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Characterization and antibacterial activity of purified microcin produced by *Klebsiella pneumoniae* K15

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Abstract

This study has interested with the characterization and antibacterial activity of purified microcin of *Klebsiella pneumoniae*. Isolates were collected and identified by morphological characteristics and biochemical tests of API 20E system. The diagnosis was confirmed by detection of 16S r RNA gene. Fifty-six (56%) of *K. pneumoniae* isolates were microcin producing, The majority of producer isolates are urine origin. Optimum conditions for production were in brain heart infusion broth with 5% Glycerol, pH 7, 37 °C / 24 hrs. The efficient producer isolate of *K. pneumoniae* (K15) was chosen for microcin extraction and purification, inhibition zone ≥16mm. Antimicrobial susceptibility test was done for microcin producing isolates. No correlation was noticed between antibiotics resistance and microcin production. Extraction and Purification of microcin by method based on retention of the protein lying on hydrophobic medium silica gel C60, eluted by methanol/water gradient from 20% - 80% concentration. Protein concentration 80 µg/ml and specific activity 1U/µg. Final purification was done by HPLC. Molecular weight was determined by SDS-PAGE; 8-9 KDa. Amino acid analysis by PITC, amino acids as following : (Asp), (Asn), (Glu), (Gln), (Ser), (Gly), (His), (Arg), (Thr), (Ala), (Pro), (Tyr), (Val), (Met), (Cys), (Trp), (Ile), (Leu), (Phe) at concentration of: 23.81, 53.44, 19.78, 17.39, 16.82, 17.73, 15.92, 15.16, 45.67, 31.17, 29.78, 28.90, 18.39, 28.29, 27.85, 22.31, 17.81, 30.47, 24.53 µg/ml respectively. Antibacterial activity of microcin(40 &80 µg/ml) against 12 local isolates were examined. In conclusion, local clinical K15 produce microcin exhibit inhibitory action on other pathogenic bacteria.

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Introduction

Microcins are gene-encoded small molecular weight (<10 kiloDalton) peptides formed by Enterobacteriaceae (Duquesne *et al.*, 2007). They are secreted under conditions of nutrient depletion through dedicated ATP-binding cassette (ABC) exporters system and exert strong inhibitory action towards strictly correlated bacteria.

Microcins are presently divided into two classes (I ,II) based on their molecular masses and posttranslational modifications: Class I [microcins B17, and J25 are encoded by plasmid(below 5kDa) that undergo extensive backbone post-translational modifications. Class II microcins include peptides with higher-molecular-mass, in the 5–10kDa range, and is itself further subdivided into 2 subclasses: Class IIa assembles 3 plasmid-encoded peptides devoid of posttranslational modifications (L, V and N). Class IIb - contains peptide encoded by chromosome that can hold or not a C-terminal modification (E492, M and H47) (Etayash *et al.*, 2015).

Lately microcins improved their capability to operate as narrow-spectrum materials to restrict the increase of Enteric bacteria through intestinal inflammation by administrating *(E. coli* Nissle 1917), a probiotic initially isolated from the human intestinal tract, a competitive benefit(Sassone-Corsi*et al.*, 2016). That is the earliest indication of the environmental function that microcins might act in the gut that aids in discovering new treatment methods (Garcia-Gutierrez *et al.*, 2018).

Microcins are manufactured by ribosomes and contribute a universal maturation course,then translated from particular mRNAs as pioneer peptides containing an N-terminal leader peptide expansion and a interior peptide, which undergo further post translational modifications before appearance the adult peptide(Duquesne *et al.* , 2007;Severinov*et al.*, 2007). Those molecules transported via (ATP-binding cassette) ABC transporter-methods or efflux pumps determined by their genetic (McIntosh *et al.*, 2009). The current study aimed to characterize the purified microcin of *K. pneumoniae* and to investigate its antibacterial activity.

Materials and methods

Bacterial isolates

One hundred isolates of *Klebsiella spp.* were collected from different hospitals at Baghdad city during the period from Feb to May 2017, from different clinical sources: urine, sputum, wounds, burns, fluids, stool and blood samples. Isolates have been identified according to cultural characteristics, microscopic examination and biochemical testes. API 20E System was used to confirm identification as well as16s r RNA gene was detected.

Antimicr\obial sensitivity

Isolates were screened for antimicrobial susceptibility towards nine different antibiotics; amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamycin, piperacillin, tetracycline, aztreonam and Imipenem depending on CLSI (2016) criteria.

Detection of microcin producing isolates

All isolates were examined for their ability to produce the microcinproteins *.E. coli* was used as indicator isolate. Cup assay method was used according to Al-Qassab and Al-Khafaji (1992) for detection of microcin production.

Extraction and purification of microcin

Potent producer isolate –K15-had been selected for microcin extraction according to the diameter of inhibition zone and productive stability. Microcin was extracted from the supernatant of bacterial culture according to De Lorenzo (1984) as the following with some modifications:

The producer isolate was grown in 5 test tubes each one contains 5ml of M9 minimal broth supplemented with sodium citrate as carbon source and incubated at 37 °C for 24 hrs. Following incubation period, bacterial suspension was added to 500 ml of M9 minimal broth medium and incubated in shaking

incubator at 37 °C for 48 hrs. Culture supernatant was separated from bacterial cells by centrifugation (10.000× g , 15 min, 4 °C), the supernatant collected in sterile flask then concentrated by dialysis tube (0-3000 Da cut off).

This concentrated supernatant passed through column (Height 18 cm×1.5cm diameter) packed with Silica gel Sep Pak C60 (Waters) on which the microcin was retained.

Elution with 95% methanol, 4 solution of methanol gradient was prepared:

The fractions collected in sterile test tubes to estimate the activity(in any solution the peptide eluted).

The peptide was concentrated by evaporation in a boiling water bath without a significant loss of its activity.

HPLC-analysis

Consequently active fractions were chromatographed lying on RP with a Waters HPLC scheme using a Hibar LiChrosorb RP18 column (Merck) and a gradient elution method methanol/ water with 0.5% TEAP buffer at pH 3.5 (Hancock and Sparrow, 1981). Amino acid analysis of purified microcin by Phenyl isothiocyanate (PITC) has been done.

Determination of microcin activity

The antibiotic power of extracted microcin was measured by the critical dilution routine (Mayr-Harting *et al.*, 1972).

A series of the sample dilutions have been prepared (usually a two-fold series has been used).

By using a sterile cork borer (5mm), wells on the surface of nutrient agar have been seeded with an inoculum of the sensitive indicator isolate have been made (3 wells for each plate).

One hundred µl from each dilution placed on the well and incubated at 37 °C for 24 hrs.

After incubation period, examination of the degree of inhibition due to each drop, and the choice of an arbitrary end-point was performed (usually the last dilution showing complete inhibition of the indicator strain).

Activity of microcinwas articulated as random units per ml where an unit was the least quantity of microcin that give a distinctregion of growth inhibition of indicator isolate in the assay plate. Microcinactivity represent the last dilution showing complete inhibition of the indicator isolate.

Specific activity

It was calculated by dividing activity unit (U) with the protein concentration as follows:

Specific activity (U/µg)= Microcin activity (U) / Protein concentration µg/ml.

Determination of protein concentration

Protein concentration was measured according to the method described by Bradford (1976), which depends on the binding of Goomassie brilliant blue G-250 stain with protein leading to the appearance of blue color, by returning to a standard curve of Bovine Serum Albumine (BSA) .

Antibacterial activity of purified microcin

Antibacterial activity of purified microcin had been examined against 12 bacterial isolates from different clinical sources using well method according to Gupta *et al.*, (1998) as follows:

Each isolate was grown in nutrient broth for 2-3 hrs and incubated at 37°C (to obtain 10^6 -10^7cell/ml).

After incubation period, a volume of 0.1 ml of each clinical isolate was spread on the surface of nutrient agar and plates were allowed to dry at 37 °C for 10 min.

Using a sterile cork borer, 5mm in diameter wells were made in previous agar layer (3 wells per plate).The agar discs were removed, 0.1 ml of purified peptide was poured to every hole.

Plates were incubated at 37 °C for 18 hrs. and appearance of inhibition zones was observed.

Results and discussion

Results of API 20E system showed that all isolates were belonged to *K. pneumoniae* species. Final identification of *K. pneumoniae* isolates was depending on the using of species-specific primers of *K. pneumoniae* where all the bacterial isolates identified to species level depending on a monoplex-PCR reaction with primer of 16SrRNA gene.

Table 1. Methanol gradient solutions.

Solution 1	3 ml Distill water: 12ml methanol
Solution 2	6 ml Distill water: 9ml methanol
Solution 3	9 ml Distill water: 6ml methanol
Solution 4	12 ml Distill water: 3ml methanol

The results showed that all bacterial isolates harbored 16SrRNA gene(Fig. 1). All bacterial isolates gave a positive result and the PCR products have been confirmed by comparing its molecular weight (bp) with 100bp DNA Ladder:

Microcin producing isolates

As shown in table- 2, the results revealed that 56 (56%) out of 100 *K. pneumoniae* isolates, were microcin producers. Fourty-six (64.28%) isolates were from urine.

Whereas the percentage of microcin producing isolates in the present study was close to that reported by Al Charrach *et al.*, (2011) who mentioned that 62.5% of *Klebsiella* isolates which isolated from environmental and clinical samples were able to produce bacteriocin on solid medium.

Table 2. Numbers and percentages of microcin-producing isolates.

Type of clinical specimens	No. of microcin- producing isolate (%)
Urine	36 (64.28%)
Blood	7 (12.5%)
Stool	5 (8.92%)
Sputum	4 (7.14%)
Burn Swabs	2 (3.57%)
Wound Swabs	1 (1.78%)
Ascitic Fluids	1 (1.78%)
Total	56 (100%)

This percentage was higher than what reported by Lafta(2010) who found that the rate of bacteriocin producing isolates of *K. pneumoniae* was 34.78%. These differences may be related to the fact that the detection of bacteriocin-producers has been shown to be influenced by the sensitivity of the indicator strains to themicrocin, the method used and the origin ofmicrocin-producer (Ozdemir*et al.*, 2011).

Table 3. The Peaks of the RP-HPLC analysis.

Peak	Retenation time min.	High	High%
1	10.396	937	8.681
2	15.853	9460	87.607
3	20.789	401	3.712
Total		10799	100.00

The determination of the productivity of isolates depends mainly on the indicator bacterial isolates and Detection of 56 isolates producer out of 100 does not mean that all isolates are not producer; it may be producer but did not show its productivity due to the absence of specific receptors for a microcin on their surfaces.

The tested isolates varied in their productivity between strong, moderate and weak producer.

Table 4. Amino acids and their concentrations (µg/ ml).

No.	Amino acids	Concentration µg/ ml
1	Aspartic acid (Asp)	23.81
2	Aspargine (Asn)	53.44
3	Glutamic acid (Glu)	19.78
4	Glutamine (Gln)	17.39
5	Serine (Ser)	16.82
6	Glycine(Gly)	17.73
7	Histadine(His)	15.92
8	Arginine(Arg)	15.16
9	Threonine(Thr)	45.67
10	Alanine(Ala)	31.17
11	Proline(Pro)	29.78
12	Tyrosine(tyr)	28.90
13	Valine(Val),	18.39
14	Methionine(Met)	28.29
15	Cystine(Cys)	27.85
16	Tryptophan(Trp)	22.31
17	Isoeucine(Ile)	17.81
18	Leucine(Leu)	30.47
19	Phenyl alanine(Phe)	24.53

Antimicrobial Susceptibility Test

Results of antimicrobials susceptibility test showed that all producer *K. pneumonia* isolates exhibited high resistance rate towards the used antibiotics where the isolates had the highest level of resistance 82.14% to piperacillin followed by (66.1%) to ceftazidium, in contrast the lowest resistance rate of *K. pneumoniae* isolates was 0% to imipenem followed by ciprofloxacin that had 26.78% as shown in Fig. 2.

By observing the resistance rate of the producer bacterial isolates it is clear that the property of antibiotic resistance is independent on microcins productivity.

Table 5. The antibacterial activity of purified microcin.

Bacterial Isolate	Source of Isolation	Diameter of inhibition zone mm at concentration	
		40 µg/ml	80 µg/ml
E. coli	Urine	11	22
Klebsiella pneumoniae	Ascitic Fluid	10	18
Enterobacter sakazaki	Blood	11	24
Serratia marcescence	Blood	8	15
Proteus mirabilis	Urine	11	18
Shigelladesentariae	Stool	0	0
Salmonella typhi	Stool	9	20
Staphylococcus aureus	Wound	13	25
Streptococcus pyogenes	Throat swab	11	19
Streptococcus pneumoniae	Sputum	8	15
Acinetobacterbaumanii	Blood	0	0
Pseudomonas aeruginosa	Ear swab	0	0

Selection of efficient producing isolate

Microcin extraction and purification was performed according to DeLorenzo, (1984). K15 was chosen as highly producer isolate, it was grown in minimal medium (M9 medium) provided with citrate as a carbon source in order to continuing extraction and purification of microcins. The microcin activity was easily detectable in the bacterial supernatants of the producer isolate liquid cultures that obtained after centrifugation and removing the cells, where the resulting extract was have activity 40 unit/ml and protein concentration 30 µg/ml.

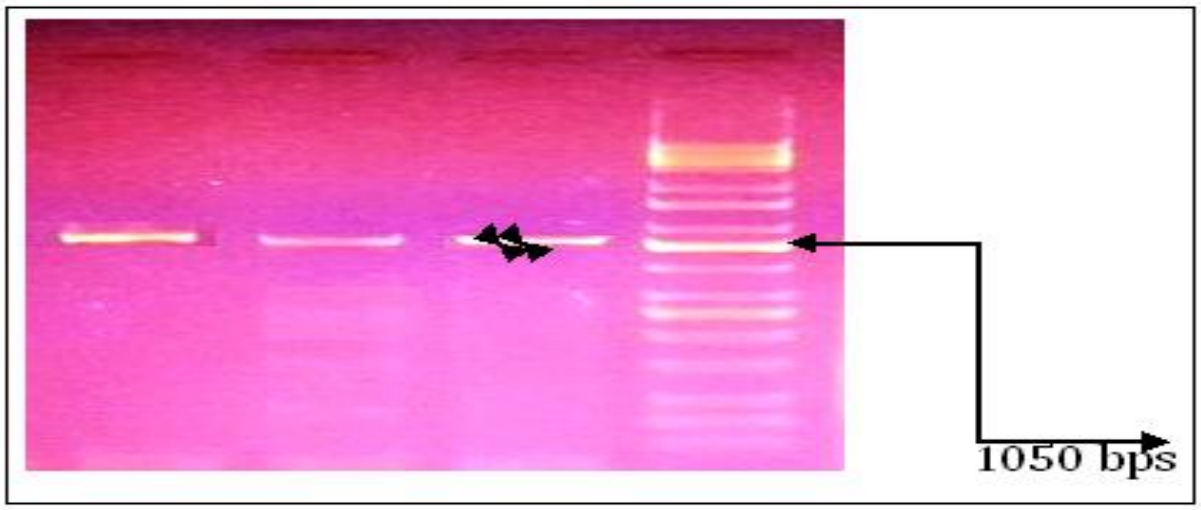

Fig. 1. The geneticdetection of16SrRNA gene, agarose gel electrophoresis (1% agarose, 7v/cm² for 60 min) for 16s rRNAgene.

The microcin was retained on the hydrophobic silica gel 60°C column then it eluted by methanol/water gradient from 20% to 80% concentration. It is found that the microcin eluted between 40% and 60% methanol concentrations, that is mean it has a hydrophobic character and it methanol soluble. It is well known that all known microcinsare soluble in organic solvents (Kolter&Moreno, 1992).

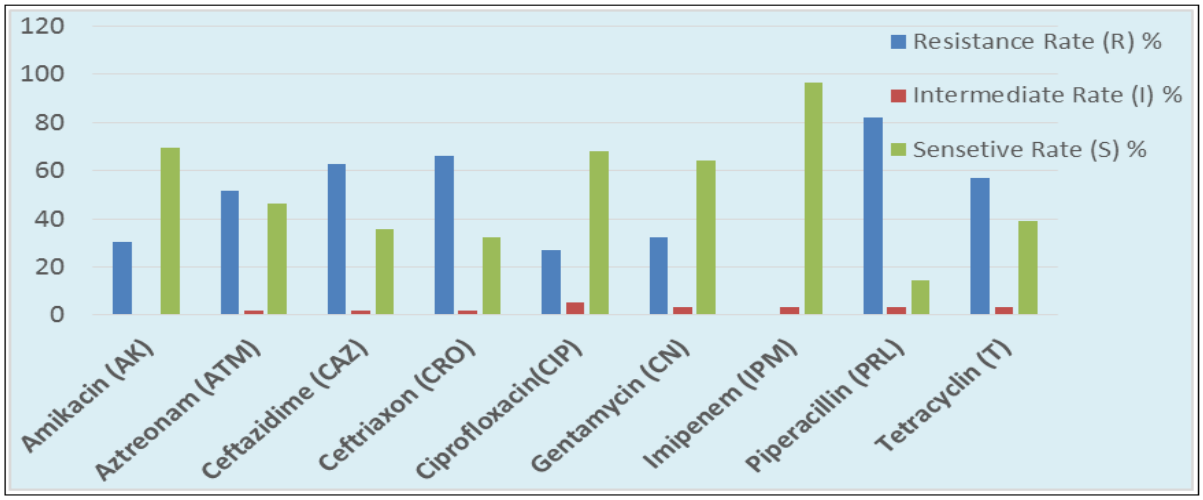

Fig. 2. Antimicrobial susceptibility for microcin-producing isolates.

The resulted semi-purified preparations of microcin, that have activity of 80 unit/ml and protein concentration 80µg/ml, were used as a starting material for HPLC analysis.

Final purification of the semi- purified peptide was done by RP- HPLC with purification system consist of methanol/water with 0.5% TEAP buffer at pH 3.5, and the result revealed that the purity of 87% as

shown in figure (3) and table (3). The adding to the solvent system of 0.5% TEAP buffer pH 3.5 was crucial to pick up active microcin by HPLC.

The microcin E492 was extracted from the supernatant of cultures of the producer strain *K. pneuminiae* RYC492 grown in citrate medium by passage of this supernatant through a cartridge of Sep Pak Cl8 (Waters) on which the microcin was retained. The peptide was eluted with 95% methanol (Lagos,*et al.*,1993), then purified by RP-HPLC, sodium dodecyl sulfate- polyacrylamide gel.

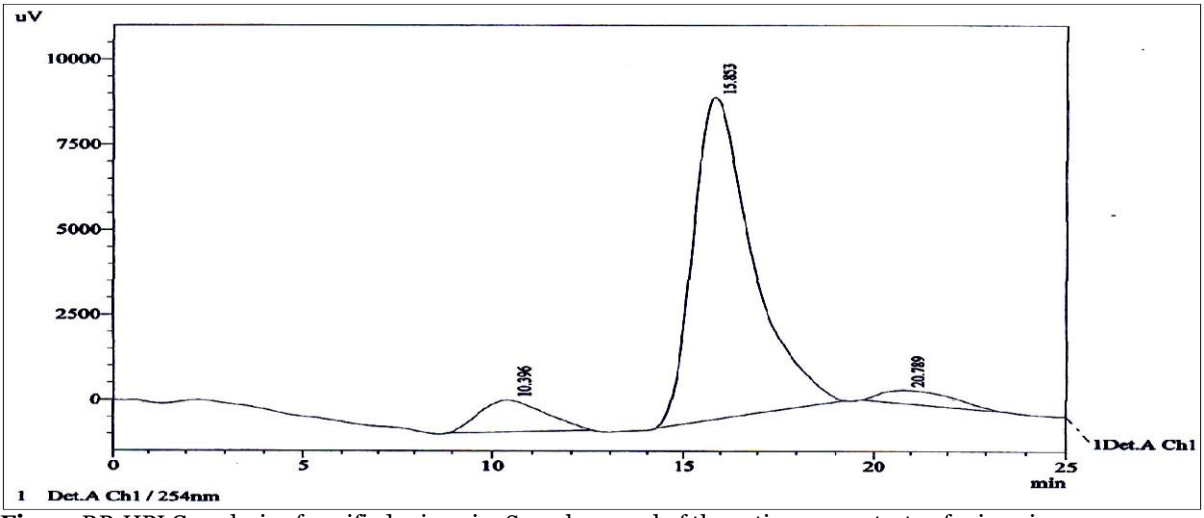

Fig. 3. RP-HPLC analysis of purified microcin, Sample = 20µl of the active concentrate of microcin.

Amino acids analysis by RP-HPLC after Derivatization with Phenyl isothiocyanate (PITC)

The resulted purified microcin was exposed to amino acids analysis by reverse-phase HPLC using pre-column derivatization reagent Phenyl isothiocyanate (PITC) as shown in table 4and Fig. (4).

Electrophoresis (SDS-PAGE)

The resulted purified microcin was electrophoresed under denaturized conditions by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and the result of SDS-PAGE showed a single band depending on staining with Goomassei brilliant blue demonstrating that the peptide was purified until homogeneity.

The molecular weight of the peptide was determined below 10 kilo Dalton depending on a comparison of the standard marker proteins available as there were no standard marker proteins below 10 Kda as shown in Fig.5.

Gaillard-Gendron *et al.*, (2000) separated the purified microcin L on a one-dimensional SDS-PAGE and after electrophoresis, gels were stained by silver stain. The electrophoresis analysis of the purified microcin L revealed a single silver-stainable protein of about 6 kDa.

The antibacterial activities of purified microcin were studied against the pathogenic bacteria.

The antibacterial activity of purified microcin with two concentrations: 40 and 80µg/ml (Table 5) showed that the purified microcin was able to inhibit the growth of nine isolates with different degrees.

The highest microcin activity was against *Staphylococcus aureus* where the diameters of inhibition zone were 13 and 25 mm at concentration of 40 and 80 µg/ml respectively followed by *Enterobacter sakazaki*;11 and 24mm, *E coli* ;11and 22mm, *Salmonella typhyi* 9 and 20 mm, *Streptococcus pyogenes*;11and 19mm, *Proteus mirabilis* 11 and 18mm, *K. pneumoniae*11 and 18mm, *Streptococcus pneumonia* 9 and 17mm and *Serratia marcescence* 8 and 15mmat concentration of 40and 80 µg/ml respectively.

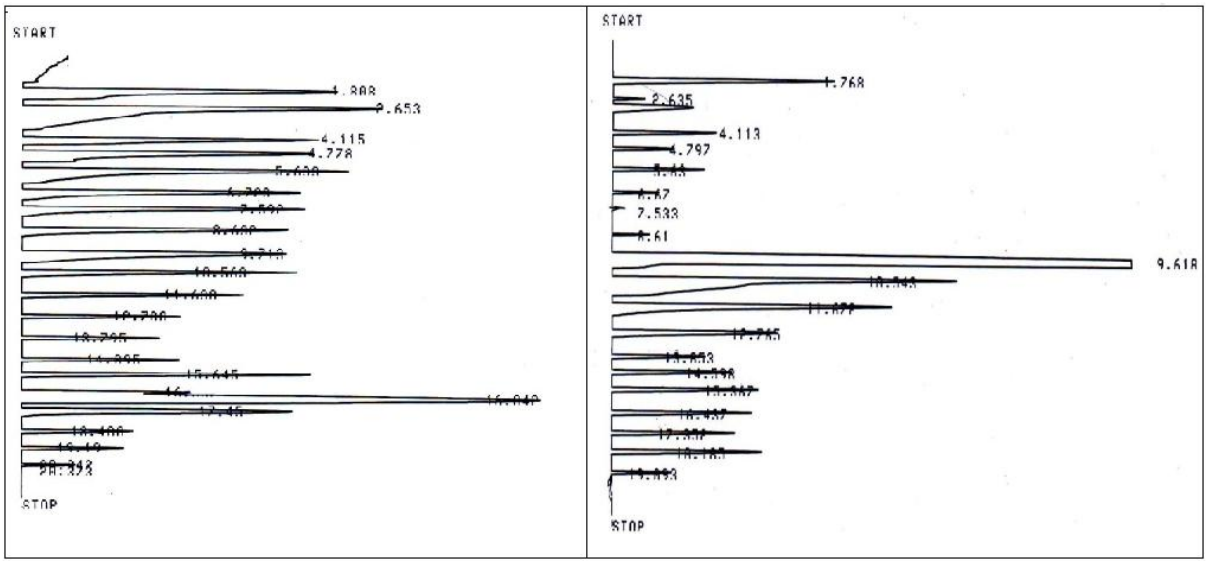

Fig. 4. RP-HPLC analysis of purified microcinusing pre-column derivatization reagent Phenyl isothiocyanate (PITC), Sample = 20µl of the active concentrate of microcin. Conditions of elution: Flow rate= 1ml/ min; detection at 254 nm; fractions of I ml. The elution formed between two solvents: solvent A 5% methanol in 0.1 N sodium acetate buffers (pH=7), solvent B methanol linear gradient from 0-20%.0.5% TEAP buffer pH 3.5.

On the other hand, three isolates are not affected by the action of the purified peptide include: *Acinetobacter baumanii*, *Shigella desentariae* and *Pseudomonas aeruginosa*.

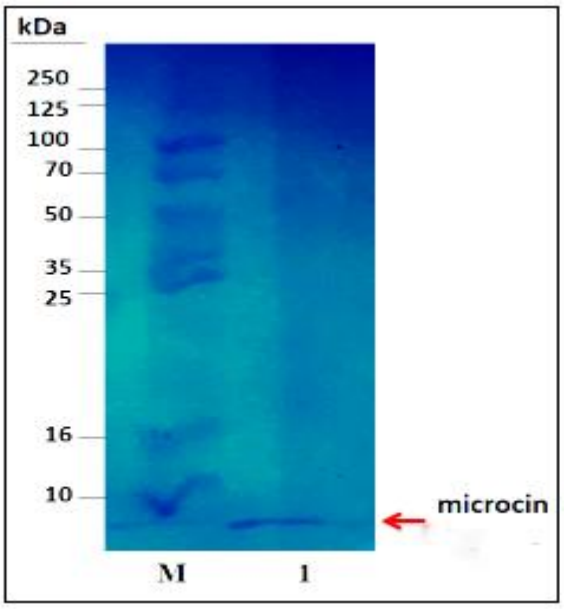

Fig. 5. One dimensional SDS-PAGE of purified microcin.

Microcins exhibit receptor-mediate ways of antibacterial action, that what exposed in their small minimal inhibitory concentrations that are found in the nanomolar range, it also clarify the narrow spectrum of activity frequently revealed by microcins, which are active against bacterial strains extremely analogous to the producing bacterial strains (Rebuffat, 2013).

The extensive variation of microcin structures lead to diversity of mechanisms of action, including the nuclease , like DNase and RNase functions, the pore-forming type, and inhibitors of protein synthesis or DNA replication, the inhibition of vital enzymatic functions and damage to the inner membrane (Duquesne *et al.*, 2007; Rebuffat, 2013).

Acuña and his colleagues incorporated enterocin CRL35 and microcin V genes to achieve a protein called Ent35-MccV. Ent35-MccV has lethal effectnext to clinically isolated *E. coli* (enterohemorrhagic type) and *L.monocytogenes*, also, several Gram-positive and negative bacteria, which are not clinically isolated, a method can fold new or multi-functional bacteriocins, which are further powerful in functionality and germicidal range. As a result, they can be widely used in medicine food and animal husbandry (Acuña *et al.*, 2012).

With the rising concerns in the technical and healthiness community over growing levels of antibiotic resistance, antimicrobial peptide

bacteriocins have emerged as hopeful substitute to usual small molecule antibiotics. Despite the fact that a large number of bacteriocins have been reported, only a few have been completely characterized and structurally elucidated (Etayash *et al.*, 2015).

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