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Characterization of antimicrobial secondary metabolites produced by *Klebsiella pneumoniae* and screening of its bioactive natural compounds using gas chromatography-mass spectrometry (GC-MS)

Sajjad Saad Zghair, Sahar Sarhan Thajeel and Imad Hadi Hameed *

Department of Medical Biotechnology, College of Biotechnology, AL-Qasim Green University, Saudi Arabia.

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Abstract

Microbial secondary metabolites are low molecular mass products with unusual structures. The structurally diverse metabolites show a variety of biological activities like antimicrobial agents. Thirty nine bioactive compounds were identified in the methanolic extract of *Klebsiella pneumoniae*. GC-MS analysis of *Klebsiella pneumoniae* revealed the existence of the Tricyclo[4.3.1.1(3.8)]undecan-1-amine, 3-Methoxybenzaldehyde semicarbazone, carboxaldehyde , 1-methyl-,oxime ,(Z)-(+), 1,5,5-Trimethyl-6-methylene-cyclohexene, 4-(2,5-Dihydro-3-methoxyphenyl)butylamine, Paromomycin , 9-Borabicyclo[3.31]nonane , 9-mercapto-, Benzenemethanol , 2-(2-aminopropoxy)-3-methyl, Acetamide , N-(6-acetylaminobenzothiazol-2-yl)-2-(adamantan, rin-6-carboxylic acid , 4-(2,5-Dihydro-3-methoxyphenyl)butylamine, N-(2,5-Dicyano-3,4-dihydro-2H-pyrrol-2-yl)-acetamide, 3,10-Dioxatricyclo [4.3.1.0(2,4)]dec-7-ene, 3-Cyclohex-3-enyl-propionic acid, Eicosanoic acid ,phenylmethyl ester, 3,7-Diazabicyclo[3.3.1]nonane , 9,9-dimethyl-, Dithiocarbamate , S-methyl-,N-(2-methyl-3-oxobutyl)-, dl-Homocysteine, 2-(2-Furyl)pyridine, 1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12-trione, 5,7-Dodecadiyn-1,12-diol, 1-(β-d-Arabinofuranosyl)-4-O-difluoromethyluracil, Uric acid, Pyrrolo[1.2-a]pyrazine-1,4-dione , hexahydro-,12-Methyl-oxa-cyclododecan-2-one, Phthalic acid , butyl undecyl ester, 9,12,15-Octadecatrienoic acid , 2,3-bis(acetyloxy)propyl ester, 1,2,4-Trioxolane-2-octanoic acid 5-octyl-, methyl ester, 12-Dimethylamino-10-oxododecanoic acid , Octahydrochromen-2-one, L-Aspartic acid , N-glycyl-,2H-Oxecin-2-one , 3,4,7,8,91,10-hexahydro-4-hydroxy-10-meth , Thiazolo[4,5-d]pyrimidine-5,7(4H,6H)-dione , 2-amino-4-(2-ph, Dec-9-en-6-oxo-1-ylamide, 3,6,12-Trimethyl-1,4,7,10,13,16-hexaaza-cyclooctadecane, 2-Iodohiistidine, 2,5-Piperazinedione ,3,6-bis(2-methylpropyl)-, 9-Octadecenamide , (Z)-, 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetra.

Clinical pathogens selected for antibacterial activity namely, *Streptococcus pneumonia*, *Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Staphylococcus epidermidis,* It were 4.09±0.013, 2.99±0.300, 4.37±0.200, 3.22±0.210, and 4.00±0.203 respectively for Bacterial products (Metabolites Produced by *Klebsiella pneumoniae*), while recorded 1.08±0.200, 0.97±0.116, 2.08±0.233, 3.04±0.261, 0.98±0.166 respectively for Bacterial products Streptomycin antibiotics, and recorded 1.02±0.180, 1.00±0.190, 2.08±0.236, 1.00±0.100, and 1.82±0.200 respectively for Kanamycin antibiotics. *Klebsiella pneumoniae* produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by *Klebsiella pneumoniae* can be useful.

Keywords: Antimicrobial; *Klebsiella pneumoniae*; Secondary Metabolites; GC-MS

* Corresponding author: Imad Hadi Hameed

1 Introduction

Klebsiella pneumoniae is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar [1-3]. Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen, is a component of the lipopolysaccharide (LPS), of which 9 varieties exist [4]. The second is K antigen, a capsular polysaccharide with more than 80 varieties [5-7]. Both contribute to pathogenicity and form the basis for serogrouping. As a free-living diazotroph, its nitrogen-fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions [8]. The most common condition caused by *Klebsiella* bacteria outside the hospital is pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a death rate around 50%, even with antimicrobial therapy. The mortality rate can be nearly 100% for people with alcoholism and bacteremia [9].

Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the *Klebsiella* genus of the Enterobacteriaceae. *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important pathogens in nosocomial infections. In addition to pneumonia, *Klebsiella* can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites [10]. The range of clinical diseases includes pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis, and bacteremia and septicemia. For patients with an invasive device in their bodies, contamination of the device becomes a risk; for example, neonatal ward devices, respiratory support equipment, and urinary catheters put patients at increased risk [11-14]. Also, the use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood.

The objectives of this study were

- Analysis of secondary metabolites produced by *Klebsiella pneumoniae* Gas Chromatography-Mass Spectrometry (GC-MS).
- Evaluation antimicrobial activity of secondary metabolites produced by *Klebsiella pneumoniae*

1.1 Enterobacteriaceae

Enterobacteriaceae is a large family of Gram-negative bacteria. It was first proposed by Rahn in 1936, and now includes over 30 genera and more than 100 species. Its classification above the level of family is still a subject of debate, but one classification places it in the order Enterobacterales of the class Gammaproteobacteria in the phylum Pseudomonadota.

Enterobacteriaceae includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Klebsiella*, and *Shigella*. Other disease-causing bacteria in this family include Enterobacter and Citrobacter. Members of the Enterobacteriaceae can be trivially referred to as enterobacteria or "enteric bacteria",.

1.2 Pathogenic bacteria

Pathogenic bacteria are bacteria that can cause disease. This article focuses on the bacteria that are pathogenic to humans. Most species of bacteria are harmless and are often beneficial but others can cause infectious diseases. The number of these pathogenic species in humans is estimated to be fewer than a hundred. By contrast, several thousand species are part of the gut flora present in the digestive tract [12].

The body is continually exposed to many species of bacteria, including beneficial commensals, which grow on the skin and mucous membranes, and saprophytes, which grow mainly in the soil and in decaying matter. The blood and tissue fluids contain nutrients sufficient to sustain the growth of many bacteria. The body has defence mechanisms that enable it to resist microbial invasion of its tissues and give it a natural immunity or innate resistance against many microorganisms.

Pathogenic bacteria are specially adapted and endowed with mechanisms for overcoming the normal body defences, and can invade parts of the body, such as the blood, where bacteria are not normally found. Some pathogens invade only the surface epithelium, skin or mucous membrane, but many travel more deeply, spreading through the tissues and disseminating by the lymphatic and blood streams. In some rare cases a pathogenic microbe can infect an entirely

healthy person, but infection usually occurs only if the body's defence mechanisms are damaged by some local trauma or an underlying debilitating disease, such as wounding, intoxication, chilling, fatigue, and malnutrition. In many cases, it is important to differentiate infection and colonization, which is when the bacteria are causing little or no harm [13].

Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Staphylococcus*, *Streptococcus* and *Pseudomonas*, and foodborne illnesses, which can be caused by bacteria such as *Shigella*, *Campylobacter*, and *Salmonella*. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy [14].

Pathogenic bacteria are also the cause of high infant mortality rates in developing countries. A GBD study estimated the global death rates from (33) bacterial pathogens, finding such infections contributed to one in 8 deaths (or ~7.7 million deaths), which could make it the second largest cause of death globally in 2019. [16,17].

Most pathogenic bacteria can be grown in cultures and identified by Gram stain and other methods. Bacteria grown in this way are often tested to find which antibiotics will be an effective treatment for the infection. For hitherto unknown pathogens, Koch's postulates are the standard to establish a causative relationship between a microbe and a disease.

1.3 Klebsiella

Klebsiella is a genus of Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule. [18] *Klebsiella* species are found everywhere in nature. This is thought to be due to distinct sublineages developing specific niche adaptations, with associated biochemical adaptations which make them better suited to a particular environment. They can be found in water, soil, plants, insects and other animals including humans [19,20].

Klebsiella is named after German-Swiss microbiologist Edwin Klebs (1834–1913). Carl Friedlander described *Klebsiella* bacillus which is why it was termed Friedlander bacillus for many years. The members of the genus *Klebsiella* are a part of the human and animal's normal flora in the nose, mouth and intestines. The species of *Klebsiella* are all gram-negative and usually non-motile. They tend to be shorter and thicker when compared to others in the family Enterobacteriaceae. The cells are rods in shape and generally measures 0.3 to 1.5 μm wide by 0.5 to 5.0 μm long. They can be found singly, in pairs, in chains or linked end to end. *Klebsiella* can grow on ordinary lab medium and do not have special growth requirements, like the other members of Enterobacteriaceae. The species are aerobic but facultatively anaerobic. Their ideal growth temperature is 35° to 37 °C, while their ideal pH level is about 7.2. [21]

Klebsiella bacteria tend to be rounder and thicker than other members of the family Enterobacteriaceae. They typically occur as straight rods with rounded or slightly pointed ends. They can be found singly, in pairs, or in short chains. Diplobacillary forms are commonly found *in vivo*. [22]

They have no specific growth requirements and grow well on standard laboratory media, but grow best between 35 and 37 °C and at pH 7.2. The species are facultative anaerobes, and most strains can survive with citrate and glucose as their sole carbon sources and ammonia as their sole nitrogen source. [23]

Members of the genus produce a prominent capsule, or slime layer, which can be used for serologic identification, but molecular serotyping may replace this method. [24]

Members of the genus *Klebsiella* typically express two types of antigens on their cell surfaces. The first, O antigen, is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for serogrouping. Based on those two major antigenic determinants several vaccines have been designed. [25]

Klebsiella species are routinely found in the human nose, mouth, and gastrointestinal tract as normal flora; however, they can also behave as opportunistic human pathogens. *Klebsiella* species are known to also infect a variety of other animals, both as normal flora and opportunistic pathogens. [26]

Klebsiella organisms can lead to a wide range of disease states, notably pneumonia, urinary tract infections, sepsis, meningitis, diarrhea, peritonitis and soft tissue infections. [27] *Klebsiella* species have also been implicated in the pathogenesis of ankylosing spondylitis and other spondyloarthropathies. The majority of human *Klebsiella* infections are caused by *K. pneumoniae*, followed by *K. oxytoca*. Infections are more common in the

very young, very old, and those with other underlying diseases, such as cancer, and most infections involve contamination of an invasive medical device. [29]

During the last 40 years, many trials for constructing effective *K. pneumoniae* vaccines have been tried, and new techniques were followed to construct vaccines against *Klebsiella*. However, currently, no *Klebsiella* vaccine has been licensed for use in the US. *K. pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections, and the second-most frequent cause of Gram-negative bacteraemia and urinary tract infections . Drug-resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays, and are especially problematic in high-impact medical areas such as intensive care units. This antimicrobial resistance is thought to be attributable mainly to multidrug efflux pumps.[30] The ability of *K. pneumoniae* to colonize the hospital environment, including carpeting, sinks, flowers, and various surfaces, as well as the skin of patients and hospital staff, has been identified as a major factor in the spread of hospital-acquired infections.[31]

1.4 Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar.

Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human and animal lungs if aspirated, specifically to the alveoli resulting in bloody, brownish or yellow colored jelly like sputum. In the clinical setting, it is the most significant member of the genus *Klebsiella* of the Enterobacteriaceae. *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important pathogens in nosocomial infections.[32]

It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. As a free-living diazotroph, its nitrogen-fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions. [33]

1.5 Secondary Metabolites

The metabolism can be defined as the sum of all the biochemical reactions carried out by an organism. Metabolites are the intermediates and products of metabolism and are usually restricted to small molecules. The term “secondary” introduced by A. Kossel in 1891 implies that while primary metabolites are present in every living cell capable of dividing, the secondary metabolites are present only incidentally and are not of paramount significance for organism’s life. Though secondary metabolites are derived from primary metabolism, they do not make up basic molecular skeleton of the organism. Its absence does not immediately curtail the life of an organism, a feature contrary to primary metabolite, but survival of the organism is impaired to a larger extent. Its presence and synthesis are observed in ecologically disadvantaged species within a phylogenetic group [34].

The difference between primary and secondary metabolite is ambiguous since many of the intermediates in primary metabolism is overlapping with the intermediates of secondary metabolites. Amino acids though considered a product of primary metabolite are definitely secondary metabolite too. Contrary to the observation that sterols are secondary metabolites that are indispensable part of many structural framework of a cell. The mosaic nature of an intermediate indicates common biochemical pathway being shared by primary and secondary metabolism [35].

The secondary metabolites serve as a buffering zone into which excess C and N can be shunted into to form inactive part of primary metabolism. The stored C and N can revert back to primary metabolite by the metabolic disintegration of secondary metabolite when on demand. There is dynamism and a delicate balance between the activities of the primary and secondary metabolism being influenced by growth, tissue differentiation and development of the cell or body, and also external pressures.

1.5.1 Production of Secondary Metabolites of Bacteria

Metabolism is a constant and collective biochemical process that occurs in every single or multicellular organism lifelong. The biochemical process largely can be classified into catabolism and anabolism. The end-products of these pathways are used for the formation of intermediates and substrates for other metabolic pathways and are known as ‘metabolites.’

Metabolites exhibit several biological properties, which are of pharmaceutical, nutritional, and agricultural importance. On the basis of functional properties and metabolic pathways, these molecules are classified into primary and secondary metabolites. The primary metabolites serve as a primary source of energy to perform various biochemical and physiological functions of live cells (e.g., amino acids, pyruvate, citric acid, and lactic acid). In contrast, the secondary metabolites are not essential for cell growth, but rather they serve as a survival strategy for the organism during adverse conditions.

The focus of this chapter is on the production of secondary metabolites by bacteria (Table 1). The secondary metabolite-producing microorganisms synthesize these bioactive and complex molecules at the late phase and stationary phase of their growth (Figure 1). The production of secondary metabolites is triggered during the exhaustion of nutrients, environmental stress, and limited growth conditions. The secondary metabolites frequently are found in bacteria, fungi, plants, and marine organisms. These organisms have the capability to produce several metabolites with various biological functions, including antibacterial agents, toxins, metal-transporting agents, sex hormones, pigments, anticancer agents, pesticides, immunomodulating agents, immunosuppressants, receptor agonists, and antagonists.

Secondary metabolic pathway reactions are conducted by an individual enzyme or multi-enzyme complexes. Intermediate or end-products of primary metabolic pathways are channeled from their systematic metabolic pathways that lead to the synthesis of secondary metabolites (Figure 1(b)).

Table 1 Biochemical and physiological properties of primary and secondary metabolites

Primary metabolites	**Secondary metabolites**
-Small molecules -Produces few intermediates or end-products -End-products are building blocks for macromolecules. -Essential for growth and cell viability -Known physiological function -Composed of simple chemical structure -End-products are used for Coenzyme synthesis -Production occurs at log phase -Primary metabolites are used in food and feed industry -Provides the energy for cellular activities	-Small molecules -Produces array of molecules -Synthesize new compounds -Not vital for the cell growth -Analysis of physiological function is difficult -Products of complex unusual chemical structure End-products are used an antibacterial agent -Production occurs at late and dormant phase -Secondary metabolites are used in food, cosmetic, agricultural and farming industry -Protects the organisms under various harsh environment

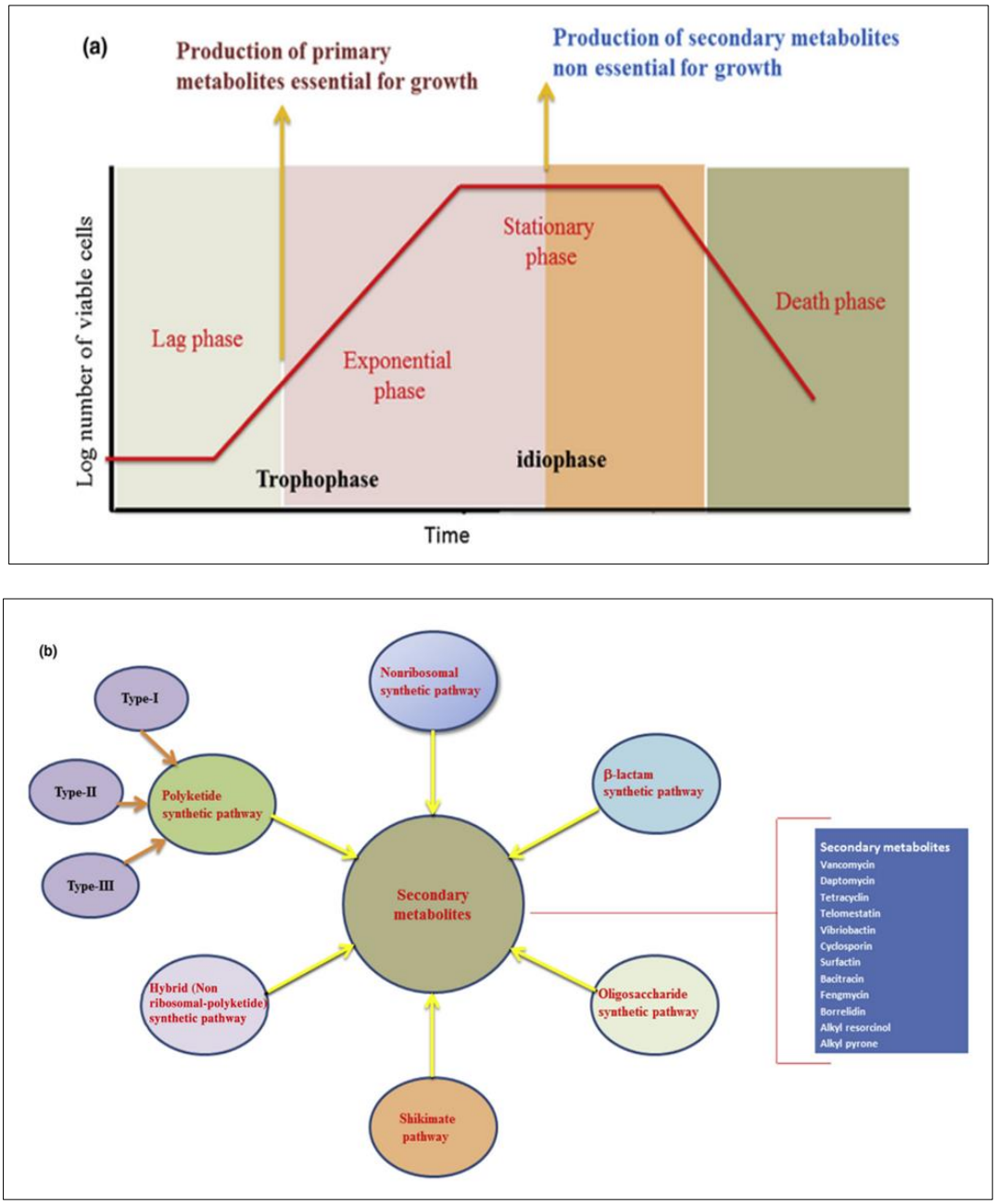

Figure 1 (a) Various phases of bacterial growth and production of metabolites. The primary metabolites production generally occurs at the late lag phase and middle of exponential phase. The secondary metabolites production occurs at the end of the stationary phase and during the persistent phase. (b) Various pathways responsible for the assembly of secondary metabolites

1.5.2 Secondary Metabolites of Microorganisms

Microbial secondary metabolites are low molecular mass products with unusual structures. The structurally diverse metabolites show a variety of biological activities like antimicrobial agents, inhibitors of enzymes and antitumors, immune-suppressives and antiparasitic agents [35,36], plant growth stimulators, herbicides, insecticides, antihelmintics, etc. They are produced during the late growth phase of the microorganisms. The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in logarithmic phase and depressed in stationary growth phases. The microbial secondary metabolites have distinctive molecular skeleton which is not found in the chemical libraries and about 40% of the microbial metabolites cannot be chemically synthesized.

1.5.3 Features of microbial secondary metabolites

- The principle and process of natural fermentation product synthesis can be successfully scaled up and employed to maximize its application in the field of medicine, agriculture, food, and environment.
- The metabolite can serve as a starting material for deriving a product of interest, extended further through chemical or biological transformation.
- New analog or templates in which secondary metabolite serve as lead compounds will lead discovery and design of new drugs.

1.5.4 Applications of Microbial Secondary Metabolites

Antibiotics

The discovery of penicillin initiated the researchers for the exploitation of microorganisms for secondary metabolite production, which revolutionized the field of microbiology [37]. With the advent of new screening and isolation techniques, a variety of β-lactam-containing molecules [38] and other types of antibiotics have been identified. About 6000 antibiotics have been described, 4000 from actinobacteria. In the prokaryotic group, unicellular bacteria Bacillus and Pseudomonas species are the most recurrent antibiotic producers. Likewise in eukaryotes, fungi are dominant antibiotic producers next to plants. In the recent years, myxobacteria and cyanobacteria species have joined these distinguished organisms as productive species.

The pharmaceutical product, especially anti-infective derivatives comprise 62% antibacterials, 13% sera, immunoglobulins, and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% nonHIV antivirals. There are over 160 antibiotics. Streptomyces hygroscopicus with over 200 antibiotics, Streptomyces griseus with 40 antibiotics, and Bacillus subtilis with over 60 compounds are the major contributors to the antibiotic market [39].

Antitumor agents

Natural product and its derivatives account for more than 60% of anticancer formulations. Actinobacteria derived antineoplastic molecules currently in use are actinomycin D, anthracyclines (daunorubicin, doxorubicin, epirubicin, pirarubicin, and valrubicin), bleomycin, mitosanes (mitomycin C), anthracenones (mithramycin, streptozotocin, and pentostatin), enediynes (calicheamicin), taxol, and epothilones [40]. Taxol is the nonactinobacterial molecule derived from plant Taxus brevifolia and endophytic fungi Taxomyces andreanae and Nodulisporium sylviforme. It interferes with microtubule breakdown, an essential event leading to cell division, thereby inhibiting rapidly dividing cancer cells. It is effective against breast and advanced form Kaposi's sarcoma. It is also found to exhibit antifungal activity against Pythium, Phytophthora, and Aphanomyces.[41]

Pharmacological and nutraceutical agents

One huge success was the discovery of the fungal statins, including compactin, lovastatin, pravastatin, and others which act as cholesterol-lowering agents. Lovastatin is produced by A. terreus. Of great importance in human medicine are the immunosuppressants such as cyclosporin A, sirolimus (rapamycin), tacrolimus, and mycophenolate mofetil. They are used for heart, liver, and kidney transplants and were responsible for the establishment of the organ transplant field. Cyclosporin A is made by the fungus Tolypocladium niveum. Mycophenolate mofetil is a semisynthetic product of the oldest known antibiotic, mycophenolic acid, and is also made by a fungus. Sirolimus and tacrolimus are products of streptomycetes [42].

Metabolites of probiotic bacteria are considered as a remedy for controlling weight gain, preventing obesity, increasing satiety, prolonging satiation, reducing food intake, reducing fat deposition, improving energy metabolism, treating and enhancing insulin sensitivity, and treating obesity. Firmicutes and Bacteroidetes are the dominant beneficial bacteria present in the normal human gastrointestinal tract, and the latter was reported in lower numbers in constipation-predominant irritable bowel syndrome patients [43].

Carotenoids of microbial origin are used as food colorant, fish feeds, nutraceuticals, cosmetics, and antioxidants. Food colorant widely used is carotene derived from Blakeslea trispora, Dunaliella salina and lycopene from B. trispora and Streptomyces chrestomyceticus, subsp. rubescens. Astaxanthin produced from Xanthophyllomyces dendrorhous is an approved fish feed. Astaxanthin, lutein, β-carotene, zeaxanthin, and canthaxanthin are used as nutraceuticals due to their excellent antioxidant property. Docosahexaenoic acid (DHA) used in infant formula as nutritional supplements is derived from microalgae Schizochytrium spp. [44].

Enzymes and enzyme inhibitors

Enzymes produced from microorganism have annual sales of US $ 2.3 billion enzymes that find application in detergents (34%), foods (27%), agriculture and feeds (16%), textiles (10%), and leather, chemicals, and pulp and paper (10%). The protease subtilisin used in detergents has an annual sale of $ 200 million. The other major enzymes include glucose isomerase (100,000 tons) and penicillin amidase (60,000 tons). Nitrilase (30,000 tons) and phytase are amounting for $135 million worth of production. Streptomyces glucose isomerase is used to isomerize D-glucose to D-fructose, to make 15 million tons per year of high fructose corn syrup, valued at $1 billion [45].

The most important enzyme inhibitors are clavulanic acid, synthesized by Streptomyces clavuligerus, the inhibitor of β-lactamases. Some of the common targets for other inhibitors are glucosidases, amylases, lipases, proteases, and xanthine oxidase. Amylase inhibitors prevent absorption of dietary starches into the body, and hence can be used for weight loss.

Agricultural and animal health products

Secondary metabolites find wide applications in the field of agriculture and animal health: kasugamycin and polyoxins are used as biopesticides; Bacillus thuringiensis crystals, nikkomycin, and spinosyns are used as bioinsecticides; bioherbicides (bialaphos) find application as bioherbicides; ivermectin and doramectin as antihelmintics and endectocides against worms, lice, ticks, and mites; ruminant growth promoters in the form of coccidiostats; plant hormones like gibberellins as growth regulators are the most common application [46].

1.6 Production of secondary metabolites from microorganisms

- Liquid fermentation Batch or fed-batch culture in submerged fermentation is employed for production of secondary metabolites. Inoculum is developed after careful strain improvement of producing organism. Initially, shake flasks culture is employed, and the culture which are in active growth phase are transferred to a small fermenter and later into a larger fermenter with production medium. Several parameters, like medium composition, pH, temperature, and agitation and aeration rate, are controlled [47,48]. An inducer such as methionine is added to cephalosporin fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in penicillin or erythromycin fermentation.
- Solid-state fermentation Solid-state fermentation, defined as a microbial culture that develops on the surface and at the interior of a solid matrix and in the absence of free water, holds an important potential for the production of secondary metabolites [49,50]. Two types of SSF can be distinguished, depending on the nature of solid phase used: (a) solid culture of one support-substrate phase solid phase and (b) solid culture of two substrate-support phase solid phase [51]. The advantages of solidstate fermentation in relation with submerged fermentation include: energy requirements of the process are relatively low, since oxygen is transferred directly to the microorganism. Secondary metabolites are often produced in much higher yields, often in shorter times, and often sterile conditions are not required.

2 Material and methods

2.1 Growth conditions and determination of metabolites

Klebsiella pneumoniae strain was isolated from bronchitis patients and obtained from Maternity and children hospital. Subcultures were obtained on the Nutrient Agar for 48 hrs. The mixture was incubated at 4ºC for 10 min and then shook for 10 min at 130 rpm. Metabolites was separated from the liquid culture and evaporated to dryness with a rotary evaporator at 45ºC.

The residue was dissolved in 1 ml methanol, filtered through a 0.2 µm syringe filter, and stored at 4ºC for 24 h before being used for GC-MS. The identification of the components was based on comparison of their mass spectra with those of NIST mass spectral library as well as on comparison of their retention indices either with those of authentic compounds or with literature values.

2.2 Spectral analysis of bioactive natural chemical compounds of *Klebsiella pneumoniae* using (GC/MS)

Analysis was conducted using GC-MS (Agilent 789 A) equipped with a DB-5MS column (30 m×0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA). The oven temperature was programmed as for the previous analysis. Helium was used as the carrier gas at the rate of 1.0 mL/min. Effluent of the GC column was introduced directly into the source of the MS via a transfer line. Ionization voltage was 70 eV and ion source temperature was 230oC. Scan range was 41- 450

amu. The components were identified by comparing their retention times to those of authentic samples of WILEY MASS SPECTRAL DATA BASE Library.

2.3 Determination of antibacterial activity

Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 25 µl of the samples solutions (Metabolites Produced by *Klebsiella pneumonia*) were delivered into the wells. The test pathogens (*Streptococcus pneumonia*, *Escherichia coli, Staphylococcus aureus, Proteus mirabilis* and *Staphylococcus epidermidis*) were swabbed in Muller Hinton agar plates. 90µl of fungal extracts was loaded on the bored wells. The wells were bored in 0.5cm in diameter. The plates were incubated at 37C° for 24 hrs and examined. Methanol was used as solvent control.

2.4 Data analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD and mean ± SE. IBM SPSS 20 version statistical software package was used for statistical analysis of percentage inhibition and disease incidence and disease severity in each case.

3 Results and discussion

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic extract of *Klebsiella pneumoniae*, shown in Figure 1-40**.** Peaks were determined to be Tricyclo[4.3.1.1(3.8)]undecan-1-amine, 3-Methoxybenzaldehyde semicarbazone, carboxaldehyde , 1-methyl-,oxime ,(Z)-(+), 1,5,5-Trimethyl-6-methylene-cyclohexene, 4-(2,5-Dihydro-3-methoxyphenyl)butylamine, Paromomycin , 9-Borabicyclo[3.31]nonane , 9-mercapto-, Benzenemethanol , 2-(2-aminopropoxy)-3-methyl, Acetamide , N-(6-acetylaminobenzothiazol-2-yl)-2-(adamantan, rin-6-carboxylic acid , 4-(2,5-Dihydro-3-methoxyphenyl)butylamine, N-(2,5-Dicyano-3,4-dihydro-2H-pyrrol-2-yl)-acetamide, 3,10-Dioxatricyclo [4.3.1.0(2,4)]dec-7-ene, 3-Cyclohex-3-enyl-propionic acid, Eicosanoic acid ,phenylmethyl ester, 3,7-Diazabicyclo[3.3.1]nonane , 9,9-dimethyl-, Dithiocarbamate , S-methyl-,N-(2-methyl-3-oxobutyl)-, dl-Homocysteine, 2-(2-Furyl)pyridine, 1,7-Dioxa-10-thia-4,13-diazacyclopentadeca-5,9,12-trione, 5,7-Dodecadiyn-1,12-diol, 1-(β-d-Arabinofuranosyl)-4-O-difluoromethyluracil, Uric acid, Pyrrolo[1.2-a]pyrazine-1,4-dione , hexahydro-,12-Methyl-oxa-cyclododecan-2-one, Phthalic acid , butyl undecyl ester, 9,12,15-Octadecatrienoic acid , 2,3-bis(acetyloxy)propyl ester, 1,2,4-Trioxolane-2-octanoic acid 5-octyl-, methyl ester, 12-Dimethylamino-10-oxododecanoic acid , Octahydrochromen-2-one, L-Aspartic acid , N-glycyl-,2H-Oxecin-2-one , 3,4,7,8,91,10-hexahydro-4-hydroxy-10-meth , Thiazolo[4,5-d]pyrimidine-5,7(4H,6H)-dione , 2-amino-4-(2-ph, Dec-9-en-6-oxo-1-ylamide, 3,6,12-Trimethyl-1,4,7,10,13,16-hexaaza-cyclooctadecane, 2-lodohiistidine, 2,5-Piperazinedione ,3,6-bis(2-methylpropyl)-, 9-Octadecenamide , (Z)-, 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetra.

3.1 Antibacterial activity

Clinical pathogens selected for antibacterial activity namely, *Streptococcus pneumonia*, *Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Staphylococcus epidermidis,* It were 4.09±0.013, 2.99±0.300, 4.37±0.200, 3.22±0.210, and 4.00±0.203 respectively for Bacterial products (Metabolites Produced by *Klebsiella pneumoniae*), while recorded 1.08±0.200, 0.97±0.116, 2.08±0.233, 3.04±0.261, 0.98±0.166 respectively for Bacterial products Streptomycin antibiotics, and recorded 1.02±0.180, 1.00±0.190, 2.08±0.236, 1.00±0.100, and 1.82±0.200 respectively for Kanamycin antibiotics.

Table 2 Bioactive chemical compounds identified in methanolic extract of *Klebsiella pneumoniae*

Bacterial products Antibiotics	**Zone of inhibition (mm)**				
	Bacteria				
	Streptococcus pneumonia	***Escherichia coli***	***Staphylococcus aureus***	***Proteus mirabilis***	***Staphylococcus epidermidis***
Bacterial products	4.09±0.013	2.99±0.300	4.37±0.200	3.22±0.210	4.00±0.203
Streptomycin	1.08±0.200	0.97±0.116	2.08±0.233	3.04±0.261	0.98±0.166
Kanamycin	1.02±0.180	1.00±0.190	2.08±0.236	1.00±0.100	1.82±0.200

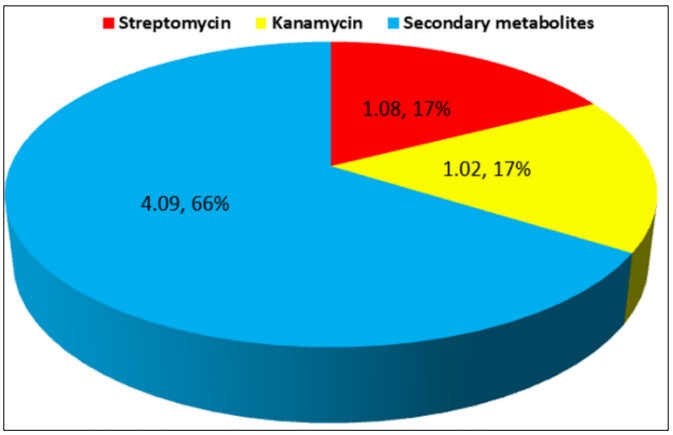

Figure 2. Metabolite products, Streptomycin and Kanamycin as anti- Bacterial activity against Streptococcus pneumonia

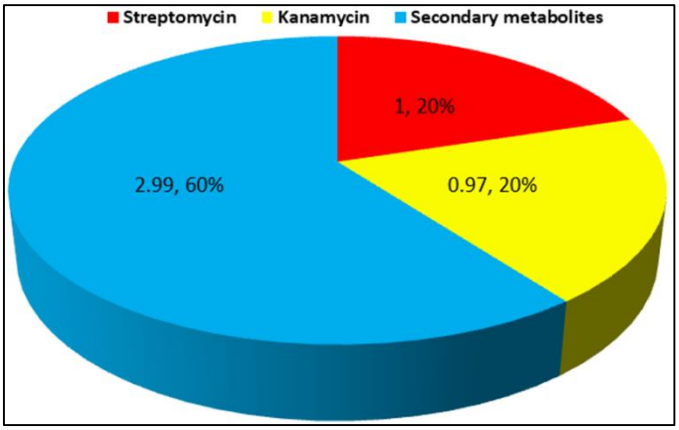

Figure 3. Metabolite products, Streptomycin and Kanamycin as anti- Bacterial activity against Escherichia coli

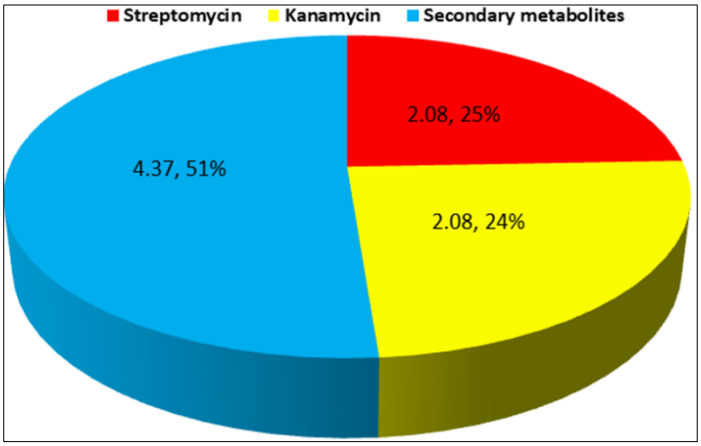

Figure 4 Metabolite products, Streptomycin and Kanamycin as anti- Bacterial activity against Streptococcus aureus

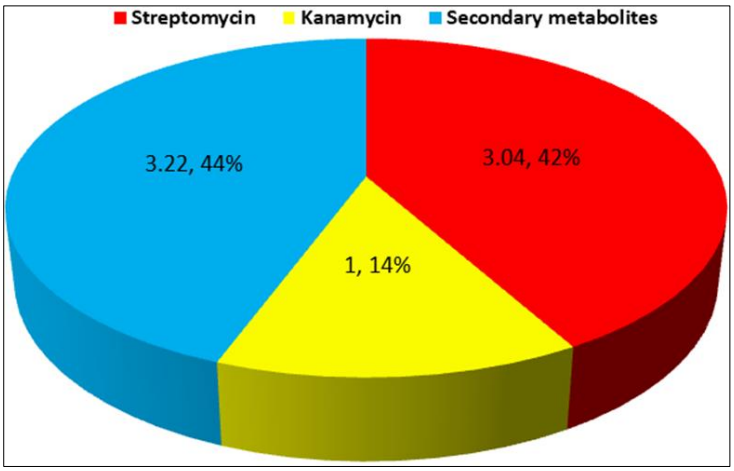

Figure 5 Metabolite products, Streptomycin and Kanamycin as anti- Bacterial activity against Proteus mirabilis

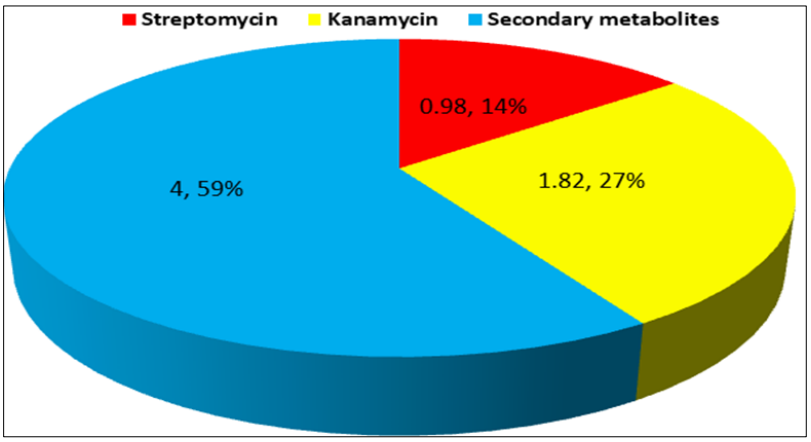

Figure 6 Metabolite products, Streptomycin and Kanamycin as anti- Bacterial activity against Streptococcus epidermidis

Maximum zone formation against *Staphylococcus aureus* (4.37±0.200) mm, Table 1. *Klebsiella pneumoniae* produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by *Klebsiella pneumonia* can be useful.

As a general rule, *Klebsiella* infections are seen mostly in people with a weakened immune system. Most often, illness affects middle-aged and older men with debilitating diseases. This patient population is believed to have impaired respiratory host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases, glucocorticoid therapy, renal failure, and certain occupational exposures (such as papermill workers). Many of these infections are obtained when a person is in the hospital for some other reason (a nosocomial infection). Feces are the most significant source of patient infection, followed by contact with contaminated instruments.

4 Conclusion

Microorganisms have the capability to produce a number of antibiotics and other pharmaceutically important drugs to treat bacterial and fungal infections, cancer, and heart-related diseases. Bacterial species exhibit a complex life cycle with a physiological and biochemical adaptability, with the capability to synthesize a great variety of secondary metabolites with complex structures using different metabolic pathways. Understanding the secondary metabolite biosynthesis and pathways will lead to progress in combinatorial biosynthesis in the pharmaceutical and biotechnology industries. Thirty nine bioactive chemical constituents have been identified from methanolic extract of the *Klebsiella*

pneumoniae by gas chromatogram mass spectrometry (GC-MS). *In vitro* antifungal and antibacterial evaluation of secondary metabolite products of *Klebsiella pneumoniae* forms a primary platform for further phytochemical and pharmacological investigation for the development of new potential antimicrobial compounds.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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