agar they appeared as small round colonies with green metallic sheen, these colonies characteristics has been similar to that describe by[13 and30], gram negative bacteria, the biochemical tests used in identification of *E. coli* showed in table (1).

Table (1): Biochemical tests of isolated *E. coli*

Test	Result
Indol production	+
Methyl red	+
Voges-proskaur test	-
Citrate utilization	-
Catalase	+
Motility	+
Urease	-

Screening of antimicrobial agent production: in primary screening, twenty three isolates showed antibacterial activity against the tested organisms expressed as a zones of growth inhibition (table, 2),this result was in line with[31 and32], who recorded the ability of *E. coli* isolated from camel's and goat's rumen to produce bioactive material in sold media which inhibite the *Staph. aureus* and *Strept.* sp.. The same table showed the ability of isolated *E. coli* to produce antimicrobial agent in fermentation media in the secondary screening of antimicrobial agent prodction, these result inagrement with[12], who reported the ability of *E. coli* isolated from alimentary tract to produced antimicrobial agent in fermentation media.

Table (2) primary and secondary screening of antimicrobial agent production.

Test organisms		*Staph. aureus* (NCTC6571)	*Bacillus subtilis*	*E. coli* (NCTC5933)	*Klebsiella spp*
Primary screening	I Z mm^{sn}	18^{11}, 17^{4}, 15^{8}	16^{13}, 15^{10}	20^{9}, 19^{6}, 17^{8}	20^{1}, 18^{7}, 17^{15}
Secondary screening	IZ mm	19	16	24	22

IZ= inhibition zon, mm= millimeter, sn= samples number

Extraction: the antimicrobial agent produced by *E. coli* was extracted from fermentation media as yellowish crystal in different amount according to incubation condition (table, 3).

Table (3): Amount of antimicrobial agent production

Method	Incubation conditions	Antimicrobial agent g\L
Direct	37C^{o}, 24h., 180rpm	1.6
	37C^{o}, 36h., 180rpm	1.1
	37C^{o}, 48h., 180rpm	0.5
In direct	37C^{o}, 24h., 180rpm	2.95
	37C^{o}, 36h., 180rpm	2.26
	37C^{o}, 48h., 180rpm	1.4

The amount of antimicrobial agent produced at 24h was larger than that produced at 36 and 48h (direct method), this result may be due to the antimicrobial agent synthesis and production induced when cultures cease exponential growth[33] and cell stop growing, because of exhaustion of glucose, ammonia or phosphate, like expression of the microcin B17 operon[34], therefore the antimicrobial agent rise to the maximum production at 24h, these result inagremente with[8 and33], they reported that the optimum production condition of extra cellular microcin at 37C^{o} in 24h incubation period. After the 24h the antimicrobial agent production decrease, because of accumulation of other metabolic and west product, which lead to death of bacteria (decline phase)[35].

In case of indirect method, the fermentation medium inoculated with active and good biomass of bacteria from seed medium, and the antimicrobial agent production begin as the cell enter the stationary phase in rich medium[34], therefore it had enough time to produced large quantity of antimicrobial agent.

Purity test: the produced antimicrobial agent appeared as a pure compound with only one spot in paper chromatography, upon exposure to 350nm ultra violate lamp blue bright was revealed, with 5.6 mm R_f value in BAW solvent system, that mean there was only one pure molecule extracted[36].

Identification: color tests (active group) for the produced antimicrobial agent were showed in table (4), milting point were recorded at 200-203C^{o} with sharp range. The antimicrobial agent was soluble in distal water, ethyl acetate and acetic acid; slightly soluble in methanol and ethanol; non soluble in petroleum ether 40-60; butanol and n-butanol.

Table (4) color tests for produced antimicrobial agent

Test	Result	Notes
ninhydrin reaction	(+) ve: blue colour was appareid	Amino acid or peptide chain present
biuret's reaction	(+)ve: violate colour was appeared	Peptide or protein found
molish test	(–)ve: violate ring did not appear	No carbohydrate found
determination of double bond	(–)ve: purple colour was stayed	No C=C bond

The infra red absorption spectrum illustrated in figure (1) and the active groups recorded in table (5), figure (2) were showed the ultra violet absorption spectrum, there are high absorption value at (210 nm) beside that other absorption value at (270 nm), all results above indicated that the produced antimicrobial agent was a peptide[37], the present results inagrement with[8 and12], who reported the ability of fecal *E. coli* isolate to produced peptide antimicrobial agent in fermentation media.

figure (1): Infra red absorption spectrum of produced antimicrobial agent

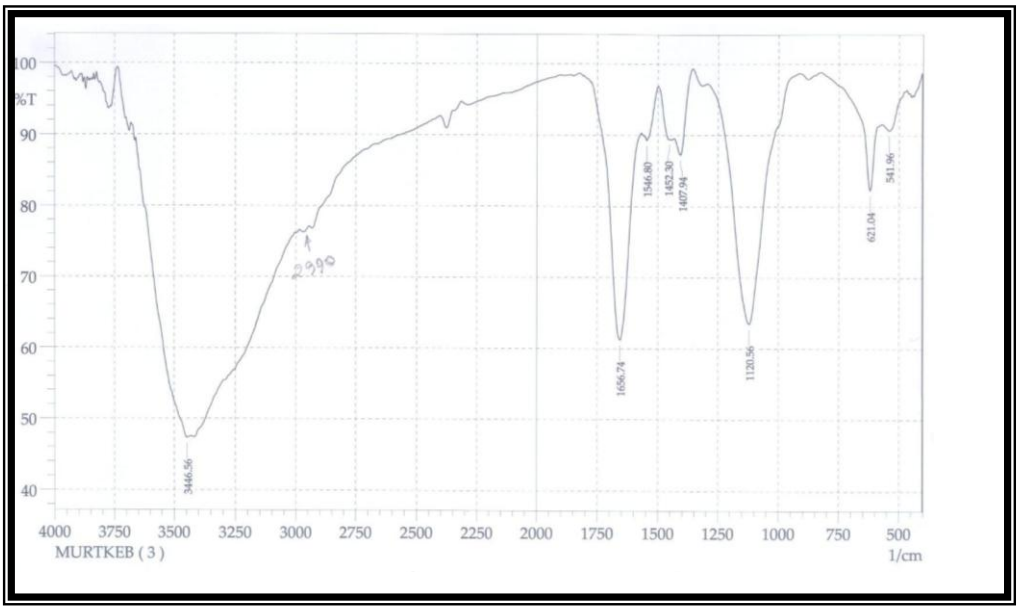
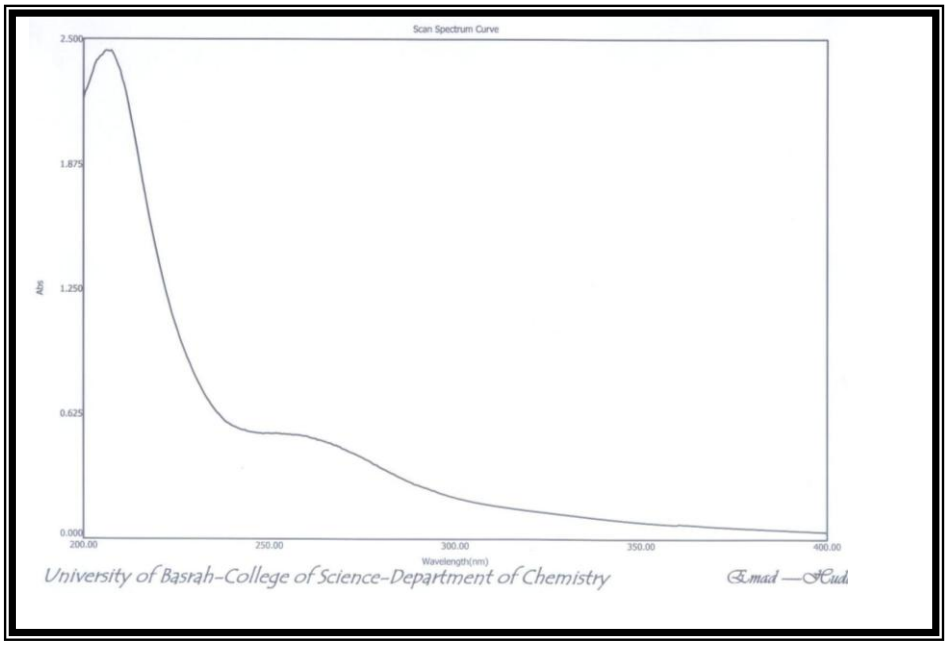


Table (5): important active groups of produced antimicrobial agent

Band Frequency (cm^{-1})	Remarks	Functional group
3446	sterch	$-NH_2$ primary amine and aminde
2990	Antisym & sym.strech	$-CH_3$ & $-CH_2$ aliphatic compound
1656	Strech	C=O primary amides
1407	Strech	C-N primary amides
1120	strech	C-NH_2 primary aliphatic amines

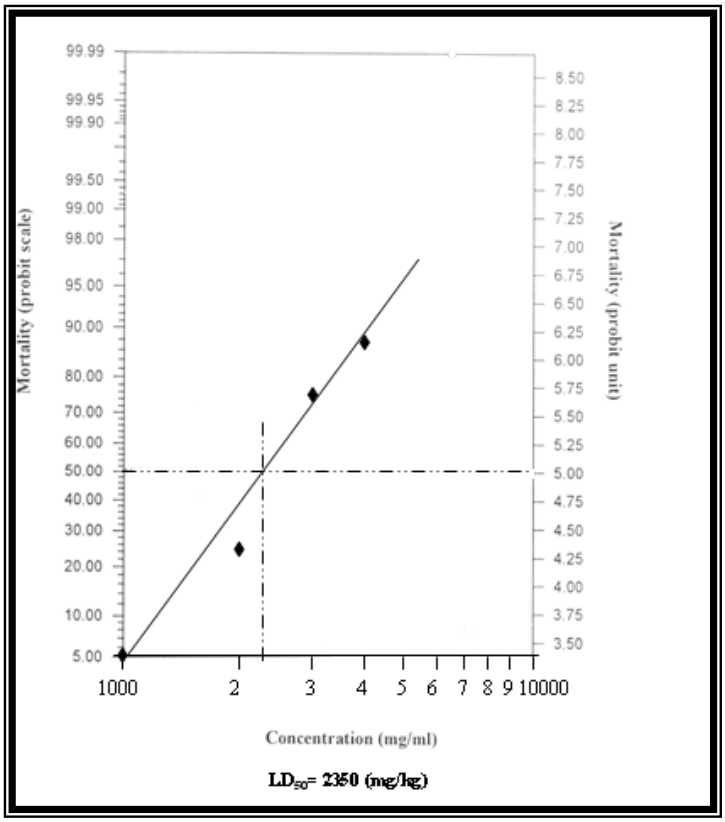
Fig(2): Ultra violet absorption spectrum



Biological properties: figure (3) showed the LD_{50} according to the probability paper and statistic test, which calculated at the 2350 mg/kg body weight, the presented result indicated that the produced antimicrobial agent had low toxicity[37], these result inline with[12], who

reported that the antimicrobial agent produced by intestinal and rumen isolated *E. coli* had low toxicity.

Fig(3): LD_{50} for produced antimicrobial agent



Conclusion: the dog's fecal *E. coli* isolates showed antibacterial activity against gram positive and gram negative bacteria.

عزل وتوصيف جرثومـــة الـ *Escherichia coli* من براز الكلاب ودراسة الصفات المميزة للمضاد الحيوي المنتج منها

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الخلاصـــة

عزلت جرثومة الـ *Escherichia coli* من 56 عينة من براز 81 كلب (69.14%). تم الكشف عن الفعالية الضد مايكروبية لهذه العزلات بواسطة الغربلة الاولية والثانوية على اوساط صلبة وسائلة. أظهرت نتائج الغربلة الاولية بان بعض عزلات *E. coli* لها فعالية ضدمايكروبية تجاه العترات القياسية من الجراثيم الموجبة والسالبة لصبغة كرام المفحوصة. وبواسطة الغربلة الثانوية ظهر بان عزلة الـ *E. coli* كانت قادرة على انتاج مضاد حيوي في وسط التخمر

وباستخدام طرق حضن مختلفة، حيث تم الحصول على اكبر كمية انتاج للمضاد الحيوي المنتج (2.95 غرام\لتر) باستخدام طريقة الوسط التخمري ووسط الاستنبات في ظروف نمو بلغت فيها درجة الحرارة 37م° ومدة حضن 24 ساعة وسرعة دوران 180 دورة\دقيقة . استخدمت عدة طرق للكشف عن النقاوه وتوصيف المضاد الحيوي المنتج فيزيائيا، كيميائيا وبايولوجيا، أظهرت نتائج اختبار النقاوة بان المضاد الحيوي المنتج ظهر بشكل مركب نقي على شكل نقطة واحدة على ورقة الكروماتوغرافية الورقية. وبينت نتائج التوصيف الفيزيائية، الكيميائية والبايولوجية بان المضاد الحيوي المنتج كان مادة ببتيدية ذات سمية واطئة بلغت 2350ملغرام\كيلوغرام من وزن الجسم.

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