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Research Article

Characterization of Antifungal Secondary Metabolites Produced by *Klebsiella pneumoniae* and Screening of its Chemical Compounds Using GC-MS

Abeer Fauzi Al-Rubaye[1], Mohanad Jawad Kadhim[2], Imad Hadi Hameed[3*]

[1]*Department of Biology, College of Science for women, University of Babylon, Iraq*
[2]*College of Biotechnology, Department of Genetic Engineering, Al-Qasim Green University, Iraq*
[3]*College of Nursing, University of Babylon, Iraq*

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ABSTRACT

Bioactives were analyzed using gas chromatography-mass spectroscopy (GC-MS) techniques, then the in vitro antibacterial and antifungal activity of the methanolic extract was evaluated. Twenty two bioactive compounds were identified in the methanolic extract of *Klebsiella pneumoniae*. GC-MS analysis of *Klebsiella pneumoniae* revealed the existence of the 6,9,12-Octadecatrienoic acid , phenylmethyl ester , (Z,Z,Z)-, 5,7-Dodecadiyn-1,12-diol, 1,4 Decadiyne, 10,12-Octadecadiynoic acid, 1-Cyclopropyl-3,4-epoxyhex-5-en-1-yne, N,N-Dimethyl-3-methoxy-4-methylphenethylamine, Ethenetricarbonitrile , 3,4-xylidino, Pentyl glycolate, 3-(1,1'-Biphenyl-4-yl)butanenitrile, 4'-Amino-6-methoxyyaurone, Ethanone , 2,2'-(octahydro-2,3-quinoxalinediylidene)bis[1-phe, 1,1'-Bicyclohexyl , 4-methoxy-4'-propyl-, [1.4]Bipiperidinyl-4'-carboxamide , 1'-(chlorobenzenesulfony, 7H-Pyrrolo[2,3-d]pyrimidin-4-amine ,Vinylsulfonamide, 1-Phenyl-2-(4-methylphenyl)diazene 1-oxide, N-Benzyl-N-ethyl-p-isopropylbenzamide, 1-phenyl-2-(4-methylphenyl)-diazene 1-oxide, 1-Benzylindole, Isophthalic acid , di(2-methoxyethyl) ester, 1-Tert , butyl -3,3-bis(trifluoromethyl)diaziridine, 4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyram. *Datura stramonium* (Alkaloids) was very highly active (6.481±0.24) mm. The results of anti-fungal activity produced by *Klebsiella pneumoniae* showed that the volatile compounds were highly effective to suppress the growth of *Aspergillus flavus* (6.287±0.30). *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.

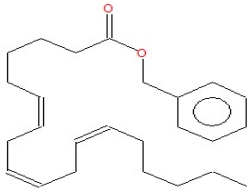
Keywords: Antifungal and antibacterial activity, *Klebsiella pneumoniae*, GC-MS, Secondary metabolites.

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[1], 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

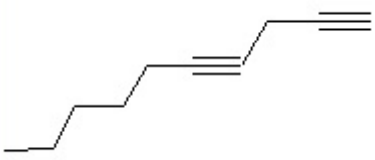
**Author for Correspondence: imad_dna@yahoo.com*

Table 1: Bioactive chemical compounds identified in methanolic extract of *Klebsiella pneumoniae*.

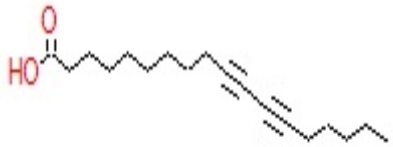


6,9,12-Octadecatrienoic acid, phenylmethyl ester , (Z,Z,Z)-
RT=1.486
Mw=368.27153
Pharmacological activity: antioxidant, anti-inflammatory, antimicrobial, pesticide and cancer preventive

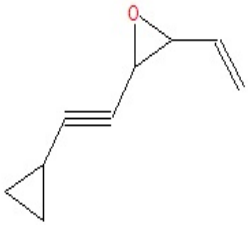
5,7-Dodecadiyn-1,12-diol
RT=1.538
Mw=194.13068
Pharmacological activity: Unknown



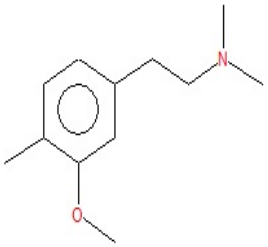
1,4-Decadiyne
RT=1.606
Mw=134.10955
Pharmacological activity: *anti*-inflammatory activity



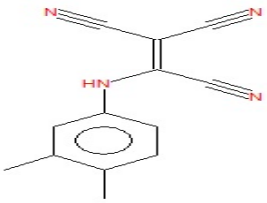
10,12-Octadecadiynoic acid
RT=1.641
Mw=276.208931
Pharmacological activity: anti-inflammatory, anticancer



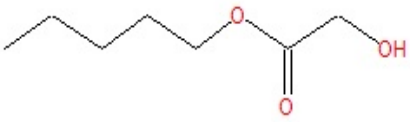
1-Cyclopropyl-3,4-epoxyhex-5-en-1-yne
RT=3.088
Mw=134.073165
Pharmacological activity: Unknown



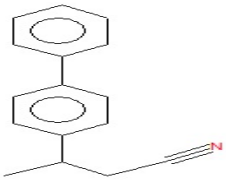
N,N-Dimethyl-3-methoxy-4-methylphenethylamine
RT=3.197
Mw=193.146665
Pharmacological activity: Unknown



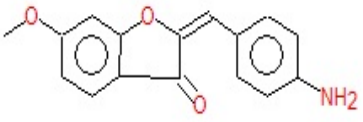
Ethenetricarbonitrile , 3,4-xylidino
RT=4.004
Mw=222.090547
Pharmacological activity: Unknown



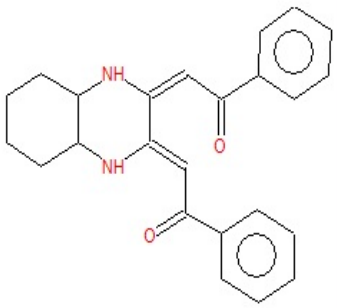
Pentyl glycolate
RT=4.462
Mw=146.094295
Pharmacological activity: Unknown



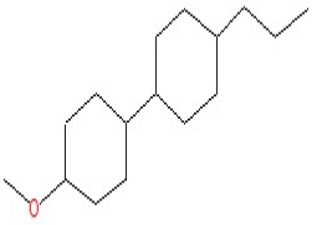
3-(1,1'-Biphenyl-4-yl)butanenitrile
RT=5.108
Mw=221.120449
Pharmacological activity: Unknown



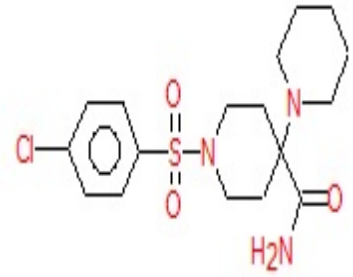
4'-Amino-6-methoxyyaurone
RT=4.919
Mw=267.089542
Pharmacological activity: anti-inflammatory



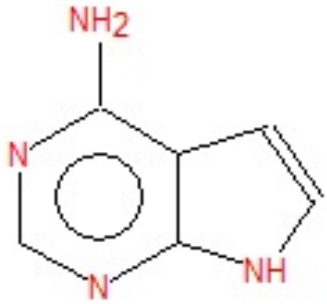
Ethanone , 2,2'-(octahydro-2,3-quinoxalinediylidene)bis[1-phe
RT=4.994
Mw=372.183779
Pharmacological activity: Unknown



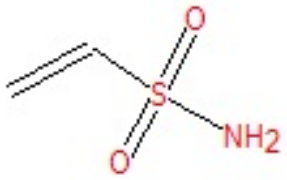
1,1'-Bicyclohexyl , 4-methoxy-4'-propyl-
RT=5.761
Mw=238.229666
Pharmacological activity: Unknown



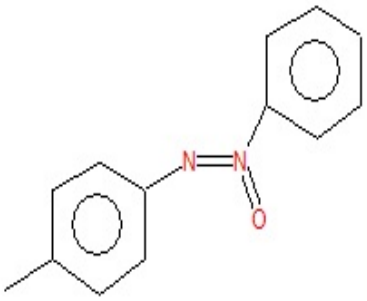
[1.4]Bipiperidinyl-4'-carboxamide , 1'-(chlorobenzenesulfony
RT=7.706
Mw=385.12269
Pharmacological activity: Unknown



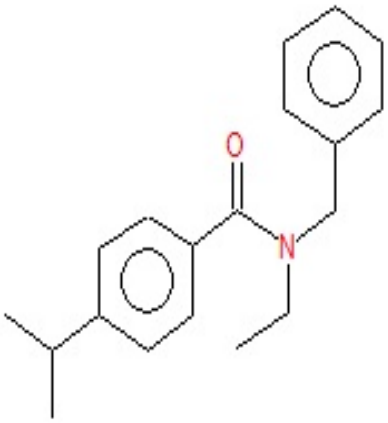
7H-Pyrrolo[2,3-d]pyrimidin-4-amine
RT=8.656
Mw=134.059246
Pharmacological activity: Anti-inflammatory activity, anticancer activity



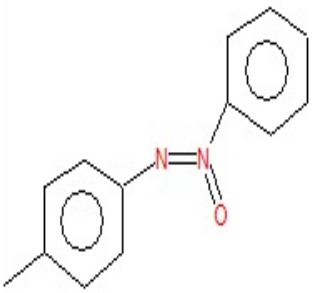
Vinylsulfonamide
RT=9.634
Mw=107.0040994
Pharmacological activity: anti-inflammatory activity



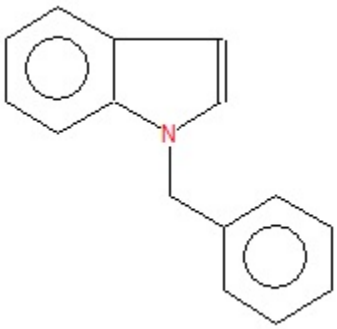
1-Phenyl-2-(4-methylphenyl)diazene 1-oxide
RT=9.806
Mw=212.094963
Pharmacological activity: anti-inflammatory



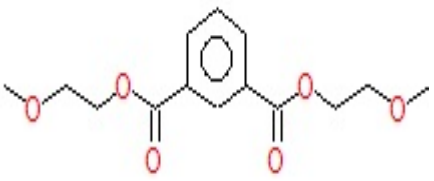
N-Benzyl-N-ethyl-p-isopropylbenzamide
RT=9.863
Mw=281.177965
Pharmacological activity: Anti-HCV activity



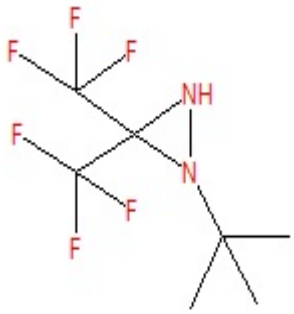
1-phenyl-2-(4-methylphenyl)-diazene 1-oxide
RT=9.943
Mw=212.094963
Pharmacological activity: anti-inflammatory activity



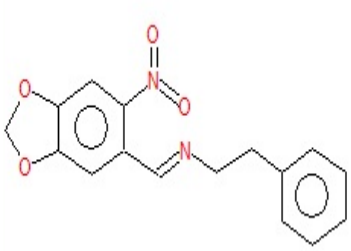
1-Benzylindole
RT=10.063
Mw=207.104799
Pharmacological activity: anti-proliferative activity



Isophthalic acid , di(2-methoxyethyl) ester
RT=10.218
Mw=282.110338
Pharmacological activity: anti-coagulant activity



1-Tert, butyl -3,3-bis(trifluoromethyl)diaziridine
RT=10.430
Mw=236.074818
Pharmacological activity:



4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyram
RT=14.807
Mw=298.095356
Pharmacological activity: anti-fungal potential

infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood.

MATERIALS AND METHODS

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. at 22℃. 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[14-23]. 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[24-29]. 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. The studied fungi, *Streptococcus faecalis*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Penicillium expansum*, *Aspergillus niger* and *Aspergillus terreus* were isolated and maintained in potato dextrose agar slants. Spores were grown in a liquid culture of potato dextrose broth (PDB) and incubated at 25ºC in a shaker for 16 days at 130 rpm. The extraction was performed by adding 25 ml methanol to 100 ml liquid culture in an Erlenmeyer flask after the infiltration of the culture[30-38].

Materials of Plants Collection and Preparation
In this study, the leaves were dried at room temperature for ten days and when properly dried the leaves were powdered using clean pestle and mortar, and the powdered plant was size reduced with a sieve[39-45]. The fine powder was then packed in airtight container to avoid the effect of humidity and then stored at room temperature.

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 (250oC). 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[46-50].

Determination of antibacterial and antifungal activity
Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 25 µl of the samples solutions *Ricinus communis* (Alkaloids), *Datura stramonium*(Alkaloids), *Linum usitatissimum* (Crude), *Anastatica hierochuntica* (Crude), *Cassia angustifolia* (Crude), *Quercus infectoria* (Crude), *Citrullus colocynthis* (Crude), *Althaea rosea* (Crude), *Coriandrum sativum* (Crude), *Origanum vulgare* (Crude), *Urtica dioica* (Crude) and *Punica granatum* (Crude) were delivered into the wells. The plates were incubated for 48 h at room temperature. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Methanol was used as solvent control. Amphotericin B and fluconazole were used as reference antifungal agent[48-53]. The tests were carried out in triplicate[51-55]. The antifungal activity was evaluated by measuring the inhibition-zone diameter observed after 48 h of incubation.

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.

RESULTS AND DISCUSSION

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic extract of *Klebsiella pneumoniae*, shown in Table 1. Peaks were determined to be 6,9,12-Octadecatrienoic acid, phenylmethyl ester, (Z,Z,Z), 5,7-Dodecadiyn-1,12-diol, 1,4 Decadiyne, 10,12-Octadecadiynoic acid, 1-Cyclopropyl-3,4-epoxyhex-5-en-1-yne, N,N-Dimethyl-3-methoxy-4-methylphenethylamine, Ethenetricarbonitrile, 3,4-xylidino, Pentyl glycolate, 3-(1,1'-Biphenyl-4-yl)butanenitrile, 4'-Amino-6-methoxyyaurone, Ethanone, 2,2'-(octahydro-2,3-quinoxalinediylidene)bis[1-phe, 1,1'-Bicyclohexyl, 4-methoxy-4'-propyl-, [1.4]Bipiperidinyl-4'-carboxamide, 1'-(chlorobenzenesulfony, 7H-Pyrrolo[2,3-d]pyrimidin-4-amine, Vinylsulfonamide, 1-Phenyl-2-(4-methylphenyl)diazene 1-oxide, N-Benzyl-N-ethyl-p-isopropylbenzamide, 1-phenyl-2-(4-methylphenyl)-diazene 1-oxide, 1-Benzylindole, Isophthalic acid, di(2-methoxyethyl) ester, 1-Tert, butyl -3,3-bis(trifluoromethyl)diaziridine, 4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyram. The results of anti-fungal activity produced by *Klebsiella pneumoniae* showed that the volatile compounds were highly effective to suppress the growth of *Aspergillus fumigatus*. *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

Table 2: Antifungal activity of *Klebsiella pneumoniae* metabolite products.

Fungi	Antibiotics / *Klebsiella pneumoniae* metabolite products			
	Klebsiella pneumoniae metabolite products	Amphotericin B	Fluconazol	Miconazole nitrate
Streptococcus faecalis	2.901±0.20 [a]	3.001±0.14	2.970±0.13	1.997±0.10
Aspergillus flavus	6.287±0.30	3.006±0.14	3.618±0.14	2.951±0.14
Aspergillus fumigatus	5.116±0.29	2.082±0.12	3.010±0.12	2.022±0.11
Candida albicans	4.000±0.19	2.998±0.13	2.911.±0.11	2.100±0.10
Penicillium expansum	4.093±0.20	3.007±0.15	2.900±0.11	2.301±0.13
Aspergillus niger	4.721±0.20	2.990±0.13	3.041±0.13	2.710±0.13
Aspergillus terreus	5.000±0.21	3.182±0.16	2.110±0.10	2.115±0.11

[a] The values (average of triplicate) are diameter of zone of inhibition at 100 mg/mL crude extract and 30 µg/mL of (Amphotericin B; Fluconazol and Miconazole nitrate).

Table 3: Zone of inhibition (mm) of test different bioactive compounds and standard antibiotics of medicinal plants to *Klebsiella pneumoniae*.

S. No.	Plant	Zone of inhibition (mm)
3.	*Ricinus communis* (Alkaloids)	4.902±0.18
4.	*Datura stramonium*(Alkaloids)	6.481±0.24
5.	*Linum usitatissimum* (Crude)	5.005±0.21
6.	*Anastatica hierochuntica* (Crude)	5.071±0.23
7.	*Cassia angustifolia* (Crude)	4.998±0.20
11.	*Quercus infectoria* (Crude)	5.830±0.22
12.	*Citrullus colocynthis* (Crude)	4.701±0.17
13.	*Althaea rosea* (Crude)	4.895±0.18
14.	*Coriandrum sativum* (Crude)	5.000±0.19
15.	*Origanum vulgare* (Crude)	5.861±0.23
16.	*Urtica dioica* (Crude)	5.001±0.18
19.	*Punica granatum* (Crude)	5.904±0.23
22.	Control	0.000

treatment of many diseases, the purification of compounds produced by *Klebsiella pneumonia* can be useful. Maximum zone formation against *Aspergillus flavus* (6.287±0.30) mm, Table 2.

ed medicinal

plants were effective against *Staphylococcus aureus*, Table 3. *Datura stramonium* (Alkaloids) was very highly active (6.481±0.24) mm against *Klebsiella pneumoniae*. *Klebsiella pneumoniae* was found to be sensitive to all test medicinal plants and mostly comparable to the standard reference antifungal drug Amphotericin B and fluconazole to some extent. Recently, it was demonstrated that volatile organic compounds (VOCs) of bacteria such as terpenoids, phenylpropanoids and fatty acid derivatives can influence the growth of some fungi and, in general, the inter- and intra-organismic communication signals. 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.

CONCLUSION

Tweny two 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.

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