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Biochemical and molecular characterization of Klebsiella species isolated from neoplastic patients under Irradiation therapy

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***Abstract*---**Neoplastic radiation therapy was able to alter the microbial flora of patients' bodies, as well as the microbial flora of radiation-exposed workers, and provided control for possible infectious consequences. *Klebsiella aerogenes* was a non-fermentative aerobic gram negative bacteria that was widely distributed in nature, requiring species identification in up to 25% of cases in the lab. Antibiotic susceptibility of *Klebsiella* species multi-resistant isolates is common, and all isolates were characterized in all cases of clinically significant infections caused by these organisms. Only 15 isolates showed significant antimicrobial activities against at least one of the tested indicator bacteria. Vancomycin is an anti-*Klebsiella* species antibiotic to which resistance is rarely seen, their use should be reserved for the treatment of species infections resistant to all other antibiotics, the emergence of widespread antibiotic-resistant bacteria enhanced the need for new sources of antimicrobial agents, and

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isolate SHM4-1 was active against all of them, with IC60 values of 3.3 and 1.1 g/ml against HCT-116 and HepG-2 cell lines, respectively. It was genotypically identified as a species with the PKS 4 biosynthetic gene cluster present. To enhance the production, mannitol, ammonium sulphate, pH 7, 2% inoculum size, and 2 days at 37°C were utilized. This permitted separation of one active antimicrobial molecule to homogeneity using HPLC, and NMR structure elucidation revealed that this chemical was a carbapenemase derivative.

Keywords---*Enterobacter*, antimicrobial, anticancer, carbapenemase, *Klebsiella* sp., *Klebsiella aerogenes*.

Introduction

Patients' microbial flora has always been thought to be mostly gram positive, but it also contains a large number of gram negative bacteria, which could be a source of the rising number of infections caused by these latter species now observed in general hospitals (Stratford et al., 2011). Periodic blood cultures to determine the existence of these strains of the host immune system are critical, as is infection control in patients who are immunological weakened (Highet et al. 2010). There has been a significant increase in colonisation of members of the Enterobacteriaceae family, *Klebsiella* species such as *K. fineumoniae, K. yiieumctiiae*, and *E.coli*. These trends are likely secondary to an increase in colonisation, particularly in males, because high moisture content promotes the growth of these organisms. Gram negative bacteria proportions in overall populations were calculated. Gram negative bacteria can be identified from virtually all anatomical location samples when leukemia patients carry them on their body organs (Jawetz et al. 2010). Due to the synthesis of several important bioactive metabolites, *Enterobacter*, particularly *Klebsiella* sp., has gotten a lot of interest around the world. A total of 60 *Enterobacter* were isolated from the microbial flora of patients' bodies in this study (Kingsbury, D.T. 2012).

Because immune insufficiency is known to occur in many patients with leukemia, all patients had been sampled before to taking any antibiotics or other disease-related medication. (Fu et al. 2017) discovered that gram negative bacteria colonised 22 of 24 transplantation patients in the gastrointestinal colon system, urinary tract infection, and skin microbial flora. Gram negative bacteria colonised the microbial flora of patients in six out of seven cases of severe illness. Those who work in environments with high temperature and humidity develop microbial flora favoured by these factors, such as gram negative organisms likely as gram positive Streptococcus pyogenes, and those who work in environments with low temperature and humidity develop microbial flora favoured by these factors, such as gram negative organisms likely as gram positive *Streptococcus pyogenes*. The need for species identification of clinically significant *Klebsiella* sp. (K.) must be emphasised because hospital workers have been shown to harbour more pathogenic organisms as transient organisms, which may become established as resident flora if these persons are exposed continuously (Katz, S. I. 2010).

Gram-negative bacteria with a non-fermentative aerobic morphology are known as *Enterobacter*. They are members of the Enterobacteriaceae family and have a complicated life cycle (Dilip et al., 2013). They are extensively spread in habitats, particularly in hospitals, where they contribute to biomaterial recycling by degrading complex structures in dead human bodies, animals, and fungi (Williams and Goodfellow, 2005). *Enterobacter*, particularly *Klebsiella* sp., are renowned for producing a variety of biologically active chemicals that are used as antibacterial, antivirals, immunomodulatory, anti-cancer medicines, and enzyme inhibitors in a variety of sectors, including medicine (Sacramento et al., 2004; Atta, 2009; Fukuchi et al., 2009; Olano et al., 2009; Ser et al., 2015). Numerous researchers are discovering antimicrobials from many natural resources, such as those produced by Enterobacter, especially those isolated from many undiscoverable or poorly investigated habitats, as a result of the advent of multi-resistant microbes to practically all current antibiotics (Undabarrena et al., 2016).

Many metabolic processes, mostly organized by polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS), create antimicrobials (Undabarrena et al., 2016). Antibacterials have a high incidence and presence of these biosynthesis genes (Donadio et al., 2007). Therefore, this study aimed to figure out the biochemical and molecular characterization of *Klebsiella* species isolated from neoplastic patients under irradiation therapy

Materials and Methods

Collection of samples and isolation of enterobacter

Using swabs, samples were taken aseptically from four separate areas in human body flora patients from the upper and lower parts of the body and delivered to the laboratory for additional isolation stages. The dilution plate technique (Williams et al., 2005) was used to isolate Enterobacter on nitrate agar (NA) and tryptone soya agar (TSA) supplemented with cefobid (10 g/ml) and Nystatin (50 g/ml) to block any bacterial or fungal contamination. In a nutshell, 1 g of each sample was diluted in 9 ml of 0.9 % saline, homogenized, and then serially diluted up to 10^4 %. A 100 L mixture of 10^2, 10^3, and 10^4 dilutions was spread on NA and TSA and incubated for 3 days at 37°C. *Enterobacter* colonies were morphologically described (Aghamirian and Ghiasian, 2009; Reddy et al., 2011), isolated using the streak plate method, and kept at 80°C in 40 % glycerol.

Screening of enterobacter for their antimicrobial activities

Antimicrobial activities of pure isolates were determined by agar diffusion method (Williams and Davies, 2005) and double layer agar method (modified spot on lawn technique) (Thakur et al., 2007; Dundar et al., 2015), against different indicator microorganisms; *Escherichia coli* (ATTC 8739), *Streptococci pyogenes* (ATTC 6546), *Enterobacter cloacae* (ATCC 13047), *Enterobacter dissolvens* (ATCC 23373), *Lipophlic diphtheroids* (clinical sample), *Klebsiella sp.* (clinical sample), and *Serratia marcescens* (environmental sample). In double layer agar method, all pure isolates were spot inoculated on NA and incubated at 37°C for 3 days and then 5 ml of molten TSA seeded with 100 µl overnight culture of indicator

microorganisms were poured on spotted plates and incubated at 37°C for 24 h. Antimicrobial activities represented in zones of inhibitions were examined.

Antibiotic susceptibility testing

According to the National Committee for Clinical Laboratory Standards methodology, the Vitek 2 system (BioMerieux) was used (NCCLS, 2015). Gram positive and Gram negative antimicrobials were tested (Vitek system, BioMerieux) Sulfamethoxazole/Trimethoprim (S/T), Tetracyclin (Gm), Nitrofurantoin (N/F), Sulfamethoxazole/Trimethoprim (S/T) (Te). Amoxicillin/clavulanate (A/C), Ciprodiazole (Cd), Gastrobiotic (G), Unictam (Un), Chloramphenicol (C), Clindamycin (Cm), Erythromycin (Em), Tavanic(Tav), Spirex(Sp), Dolabactin (Dol), and Vancomycin (Va) are all antibiotics that *Klebsiella* sp. are innately resistant to (Mukesh, 2015).

The quantity of bacteria in a liquid medium can be assessed visually by comparing the turbidity of the liquid medium to a standard that reflects a known number of bacteria in suspension. Turbidity standards are made by combining compounds that precipitate to generate a turbidity solution that is repeatable. MDR isolates were defined as those that were resistant to three or more antibiotic classes (excluding nitrofurantoin and amoxicillin/clavulanate). McFarland produced barium sulfate-based solutions to estimate the amount of bacteria in solutions with equal turbidity as indicated by colony counts.

Bacterial identification and antibiotic susceptibility testing (AST) BioMerieux minimum inhibitory concentration (MIC)

For the identification of different groups of bacteria, samples from various types of colonies were chosen. The flow charts displaying fundamental identifying factors for bacteria were used to classify the organisms. Bacterial growth and/or morphology on selective or differential media aided the preceding basic parameters for identification. Identification and control of antimicrobial resistance Microbiology Vitek 2. Infections in healthcare, bacterial infections, and diseases transmitted via the body such as urinary tract infections, gastrointestinal colon infections, and skin flora (Heubner & Goldmann, 1999). The BioMerieux API 25 strips can be used to identify Gram negative, non-fermenting rod-shaped bacteria in the range of 30 species.

Fermentation and extraction of secondary metabolites and total proteins

Fermentation was taking place with the strongest *Enterobacter*, which had high antibacterial activity. In a nutshell, *Enterobacter* was subcultured in tryptone soya broth (TSB) for 3 days before being inoculated with 2% of the beginning inoculum in 1 L of International *Klebsiella* Project 2 (IKP4) broth in a 2 L Erlenmeyer flask and incubated at 37°C for 4 days in a rotary shaker incubator (200 RPM). After centrifugation at 13,000 g for 20 minutes, the cell-free supernatant from each flask was collected and split into two parts: one for total secondary metabolites and the other for total proteins. For total metabolite extraction, a 1:1 v/v ethyl acetate solution was added to the cell-free culture supernatant and vigorously shook for 1 hour. A rotary evaporator was used to

separate the organic phase and evaporate it to dryness (Romankova et al., 2011; Selvameenal et al., 2012). Total extract residues were weighed and diluted in 5 mL ethyl acetate before being stored at 4°C in the refrigerator. To extract total proteins, a 40 % concentration of ammonium sulfate was added to the cell-free culture supernatant. Protein was separated by centrifugation at 14,000 g for 30 minutes at 4°C, then dissolved in 5 mL distilled water and stored at 4°C.

Invitro **Anti-tumor Cytotoxicity**

Tissue culture was used to test the cytotoxicity of ethyl acetate extracts and total proteins from the most virulent Enterobacter isolates with substantial antibacterial activity. El-Hussein, oncology Department, Al-Azhar University, Cairo, Egypt, provided HepG2 (hepatocellular carcinoma cell line) and HCT 116 (human colon cancer). The cells were kept at 37°C and 5% CO2 in DMEM media with 10% fetal calf serum, sodium pyruvate, 100 U/ml cefobid, and 100 mg/ml vancomycin until the cytotoxicity bioassay was performed. Skehan et alapproach.'s was used to assess the potential cytotoxicity of four site samples (2010). To allow cells to adhere to the plate wall, 100 cells/well were plated into 96-well dishes overnight before being treated with the investigated chemicals. Each investigated substance was applied to the cell monolayer at different doses (0, 6.25, 12.5, 25, 50, 100 g/ml), with triplicate wells utilized for each dosage. At 37°C and 5% CO2, monolayer cells were treated with the tested agent(s) for 48 hours. The cells were fixed and stained with sulforhodamine B dissolved in acetic acid at the conclusion of the incubation time. The protein-bound dye was extracted using Tris-EDTA buffer after the unbound stain was removed four times with 1 percent acetic acid. An ELISA reader was used to measure the absorbance. The survival curve of each tumor cell line and the IC60 were created by plotting the relationship between surviving fraction and chemical concentration. The survival curve was used to determine the concentration of an agent that induces a 50% growth inhibition for each tested compound using each cell line (Skehan et al., 2010).

Phenotypic Characterization of SHM4-1 Isolate

The physiological, biochemical, and culture properties of SHM4-1, a gifted isolate with extensive antibacterial and cytotoxic activity, were studied in depth. IKP-3, IKP-4, IKP-5, Czapek Dox, Sato, nutritional agar, and NA were used to test the growth potential, production, and colour of the substrate. Visual comparisons with chips from the ISCC-NBS centroid colour charts were used to identify the colour (Williams et al., 2005). According to Williams et al., production of catalase, protease, lipase, amylase, hydrogen sulphide, nitrate reductase, and urease, as well as screening for utilisation of different nitrogen sources (protease peptone, potassium nitrate, and ammonium sulphate), different carbon sources (glucose, sucrose, fructose, and mannitol), and ability to grow (2005).

Genomic DNA Extraction and Purification

According to Sinha et al. (2004) and Aly et al. (2016) with some modifications. Briefly, a 1.5 ml of culture was centrifuged for 10 min at 3,000 *g*, the supernatant was discarded and were suspended in 200 μl spheroblast buffer (10% sucrose, 25

mM Tris pH 8.4, 25 mM EDTA pH 8.0, 2 mg/mL lysozyme and 0.4 mg/ml RNase A), vortexes and incubated at 37°C for 10 min until cell lysis occurred. Then, 50 µl of 5% SDS (lysis buffer 1) and 5 M NaCl (lysis buffer 2) were added, mixed and incubated at 65°C for 5 min. A 100 µl neutralizing buffer (60 ml 5 M Potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml dH_2O) was then added and put on ice for 5 min before centrifugation at 18,000 *g* at 4°C for 15 min. The supernatant (approximately 400 µl) was transferred to a new tube, mixed with equal volume of isopropanol, left 5 min at room temperature and centrifuged at 18,000 *g* at room temperature for 15 min to precipitate the DNA. The resulting was washed with 70% ethanol by centrifugation at 18,000 *g* at room temperature for 5 min. The final pellet was air dried suspended 50 µl 1x TE buffer pH 8 and stored in the refrigerator at 4°C.

According to PCR amplification and sequencing of 16S rRNA Gene, the primers used for amplification of the 16S rRNA gene were 11F: 5'-TAACACATGCAAGTCGAACG-3' (Birri et al., 2013; Hong-Thao et al., 2016) and 12R: 5'-AGGGTTGCGCTCGTTG3' (Stackebrandt and Charfreitag, 2010; Isik et al., 2014). PCR was carried out in 50 µl reaction volume in sterile 200 µl PCR tube. The PCR reaction mixture consisted of 500 ng genomic DNA, 10 mM dNTPs mixture, 1 µl (20 uM of each primer), 2.5 units of Taq DNA polymerase enzyme and 10 µl 5x reaction buffer. The PCR program included template denaturation at 94°C (3 min), followed by 34 cycles of denaturing at 94°C (30 s), annealing at 56°C (30 s), and extension at 72°C (60 s), and followed by completion of DNA synthesis at 72°C (5 min). Primers were removed from the final PCR product prior to sequencing using QIA quick PCR purification kit. The PCR product of interest was detected and purified by agarose gel electrophoresis using 1% (w/v) agarose gels with reference to 1 kbp DNA ladder. DNA was sequenced using the ABI Prism Big Dye terminator sequencing ready reaction kit version 3.1 and analyzed with the ABI Prism 3100 generic analyzer.

Sequence Manipulation and Phylogenetic Analysis

The whole ethyl acetate extract of SHM4-1 was concentrated and chromatographed using high performance liquid chromatography (HPLC) (Dionex Ultimate 3000 model HPLC equipment at the BLAST facility). MEGA7 programme was used to assess multiple sequence alignment and molecular phylogeny (Tamura et al., 2007).

PCR Screening for Antibiotic Biosynthetic Gene Clusters

The genomic DNA of SHM4-1 isolate was screened for the presence of the biosynthetic genes involved in the production of type I polyketide synthase (PKS I), type II polyketide synthase (PKS II), NRPS and glycopeptide antibiotics. This was achieved by PCR amplification of these genes using the following primers; PKS/K1 F: 5′-TSAAGTCSAACATCCGBCA-3′ and PKS/M6 R: 5 ′ -CGCAGGTTSCSGTACCAGTA-3 ′ to amplify the PKS I gene with expected product size of 1200–1400 bp (Passari et al., 2015), ARO-PKS-F: 5 ′ -GGCAGCGGITTCGGC GGITTCCAG-3 ′ and ARO-PKS-R: 5 ′ -CGITGTTIACIGCG TAGAACCAGGCG-3 ′ to amplify the PKS II gene with expected product size of 492–630 bp (Wood et al., 2007), NRPS/A3 F: 5 ′ -

GCSTACSYSATSTACACSTCSGG-3 ′ and NRPS/A7 R: 5 ′ - SASGTCVCCSGTSGCGTAS-3 ′ to amplify the NRPS gene with expected product size of 700 bp (Passari et al., 2015), and finally oxyB, F: 5′-CTGGTCGGCAACCTGATGGAC-3′ and oxyB R:5′-CAGGTACCGGATCAGCTCGTC-3 ′ to amplify the glycopeptide antibiotic gene with expected product size of 696 bp (Wood et al., 2007). The PCR program included template denaturation at 95◦C (5 min), followed by 40 cycles of denaturing at 95°C (30 s), annealing for PKS I, PKS II, NRPS and glycopeptide primers at 55, 64, 59, and 60°C, respectively (60 s), extension at 72°C (2 min), and followed by completion of DNA synthesis at 72°C (10 min) (Baker et al., 2003).

Optimization of antimicrobial production

In order to maximize the production of secondary metabolites by the isolate SHM4-1, the effect of different carbon sources; fructose, glucose, mannitol, starch, and sucrose (20 g/l) in basal nitrate salt medium were studied (Selvin et al., 2009). The effect of different nitrogen sources, ammonium sulfate, protease peptone, and KNO3 (2 g/l) in the basal salt medium were also studied. The most effective carbon and nitrogen sources were further used in different concentrations at (1, 1.5, 2, 2.5, 3 g/100 ml) and (0.1, 0.15, 0.2, 0.25, 0.3 g/100 ml) respectively. The effects of cultural conditions like different incubation time (2–14 days), different starting pH (5, 6, 7, 8, 9, 10, and 11), and different starting inoculum (0.01, 0.1, 2, 5, 10, and 15%) were also examined (Kadiri and Yarla, 2016). The antimicrobial activity assay using cup technique against sensitive indicator *Serratia marcescens*, was managed after each experiment, and zones of inhibitions were measured after incubation of plates at 37°C for 24 h.

Fermentation using optimized conditions and extraction of the antimicrobial compound

To extract antimicrobial compound, a 20 ml of 3 days sub-cultured broth was inoculated in 2 L flasks containing 1 L of liquid mannitol – ammonium sulfate medium (tow flasks). These flasks were incubated in a rotary shaker (160 RPM) at 30°C for 6 days. A 2-L total volume was filtered through Whatman No. 1 filter. After filtration, the total culture filtrate was extracted with ethyl acetate in a ratio of (1:1 v/v) and shaken vigorously in a separating funnel. Then, the organic layer was collected and the solvent extracts were concentrated to dryness using rotary evaporator and tested for their antimicrobial activity against various indicator microorganisms.

Purification by HPLC and spectroscopic characterization

A Nucleosyl C18 column was used. Elution was performed using a flow rate of 3 ml/min of 10–100% acetonitrile in water and a total run time of 25 minutes. A total of 23 fractions, each with a volume of 3 mL, were collected. Fractions were purified, dried, weighed, and dissolved in DMSO before being evaluated for antimicrobial activity on a Serratia marcescens lawn and for minimum inhibitory concentrations against different indicator microbes using the broth micro-dilution technique. The nuclear magnetic resonance (NMR) spectrum was measured at Al-Azhar University's college of pharmacy (El-Mahdy et al., 2015).

Results

Klebsiella species isolated from the research groups were identified as follows: The most common *Klebsiella* sp. was identified as K. *aerogenes* (81 % of all isolates), with 18 cases in the control group, 16 cases in occupationally radiation-exposed personnel, and 15 cases in radiation-treated cancer patients. Other *Klebsiella* sp. species identified were K. *fineumoniae* (8 % of all isolates) and K. *yiieumciiae* (8% of all isolates) (11% of all isolates) Table (1).

Table (1)
Species identification of *Klebsiella* sp. isolated from the study groups

Species (N° of isolates)	N° of cases		
	Control group	Occup. Exposed p.	Cancer patient
K. aerogenes	18	16	15
K. fineumoniae	_	1	3
K. yiieumciiae	2	3	2

Frequency percentage of resistant cases to different antimicrobial in *Klebsiella* sp. Table (2). Vancomycin is an anti-*Klebsiella* species drug to which resistance has already been observed; it should only be used to treat *Klebsiella* sp. infections that have become resistant to all other antibiotics.

Table (2)
Antimicrobial susceptibility testing of *Klebsiella* sp.

Species	Frequency percentage of resistant cases to different antimicrobials.														
	A/C	Sp	Cd	Dol	Cm	G	Tav	Un	Em	C	N/F	Gm	S/T	Te	Va
K. aerogenes	100	50	70	100	55	95	50	65	75	90	30	40	50	45	0
K. fineumoniae	100	70	85	100	65	100	55	95	50	90	15	65	85	50	0
K. yiieumciiae	100	65	95	100	60	100	60	90	90	95	25	60	70	70	0

Table (3)
Percent incidence of microorganisms at the four sample sites of Radiation-treated cancer patients

Sample sites	Percent incidence of microorganisms (cancer patient).					
	K. aerogenes	*K. fineumoniae*	*K. yiieumciiae*	*E. coli*	*Lipophilic diphtheroid*	*Streptococci*
Gastrointestinal colon tract	35	30	35	40	100	5
Urinary tract infection	20	100	10	40	60	30
Upper skin flora	20	75	25	100	35	5
Lower skin flora	20	100	15	45	65	25

Percent incidence of microorganisms at the four sample sites of Radiation-treated cancer patients in Table (3). Lipophilic *diphtheroids* (100%) were the most common bacteria in the GI colon tract, followed by both K. *fineumoniae* (30%) and K. *yiieumciiae* (35%), E. *coli* (40%), K. *aerogenes* (35%), and *Streptococci* (5%), as shown in Figure 1 (A,B,C, D and E).

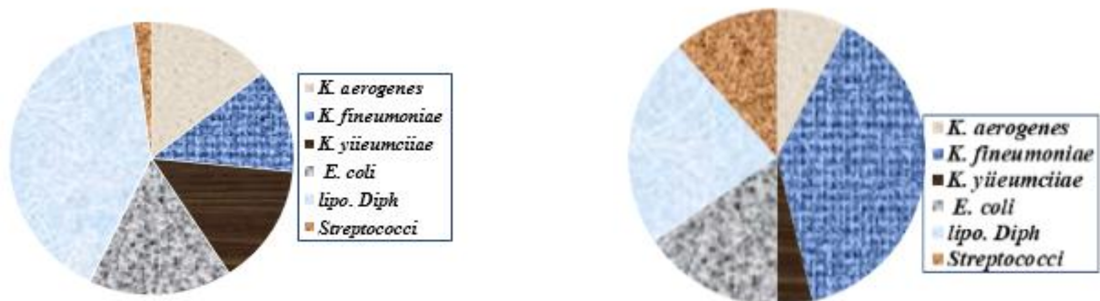


Fig.1 (A &B): Percent incidence of microorganisms at the Gastrointestinal Colon and urinary tract of radiation-treated cancer patient

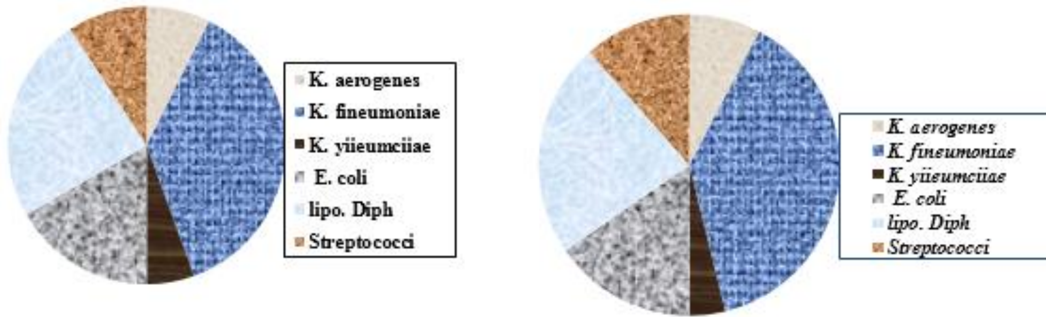


Fig.1 (C & D): Percent incidence of microorganisms at the upper and lower skin flora of radiation - treated cancer patient

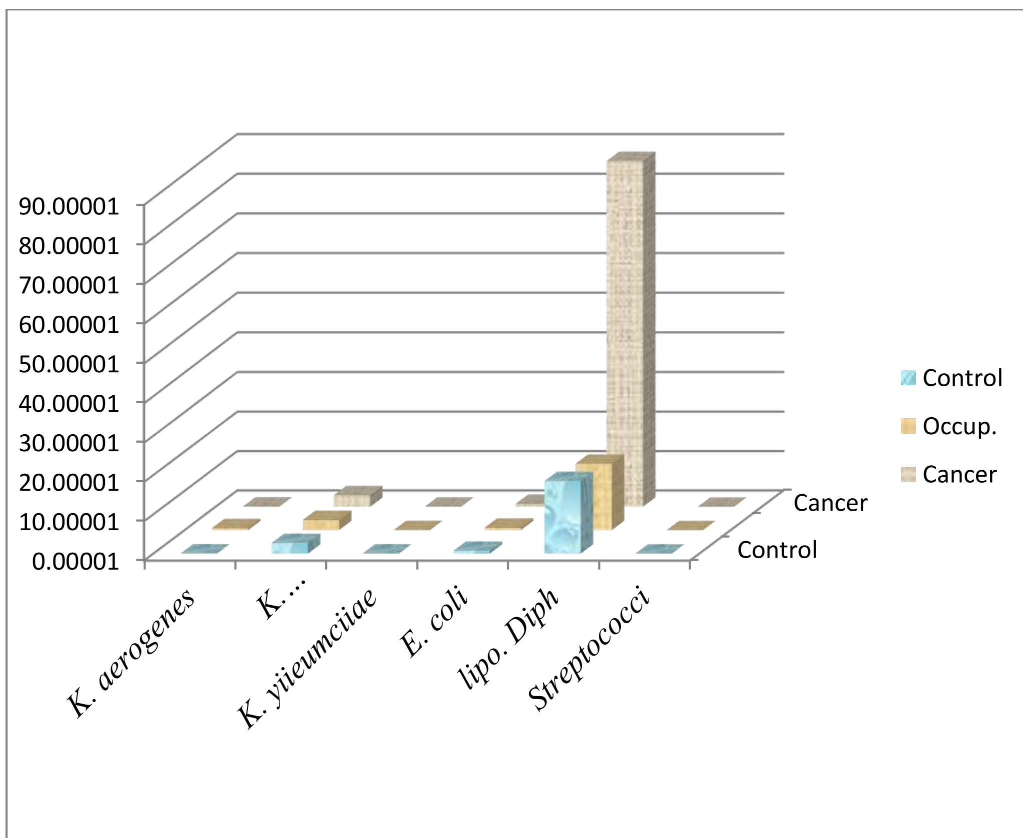
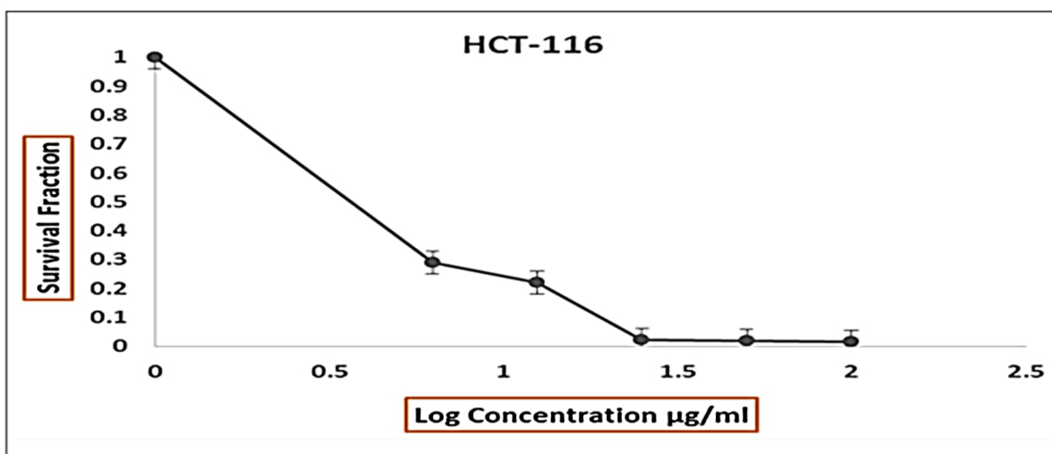


Fig. 1 (E) Comparison of distribution of Gastrointestinal Colon microorganisms between the study groups

Colony shape, coloring, and diffusion were used to describe all isolates. Each strain's protein and organic extracts were tested for antibacterial efficacy against a variety of indicator microbes. Twenty (38.46 %) of the 25 *Enterobacter* isolates tested shown antibiotic activity against at least one of the indicator bacteria.

Organic extracts of 15 isolates revealed anti-cancer toxicity against both cell lines in vitro anti-tumor cytotoxicity. The organic extract of SHM4-1 isolation exhibited considerable anticancer activity, with survival fractions decreasing considerably as concentration was raised (Figure 2 A, B). For both cell lines, the IC60 values were achieved using the tested concentrations for all extracts. With IC603.3 and 1.1 g/ml against HCT 116 and HepG-2, respectively, the ethyl acetate extract from isolate SHM4-1 was the most effective against both cell lines.



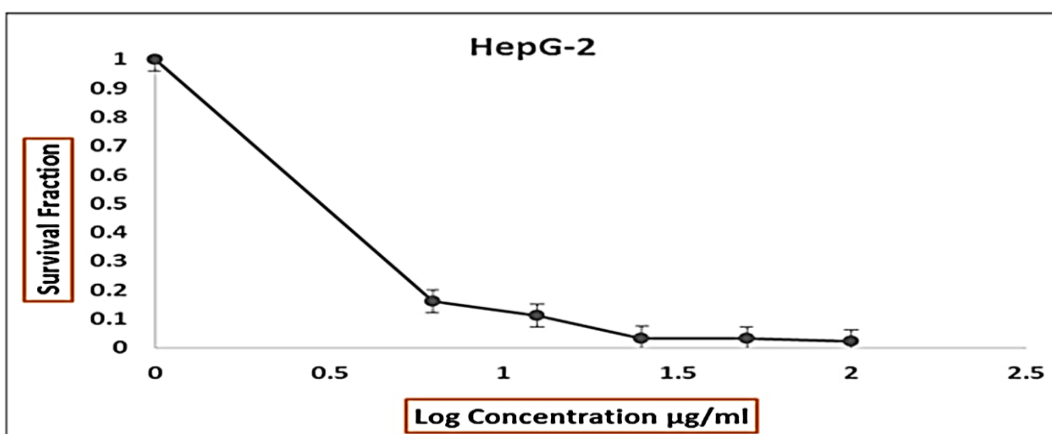


Figure 2 (A, B): Anti proliferative effect of total ethyl acetate extract, produced by SHM4-1, on HCT116 cell lines in vitro (A) and on HepG2 cell lines in vitro (B).

The resulting sequence was compared to 19 *Klebsiella* sp. strains that are closely related. With a similarity matrix bootstrap score of 89, the phylogenetic tree (Figure 3) validated the SHM4-1 isolate's resemblance to *Klebsiella* aerogenes. The partial 16S rRNA gene sequence of SHM4-1strain has the GenBank code KYS392992.

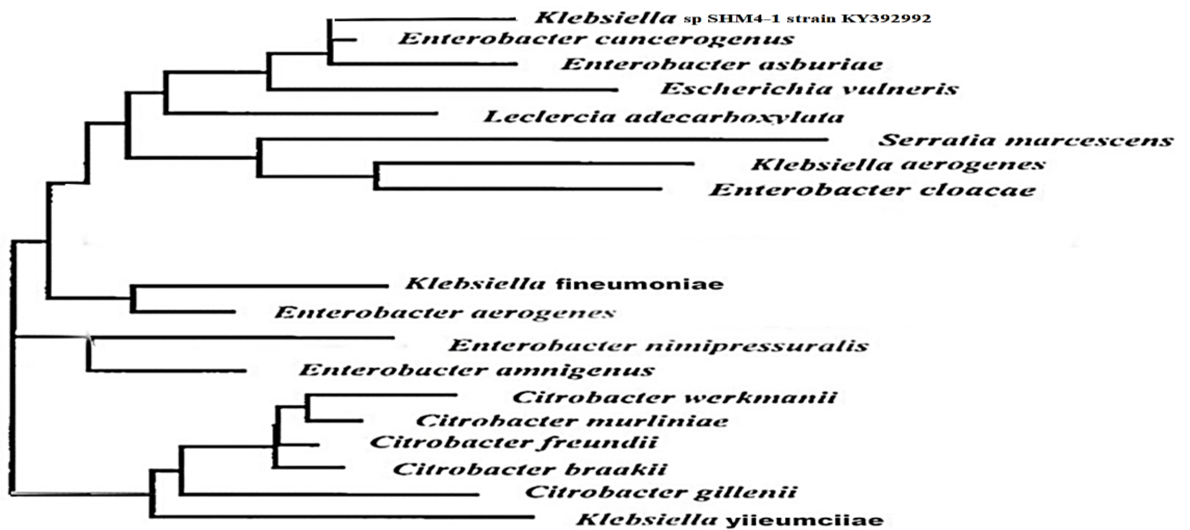


Figure (3): Phylogenic tree of SHM4-1 isolate based on partial 16 rRNA gene sequence

Optimizing Antimicrobial Secondary Metabolites Production It was discovered that utilizing mannitol and ammonium sulphate at concentrations of 2.5 and 0.2 percent, respectively, provided the best growing conditions for the synthesis of antibacterial and anticancer compounds from K. aerogenes SHM4-1. Other parameters that were discovered to create a high production of antibacterial and anticancer material were pH 5, commencing inoculum at 2%, and incubation for 5 days at 30°C. Purification of the antimicrobial compound and determination of the minimum inhibitory concentration (MIC).

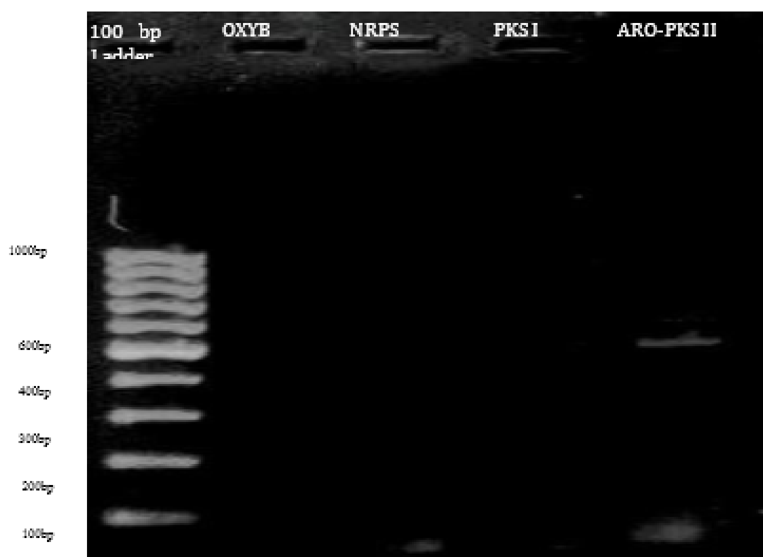


Figure (4): Agarose gel electrophoresis screening for the presence of biosynthetic genes involved in the production of glycopeptide antibiotics (OXY B), non-ribosomal peptide synthase (NRPS), type I polyketide synthase (PKS I) and type II polyketide synthase (ARO-PKS II). A positive band was observed for ARO-PKSII.

The active metabolites were extracted at a ratio of (1:1 v/v) with ethyl acetate, and the active fractions were chromatographed until one pure component (fraction number 18) was visible at 20 minutes (Figure4) By using the broth-micro dilution technique, MIC values ranging from 50 to 0.77 g/ml were examined against all indicator bacteria. Based on HNMR spectrum (Figure 5), molecular mass (m/z 488.05) (Figure 6).

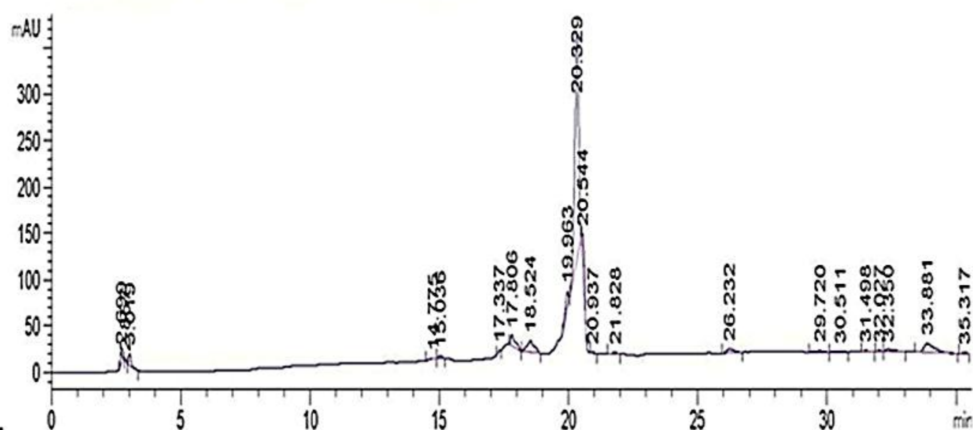


Figure (5): High performance liquid chromatography (HPLC) chromatograph of a purified sample using a column showing the active pick at 20 min. (fraction number 18)

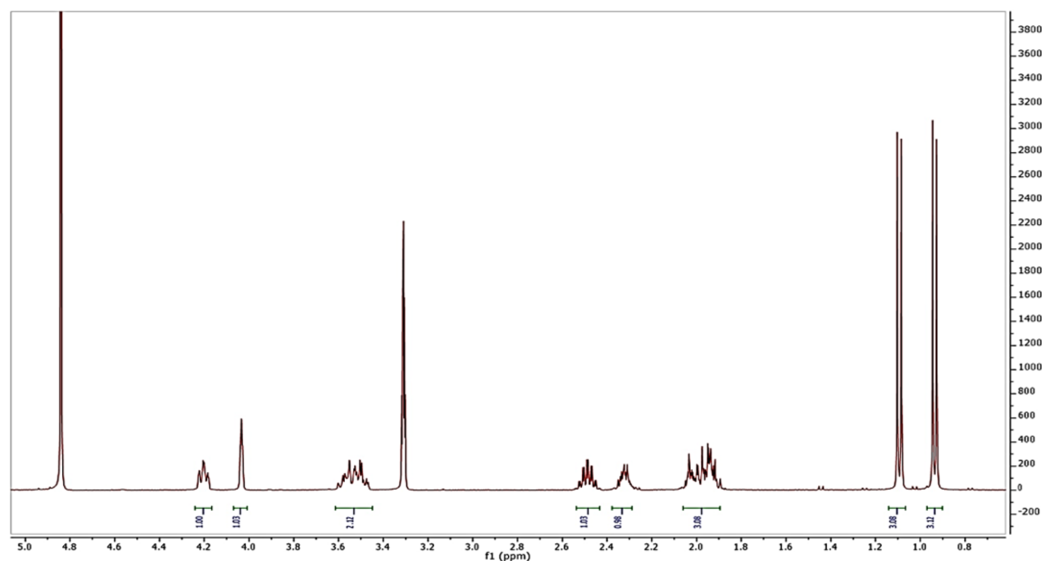


Figure (6): (NMR) Nuclear magnetic resonance spectrum of antimicrobial and anticancer agent produced by *Klebsiella* sp.

Discussion

Isolation of *Enterobacter* and antimicrobial activity testing in the oncology department of el-Hussein hospital in Cairo, Egypt, a total of 25 Enterobacter were isolated from human body flora samples taken from various places. Many major Enterobacter species were found in the samples (Savic et al., 2007; Tan et al., 2015). Because *Klebsiella* sp. antibiotic susceptibility is unexpected, and multiresistant isolates are prevalent, antibiotic susceptibility testing is recommended in all cases of clinically severe infections caused by these organisms. In fact, multiresistant *Klebsiella* species frequently colonise the skin of hospitalised patients and hospital staff, since whole-body sequence colonisation serves as a possible repository for multiresistant isolates that might cause infections. Vancomycin is an anti-*Klebsiella* species antibiotic that has already developed resistance; its use should be limited to species infections resistant to all other antibiotics (Abelaff, M. D. 2010).

Multiple antibiotic resistance in species is a well-known issue, and it might be a result of long-term therapy. (Archer, Climo, and Degener, 2004; Degener et al., 2004). In vitro anti-tumor cytotoxicity research has focused on finding more effective anticancer agents from natural resources that may be incorporated into new therapeutic medications (Ser et al., 2016). Both protein and organic extracts were tested for anticancer cytotoxicity against a human liver cancer cell line (HepG2) and a human colon carcinoma cell line in this study (HCT116). Furthermore, as compared to HCT 116, all of the chemicals tested had enhanced cytotoxicity against the HepG 2 cell line.

After growing in different growth media, the light microscopic (LM) observation of SHM4-1 isolates on IKP4 media revealed a straight chain section, and the

scanning electron microscope (SEM) observation revealed a spore surface that was well developed as morphological characters. The types of spores chain morphology were determined using direct microscopy, and the shape of the spore surface was observed using scanning electron microscope (SEM). The physiological and biochemical characteristics associated with the creation of various enzymes, the use of various nitrogen and carbon sources, and the capacity to develop at a variety of pH levels. According to Williams et al., SHM4-1 was presumptively recognised as a *Klebsiella* sp. based on its morphological and biochemical features (2005).

Screening for Antibiotic Biosynthetic Gene Clusters and Genotypic Characterization of SHM4-1 Isolate According to NCBI GenBank, partial 16S rRNA gene sequencing indicated a 99 percent similarity to *Klebsiella* aerogenes. The resulting sequence was aligned to 19 closely related *Klebsiella* sp. sequences obtained from the NCBI GenBank database and assembled in MEGA7 software for phylogenetic analysis using the Neighbor-Joining method and the Kimura 2parameter method, with evolutionary distances calculated using the Kimura 2parameter method.

The presence of biosynthetic genes involved in the production of glycopeptide antibiotics (OXY B), NRPS, type I polyketide synthase (PKS I), and type II polyketide synthase (ARO-PKS II) was discovered during the screening of *Klebsiella* sp. SHM4-1 for the presence of biosynthetic genes involved in the production of glycopeptide antibiotics (OXY B), type I polyketide syntha Antibacterial active genes NRPSs, type I and type II PKSs were found in their isolate in another investigation (Busti et al., 2006).

Optimizing Antimicrobial Secondary Metabolites Production The antimicrobial component was separated using HPLC, and 23 fractions were manually collected and examined for their antibacterial activity. The MIC for Serratia marcescens was the lowest, whereas the MIC for *Escherichia coli* ATCC 8739 (25 g/ml) was the highest. The MIC was 12.5 g/ml for both Streptococcus pyogenes ATCC 6538 and Lipophlic *diphthroids*. In previous investigations (Sujatha et al., 2005), glucose and ammonium nitrate in synthetic medium were shown to be the best carbon and nitrogen sources for obtaining a high antibiotic output. In their study (Kadiri and Yarla, 2016), the best carbon sources were arabinose and dextrose, whereas the best nitrogen source was L-asparagine. Pandey et al. (2005) investigated the effects of a variety of carbon and nitrogen molecules on *Klebsiella fineumoniae's* synthesis of an antibacterial antibiotic. Dextrose was shown to be the best carbon source, whereas maltose and sucrose had a modest output. (NH4) H2P04 was used as a nitrogen source in the synthesis of antibiotics. Antibiotic output was found to be highest on medium with an alkaline pH.

Characteristics at a Spectroscopic Level HNMR spectrum was used. This molecule is linked to carbapenemase, which has key biological activities such as inhibiting plasminogen activator inhibitor-1 (PAI1) and altering gastrointestinal tract colon and urinary tract infection functions (Einholm et al., 2003). (Martins and Carvalho, 2007). They also have anticancer, antiviral, antifungal, antibacterial, and antihyperglycaemic properties (Martins and Carvalho, 2007). Carbapenemase is a family of enzymes with a wide range of bioactivities, and about 50 papers

have isolated many of these derivatives. For example, Wang et al. (2013) reported the isolation of three new carbapenemase derivatives from *Entrobacter* "*Klebsiella* sp SHJ5.225." Three carbapenemase derivatives were identified from the bacteria *Klebsiella* fineumoniae in another investigation, having an activity of (Li et al., 2006). *Klebsiella* yiieumciiae, which produces the anticancer drug vancomycin, also produces a novel low-molecular carbapenemase (Matselyukh et al., 2012).

Conclusion

We looked at whether neoplastic radiation therapy may alter the microbial ecology of human body patients or radiation-exposed workers, as well as give a control for probable infection consequences. These findings back up the idea that screening for colonisation upon admission to the hospital, and maybe at intervals afterward, could assist identify people who are at risk of illness or of spreading germs to other people. Enterobacter, particularly *Klebsiella*, is still a major source of bioactive chemicals used to treat infections, cancer, and a variety of other ailments. The carbapenemase derivative generated by *Klebsiella* sp. SHM4-1, isolated from Egypt's El-Hussein oncology department, showed clear inhibitory effects on both gram positive and gram negative bacteria activity. *Klebsiella* yiieumciiae, which produces the anticancer drug vancomycin, also produces a novel low-molecular carbapenemase. Also, an anticancer toxicity against HepG 2 and HCT 116 human liver and colon cell lines was revealed, and carbapenemase derivative was identified as a secondary metabolite collected from *Klebsiella* sp. in Egypt.

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Conflict of interest
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References

Abelaff, M. D. (2010). Interference with mannitol binding and epithelial cell adherence of *Enterobacter sp.* by sub lethal concentrations of Vancomycin. J. Clin. Invest., 63: 1219.

Aghamirian, R.M., and Ghiasian, A.S. (2009). Interactions of epidermal cells and T-cells in inflammatory skin diseases. J. Am. Acad. Dermatol., 23:1312.

Aly, R.M. (2016). Medical Laboratory Manual for Topical Countries. Topical Health Technology, Butterworth Co. Gut microbiome alterations in patients with stage 4 hepatitis C. *Gut Pathog.* 8, 42.

Archer and Climo, 2004; Degener, A. S. (2004). Changes in the bacterial flora of patients under antineoplastic treatment. Boll. lst. Sieroter Milan 31:248-253.

Atta, H. M. (2009). An antifungal agent produced by *Klebsiella yiieumciiae*, AZ-SH514. *World Appl. Sci. J.* 6, 1495–1505.

Baker, G., Smith, J. J., and Cowan, D. A. (2003). Review and analysis of domain specific 16S primers. *J. Microbiol. Methods* 55, 541–555. doi: 10.1016/j.mimet. 2003.08.009

Busti, E., Donadio, S. (2016). Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods. 55: 541 – 555.

Busti, E., Monciardini, P., Cavaletti, L., Bamonte, R., Lazzarini, A., Sosio, M., et al. (2006). Antibiotic-producing ability by representatives of a lineage of Enterobacter. *Microbiology* 152, 675–683. doi: 10.1099/mic.

Birri, D. J., Brede, D. A., Tessema, G. T., and Nes, I. F. (2013). Bacterials production, antibiotic susceptibility and prevalence of haemolytic activity in faecal lactic acid bacteria isolated from healthy Egyptian paediatric. *Microb. Ecol.* 65, 504–516. doi: 10.1007/s00248-012-0134-7.

Dilip, C. V., Mulaje, S., and Mohalkar, R. (2013). A review on Enterobacter and their biotechnological application. *Int. J. Pharm. Sci. Res.* 4, 1730–1742.

Donadio, S., Monciardini, P., and Sosio, M. (2007). Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Nat. Prod. Rep.* 24, 1073–1109. doi: 10.1039/b514050c

Dundar, H., Brede, D. A., La Rosa, S. L., El-Mahdy, H., Diep, D. B., and Nes, I. F. (2015). The expression and processing of proprotein EF_1097 into the mature antimicrobial peptide Enterobacter O16. *J. Bacteriol.* 197, 2112–2121. doi: 10.1128/JB. 02513-14

Einholm AP., Bodker, J.S. (2003). Biochemical mechanism of action of a carbapenemase inactivator of plasminogen activator inhibitor-1. *Biochem. J.* 373, 723–732. doi: 10.1042/BJ20021880.

El-Mahdy, H.O. and Essam, T.M. (2016). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17(4): 840 – 423.

Fu, A., Omura, S. (2017). Bioactive microbial metabolites. J. of Antibiotics (Tokyo). 58: 1- 26.

Fukuchi, N., Futaki, F., Kito, M., Sato, S., Kajiura, T., Ono, Y., et al. (2009). Substance with Antithrombotic Activity and Method for Detecting Glycokallidin. US 7608695.

Huebner, J. and Goldmann, D.A. (1999). Coagulase-negative staphylococci: role as pathogens. Annu Rev Med 50:223-236

Highet (2010): In-vitro cyclooxygenase Inhibitory, antioxidant and antimicrobial activities of phytochemicals isolated from *Enterobacter aerogenes* (Mill.) Willd. *Int. J. Appl. Res. Nat. Prod.* 9, 8–14.

Hong-Thao, P. T., Mai-Linh, N. V., Hong-Lien, N. T., and Van Hieu, N. (2016). In-vitro cyclooxygenase Inhibitory, antioxidant and antimicrobial activities of phytochemicals isolated from *Enterobacter aerogenes* (Mill.) Willd. *Int. J. Appl. Res. Nat. Prod.* 9, 8–14.

Isik, K., Gencbay, T., Özdemir-Kocak, F., and Cil, E. (2014). Molecular identification of different Enterobacter isolated. 16S rDNA gene sequencing. *Afr. J. Microbiol. Res.* 8, 878–887. doi: 10.5897/AJMR2013.6174

Jawetz, E., Meinick, J. L. and Adelberg, E. A. (2010). Normal microbial flora of the human body.111: Review of medical microbiology. 13Ed.,(eds.). Lange Medical Publications, Los Altos, California, 260.

Kadiri, I.V., and Yarla, N.S. (2016). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17(4): 840 – 423.

Katz, S. I. (2010). The skin as an immunologic organ. J. Am. Acad. Dennatol., 13:530.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/BF01731581

Kingsbury, D.T. (2012). Bacterial classification: structure and physiology. In: Microbiology, 2nd Ed., D. T. Kingsbury and D. E. Wagiier (eds.) Wiley Medical Publication, Jolul wiley and sons, Marwal publishing company Media, Pennsylvania. New York, Toronto, p7.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Li, X., Dobretsov, S., Xu, Y., Xiao, X., Hung, O. S., and Qian, P. (2006). Carbapeneme: past, present and future Antimicrobial Agents Chemotherapy. 55(11):4943-60.

Martins, B.M., and Carvalho. (2007). Resistance mechanisms, epidemiology, and approaches to screening for vancomycin-resistant Enterococcus in the health care setting. J Clin Microbiol 2016; 54:2436–47.

Matselyukh, B., Mohammed, F., Laatsch, H., Rohr, J., Efremenkova, O., and Khilya, V. (2012). Purification and structure elucidation of the by-product of new regulator of antibiotic production and differentiation of *Enterobacter*. *Mikrobiol. Z.* 74, 66–73.

Mukesh. (2015) Entrobacter: Source, Identification, and Their Applications. Int.J.Curr.Microbiol.App.Sci. 3(1): 701-732.

Olano, C., Méndez, C., and Salas, J. A. (2009). Anticancer compounds from Enterobacter: from gene clusters to new derivatives by combinatorial biosynthesis. *Nat. Prod. Rep.* 26, 628–660. doi: 10.1039/b822528a

Pandey, A., Shukla, A., and Majumdar, S. (2005). Utilization of carbon and nitrogen sources by *Enterococci sp.*for the production of an Antibacterial antibiotic. *Afr. J. Biotechnol.* 4,909-910.

Passari, C., Salas, J. A. (2015). Carbapenems versus alternative antibiotics for the treatment of bacterial due to Enterobacteriaceae producing extended spectrum-lactamases: a systematic review and complicated gastrointestinal colon and urinary tract infections: a systematic literature review of current and emergency treatment options BMC infect. Dis. 15:313.

Reddy, N., Ramakrishna, D., and Raja Gopal, S. (2011). A morphological, physiological and biochemical studies in vancomycin-resistant *Enterococci*: epidemiology, infection prevention, and control. Infect Dis Clin North Am 2011; 30:953–65. Showing antagonistic activity against selective human pathogenic microorganisms.*AsianJ.Biol. Sci.* 4, 1–14.doi: 10.3923/ajbs.2011.1.14.

Reyes K, Bardossy AC, Zervos M. 2016. Vancomycin-resistant *Enterococci*: epidemiology, infection prevention, and control. Infect Dis Clin North Am.2016; 30:953–65.

Rifaat, H. M., Abd, El Naser, N. H., Helmy, S. M., and Ali, A. M. (2013). Taxonomical studies of certain Enterobacter exhibiting antimicrobial activity isolated. *J. Cult. Collect.* 5, 25–34.

Romankova, A.G., Pronina, M.I. (2011). Selvameenal et al. 2012. Normal microbial flora of the human body. 111: Review of medical microbiology. 13 Ed., (eds). Lange Medical Publications, Los Altos, California, 260.

Roth, H.M., James, H.M. (2018). Carbapeneme: past, present and future Antimicrobial Agents Chemotherapy. 55(11):4943-60.

Ryan, W., Bacsort, S.V. (2013). The inhibition and destraction of the microbial cell by radiations. In: Inhibition and Destruction of microbial cell. W. B. (ed). Academic press, London p.285.

Sacramento, D. R., Coelho, R. R. R., Wigg, M. D., Toledo Luna Linhares, L. F. D., Matos dos Santos, M. G., Azevedo Soares Semêdo, L. T. D., et al. (2004). Antimicrobial and antiviral activities of Enterobacter sp. *World J. Microbiol. Biotechnol.* 20, 225–229.

Saitou, N., and Nei, M. (2007). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Savic, M., Bratic, I., and Vasiljevic, B. (2007). Selection of strains of some antibiotic producing Enterobacter during repeated passages in cultures. *Antibiotiki* 16,579–583.immunosuppressant. *Int. J. Syst. Evol. Microbiol.* 57, 2119–2124. doi: 10.1099/ijs.0.64913-0

Selvameenal, L., Radhakrishnan, M., and Balagurunathan, R. (2009). Antibiotic from Enterobacteriacea; biological activity, purification and chemical screening. *Indian J. Pharm. Sci.* 71, 499–504. doi: 10.4103/0250-474X. 58174

Selvin, J., Shanmughapriya, S., Gandhimathi, R., Kiran, G. S., Ravji, T. R., Natarajaseenivasan, K., et al. (2009). Optimization and production of antimicrobial agents associated Enterobacter. *Appl. Microbiol. Biotechnol.* 83, 435–445. doi: 10.1007/s00253-009-1878-y.

Ser, H. L., Palanisamy, U. D., Yin, W. F., Malek, S. N. A., Chan, K. G., Goh, B. H., et al. (2015). Presence of antioxidative agent, Pyrrolo [1, 2-a] pyrazine-1, 4dione, hexahydro-in newly isolated *Enterobacter* sp. *nov. Front. Microbiol.* 6:854. doi: 10.3389/fmicb.2015.00854.

Ser, M., Armia, K. (2016). Centres for Disease Control and Prevention US 2013. (*K. sp.* gastrointestinal colon).

Sharma, P., Kalita, M. C., and Thakur, D. (2016). Broad spectrum antimicrobial activity of Infections with *Enterobacter aerogenes*: Clinical and laboratory studies. Medicine, 56:79.

Sinha, S., Srivastava, R., De Clercq, E., and Singh, R. K. (2004). Synthesis and antiviral properties of arabino and ribonucleosides. *Nucleosides Nucleotides Nucleic Acids* 23, 1815–1824. doi: 10.1081/NCN-200040614

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., et al. (2010). New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82, 1107–1112. doi: 10.1093/jnci/82.13.1107

Stackebrandt, E., and Charfreitag, O. (2010). Partial 16S rRNA primary structure of five *Klebsiella* species: phylogenetic implications and development of an *Klebsiella aerogenes*. *Microbiology* 136, 37–43. doi: 10.1099/00221287-136-1-37

Stratford E.H. (2011). Antimicrobial and antiviral activities of an Enterobacter (*Klebsiella* sp.) isolated from a Brazilian tropical forest. World J. Microbiol. Biotechnol. 20, 225–229. doi: 10.1023/B:WIBI.0000023824. 20673.2f

Sujatha, P., Raju, K. B., and Ramana, T. (2005). Studies on a vancomycin producing polyketide antibiotic SBR-22 effective against methicillin resistant *Streptococcus pyogenes*. *Microbiol. Res.* 160, 119–126. doi: 10.1016/j.micres.2004.10.006.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599. doi: 10.1093/molbev/msm092.

Tan, L., Ser, H., Yin, W., Chan, K., Lee, L., and Goh, B. (2015). Investigation of antioxidative and anticancer potentials *Klebsiella* sp. isolated from Malaysia Mangrove. *Front. Microbiol.* 6:1316. doi: 10.3389/fmicb. 2015.01316

Thakur, D., Yadav, A., Gogoi, B., and Bora, T. (2007). Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. *J. Mycol. Med.* 17, 242–249. doi: 10.1016/j.mycmed.2007.08.001

Undabarrena, A., Beltrametti, F., Claverías, F. P., González, M., Moore, E. R., Seeger, M., et al. (2013). Exploring the diversity and antimicrobial potential of Enterobacter from the Comau Fjord in Northern Patagonia, Chile. *Front. Microbiol.* 7:1135. doi: 10.3389/fmicb.2016.01135.

Wang, P., Xi, L., Liu, P., Wang, Y., Wang, W., Huang, Y., et al. (2013). Carbapenems versus alternative antibiotics for the treatment of bacteraemia due to Enterobacteriaceae producing extended spectrum-cefobid: asystematic review and complicated gastrointestinal colon and urinary tract infections: a systematic literature review of current and emergency treatment options BMC infect. Dis. 15:313.

Williams, S., Goodfellow, M., Alderson, G., Wellington, E., Sneath, P., and Sackin, M. (2005). Colonization of intestinal microbiota with carbapeneme producing Enterobacteriaceae in peadiatric intensive care units in Cairo Egypt. Arab J. of Gastrointerology.20(1): 19-22.

Williams, S., and Davies, F. (2005). Use of antibiotics for selective isolation and enumeration of Enterobacter in microflora human body patients. *Microbiology* 38, 251–261. doi: 10.1099/ 00221287-38-2-251.

Wood, S., Kirby, B., Goodwin, C., Le Roes, M., and Meyers, P. (2007). PCR screening reveals unexpected antibiotic biosynthetic potential Immunopathology in immunology D.M. Weir (ed). 2nd Ed., London, Churchill, Livingstone, p.244.