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EXTRACTION AND CHARACTERIZATION OF LIPOPOLYSACCHARIDE FROM SERRATIA RUBIDAEA AND ITS CYTOTOXICITY ON LUNG CANCER CELL LINE-NCI-H69

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ABSTRACT: *The present study was carried out on Lipopolysaccride from Serratia rubidaea followed by its partial purification, characterization, evaluation of its Cytotoxicity on Lung Cancer Cell Line. LPS was extracted from Serratia rubidaea and detected by the addition of Schiff's reagent. The various subgroups such as 2-KDO, uronic acid and 4-amino arabinose were detected by paper chromatography and the subgroups such as Lipid A and free amino groups were detected by Thin layer chromatography. Partial purification of the extracted LPS was done by SDS-PAGE in which bands were obtained in ladder like patterns. The presence of fatty acids, esters, phosphates in LPS of S.rubidaea were revealed by GC-MS analysis. LPS was found to exhibit cytotoxicity on the lung cancer cell line NCI-H69.*
KEYWORDS: *LPS, Serratia rubidaea, NCI-H69, Cytotoxicity*

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the outer membrane contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. LPS is an endotoxin, and induces a strong response to animal immune systems. Lipopolysaccharides are of crucial importance to gram negative bacteria, and are therefore candidate targets for new antimicrobial agents. Gram-negative bacteria produce a variety of polysaccharides that are important in determining plant and animal-microbe interactions.

These polysaccharides include extracellular, capsular and the cell wall lipopolysaccharides (EPSs, CPSs, and LPSs, respectively). Bacterial lipopolysaccharides are commercially important in different industrial area as such as petroleum, textile, pharmaceutical, and fine chemical industry Sanford [1]. Bishop [2] large number of lipopolysaccrides is medically important, since they are immunogenic. LPSs are the major structural component of cells walls of gram-negative bacteria. They are unique biopolymers whose molecule contains a hydrophobic moiety (lipid A), which consists of fatty acids linked to a phosphorylated disaccharide (comprising two glucosamine residues), and hydrophilic carbohydrate domains. The latter include the core oligosaccharide (the central part of the LPS molecule), consisting of a certain number of monosaccharide residues (approximately10), and O-specific polysaccharide chains, consisting of repeated oligosaccharide units, whose structure may significantly vary in different strains of the same species. Serratia a genus of bacteria (tribe Serratieae) gram-negative, rod which produces a red pigment. Lipopolysaccride exhibit a numerous biological activity as antibiotics, helps in immune response, and anticancer property. Lipopolysaccharide exhibits a cytotoxic action on various types of cells via apoptosis[3-4]. In the present study LPS were extracted and partial purification were performed from selected species of S. rubidaea. The components present in LPS were analyzed by GC-MS. The anti cancer property of LPS were determined against lung cancer cell line.

MATERIALS AND METHODS

GROWTH OF BACTERIA AND EXTRACTION OF LPS

The selected Gram-negative isolate was grown in Brain heart infusion broth Fukushi et al. [5] for 24 hours at 37°C and the cells were harvested by centrifugation at 5000 rpm for 10 minutes. Extraction of LPS was carried by Westphal and Jann[6]. The bacterial suspension (5 g dry biomass in 100 ml), was mixed with of 90% phenol previously heated at 67°C, and incubated at 37°C in an orbital shaker at 160 rpm for 15 minutes. The mixture was then placed on ice to facilitate the separation of phases and centrifuged for 20 minutes at 5000 rpm.

The aqueous phase was collected. A second extraction was made on the mixture of phenol and the cellular pellet by addition of distilled water at 67°C. Both aqueous phases were combined and dialyzed against distilled water until the phenol was completely eliminated. The sample was then clarified by centrifugation at 10,000 rpm for 20 minutes at room temperature to eliminate the insoluble material.

LPS was concentrated by alcohol precipitation as follows; Sodium acetate was added at a final concentration of 0.15 M followed by addition of ice cold 96% ethanol drop by drop for a final sample to

ethanol proportion of 1:4. The mixture was incubated for 24 hours at -20°C. The pellet was then collected by centrifugation at 4000 rpm, and suspended at a concentration of 25 mg/mL in distilled water and lyophilized.

- **ANALYTICAL METHODS**

The subgroup present in LPS includes 2-Keto-3-deoxyoctonate (2-KDO), Uronic acids, 4-Amino-arabinose, free amino group and Lipid A. The methods used for identification of sub groups present in LPS are as follows

- **PAPER CHROMATOGRAPHY**

The sample was treated with 1% acetic acid at 100°C for 3hours. The treated sample was gently spotted in Whatman No.1 filter paper and the mobile phase (pyridine, acetic acid, and water in the ratio, 10:4:86, vol/vol/vol; pH 5.3) was allowed to run till it reaches three-fourth of the paper. The spots were detected by spraying 5% thiobarbituric acid for the presence of 2-Keto-3-deoxyoctonate as given by Weissbach and Hurwitz [7].

The sample was treated with 1N H_2SO_4 at 100°C for 4 h. The treated sample was gently spotted in Whatman No.1 filter paper and the mobile phase (pyridine, acetic acid, and water in the ratio, 10:4:86, vol/vol/vol; pH 5.3), chromatograms were developed by spraying 2% alkaline silver nitrate solution to confirm the presence of Uronic acids Dische[8]

The sample was treated with 10 N HC1 at 100°C for 15 seconds. The treated sample was gently spotted in Whatman No.1 filter paper and the mobile phase (pyridine, formic acid, acetic acid, and water in the ratio, 1:1.5:10:90 v/v/v/v; pH 2.8, the spots were detected by spraying ninhydrin solution to confirm the presence of 4-Amino-arabinose Rondle and Morgan [9].

- **TLC ANALYSIS**

Silica gel plate was spotted with 10 µl of sample containing extracted LPS and kept in a chamber containing phosphate buffer solution of pH 9-11, which act as a mobile phase. When the solvent run up to 10 cm the plate was removed and air dried for 10 minutes. The spots were then detected by spraying bromocresol green over the chromatogram to confirm the presence of Lipopolysaccharides Caroff et al.[10]

The Mobile phase for the amino group Kapustina et al. [11](chloroform, methanol, water and ammonium hydroxide, 100: 50: 8: 4 v/v). Bands were visualized by spraying 20%H_2SO_4 in methanol on the chromatogram followed by heating at 130°C for 10 minutes.

For Lipid A the solvent mixture containing chloroform, pyridine, 88% formic acid, methanol and water (60:35:10:5:2 v/v/v/v). When the solvent reaches 10 cm the plate was removed and air dried. The plate was then observed for the formation of waves Nanette et al.[12]

- **SDS-PAGE FOR LPS** (Modified procedure of Formsgaard et al. [13])

SDS-polyacrylamide gel (10 cm by 10 cm by 0.75 mm) containing 4% and 12.5% acrylamide in the stacking and separating gel was prepared and electrophoresis was done at 50mA for stacking gel and 100mA for separating gel until the tracking dye Bromophenol blue run about 10 cm. SDS-PAGE-fractionated LPS preparations were stained by the silver staining Gromova and Celis. [14]

- **GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS**

The LPS was analyzed by GC-MS (JEOL GCMATE II GC-MS and Finnigan Mat 8230 Mass Spectrometer). The GC column dimension used was 30X0.25mmX0.5mm AB-35MS fused silica capillary column. For GC the injector temperature was about 250°C, column temperature isothermal at 100°C then programmed to rise up to 250°C at 6°C/min and be held at this temperature for 10 minutes. The ion source temperature was 200°C and the interface temperature was 250°C. Helium gas was engaged as a carrier gas at the rate of 1ml/minutes. The spectra were obtained in the EI mode with 70eV ionization energy. The compounds were identified by comparing with the mass spectrum and matched with the inbuilt library.

- **CYTOTOXICITY OF LPS ON LUNG CANCER CELL LINE**

The Cytotoxic effect of LPS against NCI-H69 (small lung carcinoma) was assayed by MTT {3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazoliumbromide}. Cells were plated at 37°C for 24 hours on 24 well plate at a density of 10^3-10^4-cells per well, with Minimal Essential Medium (MEM) supplemented with 10% Fetal Calf Serum (FCS). Penicillin (100 units/ml), Streptomycin (100µg/ml) and Amphotericin B (5µg/ml) were added to the medium and maintained in a humidified atmosphere (5% CO_2) at 36°C. After 24 hours the cells were exposed to different concentration of LPS, 100µg/ml, 150µg/ml and 250µg/ml which were prepared by diluting from stock solution of 5mg/ml. The cells were incubated at 36°C in humidified incubator with 5% CO_2 for a period of 72 hours. Morphological changes of the cell culture were examined using an inverted microscope. After 72 hours cell viability was determined. Cytotoxicity of LPS was determined by plotting percentage cell viability against concentration of LPS.

RESULTS AND DISCUSSIONS

The bacterium used in this study was isolated from soil sample by serial dilution. Pink colored colonies were obtained on nutrient agar. The morphological and biochemical characterization of the bacterium revealed the isolated organism to be Serratia rubidaea. The selected organism was inoculated in brain heart infusion broth for the LPS extraction by

hot phenol water extraction method. LPS was concentrated by alcohol precipitation. The extracted LPS were preliminarily detected by using Schiff's reagent. On adding Schiff's reagent to the extracted sample, the color changed from pink to red indicating the presence of LPS. The presence of impurities such as protein, DNA and RNA were also analyzed in the sample. Protein was found to be present in negligible amount whereas DNA and RNA were found to be absent in the sample.

The subgroups such as 2-KDO, uronic acid, 4-amino arabinose, were identified using paper chromatographic techniques. The LPS, free amino group, Lipid A were identified by using TLC. The presence of 2-KDO was confirmed by color change to light yellow on spraying with 5% thiobarbituric acid. Uronic acid was detected by spraying alkaline silver nitrate solution during which orangish red colored spots were developed. On spraying ninhydrin solution, amino sugar formed brown wave's pattern.

LPS was detected by spraying bromocresol green over TLC chromatogram. The extracted LPS formed clear round zones which confirmed its presence in the sample. Charring was observed when TLC plate loaded with sample was sprayed with 20%H_2SO_4 in methanol followed by heating at 130°C.Charring resulted due to the presence of free amino groups. The presence of Lipid A was confirmed by the formation of reddish brown wave like patterns due to reaction of the loaded sample with the solvent system containing chloroform, pyridine, 88% formic acid, methanol and water.

The presence of the LPS was confirmed by SDS-PAGE. The SDS-PAGE procedure was standardized for LPS and a modified silver staining method specific for LPS was employed. After running the gel staining was done. Ladder like patterns of bands were obtained which is characteristic of LPS Formsgaard et al. [15] The whole gel became light brown because of cold staining solution Plate 1.

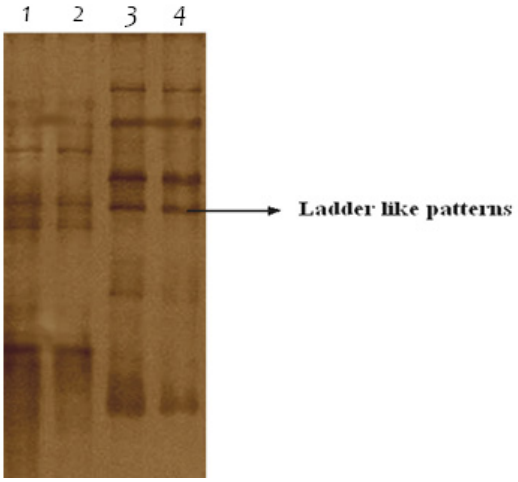

Plate 1. Lipopolysaccride separation by SDS-PAGE
Lane 1-20µl, Lane 2-25µl, Lane 3-30µl, Lane 4-30µl

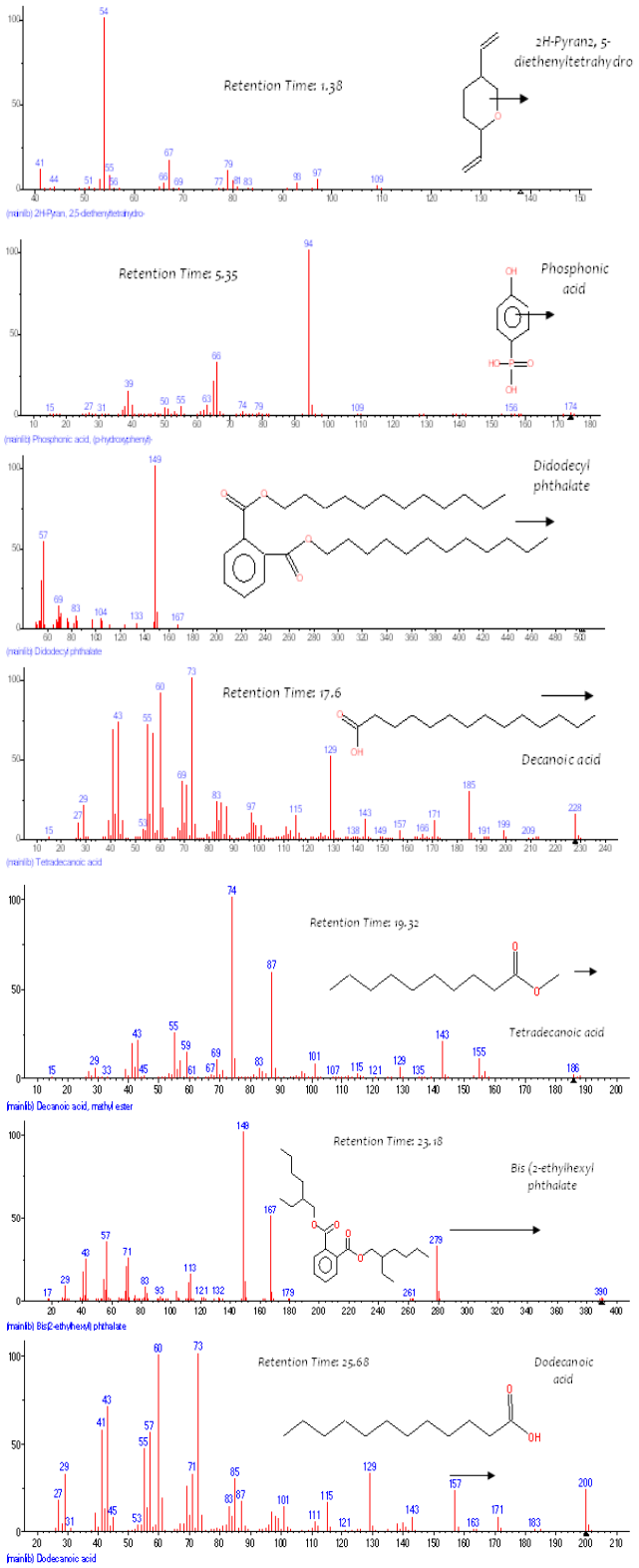

Figure 1: Mass spectra of lipopolysaccharide

The components of LPS were characterized by using GC-MS. The amount of LPS obtained from 5 g of S.rubidaea was found to be 0.25 mg. The GC-MS analysis resulted in chromatogram and m/z spectrum. The chromatogram was analyzed for the Hit compounds by comparison with the Hit library with the help of NIST'08 software. The component present in LPS of S. rubidaea was determined by comparison of m/z (mass/energy) spectrum as well as with the hit Library given in Figure 1. The components were found

to be 2H-Pyaran,2, 5-diethenyltetrahydro(rt-1.38), Phosphonic acid (rt-5.32), Didodecyl phthalate(rt - 12.62), Decanoic acid (methyl ester) (rt-17.6), Tetradecanoic- acid (rt-19.32), Bis(2-ethylhexyl) phthalate (rt-23.18), Dodecanoic acid (rt-25.68).

The area- percentage, chemical formula and the molecular weight of the compounds are shown in Table 1. The mass spectra of each peak are given in Figure 2. As seen earlier with case of E. coli Re-mutant LPS, the initially eluting molecular species contained 3-hydroxytetradecanoic and dodecanoic acid. In later fractions tetradecanoic and hexadecanoic acids appeared Werner [16].

Table 1: Compounds Present In LPS

Retention Time	Compound Name	Formula	Molecular Weight	Area (%)
1.38	2H-Pyaran,2,5-diethenyltetrahydro	$C_9H_{14}O$	138	6.8
5.35	Phosphonic acid	$C_6H_7O_4P$	174	1.7
12.62	Didodecyl phthalate	$C_{32}H_{54}O_4$	502	6.84
17.6	Decanoic acid, (methyl ester)	$C_{11}H_{22}O_2$	186	7.83
19.32	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	2.31
23.18	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	6.50
25.68	Dodecanoic acid	$C_{12}H_{24}O_2$	200	4.58

The Cytotoxicity of LPS was analyzed by treating the lung cancer cell line-NCI-H69 with different concentrations of LPS such as 100 µg, 150 µg, and 250 µg. MTT assay was followed to determine the Cytotoxicity. Before carrying out the Cytotoxicity assay, all the samples were filtered through 0.45 µm syringe filter.

The cell viability was gradually decreased from 100µg/ml concentration to 250 µg/ml concentrations. The lowest cell viability of 48.88 was observed at 250 µg/ml concentration.. The picture of LPS untreated cell line (control) and LPS treated cell line are shown in Plate 2A, B, C, D. Prakash et al., [17]investigated the effect of β-carotene on the morphology of NCI-H69 small lung cancer cell line.

Muto et al. [18] studied the effect of β-carotene at a concentration of 10 µmol/L on human cervical cancer cell line, CICCN-2. They reported that β-carotene caused chromatin condensation a characteristic property of apoptosis. Stryokova[19] studied the effect of LPS prepared from a strain Salmonella typhi isolated from a carrier on sheep erythrocytes. Erythrocytes were treated with alkali-hydrolyzed, peroxidase labeled LPS.

The membrane morphology of LPS treated erythrocytes was studied by electron microscopy. The study revealed a change of typical shape of erythrocyte to oval shape.

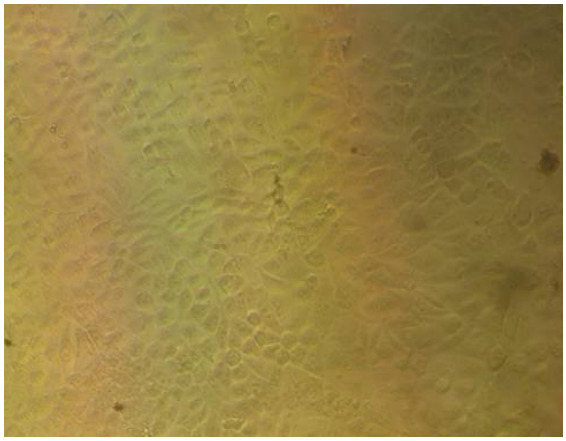

Plate 2A-Control

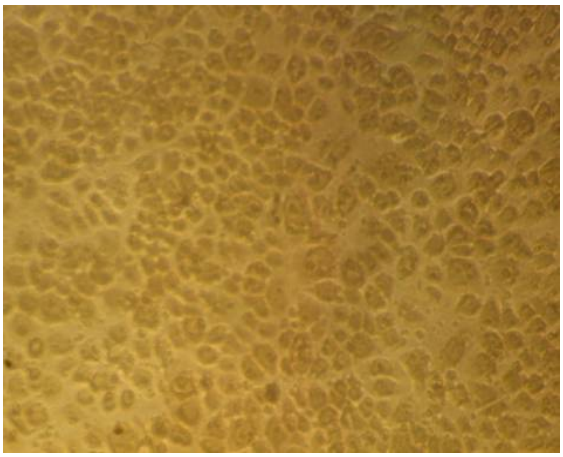

Plate 2B (Conc-100µgLPS/ml)

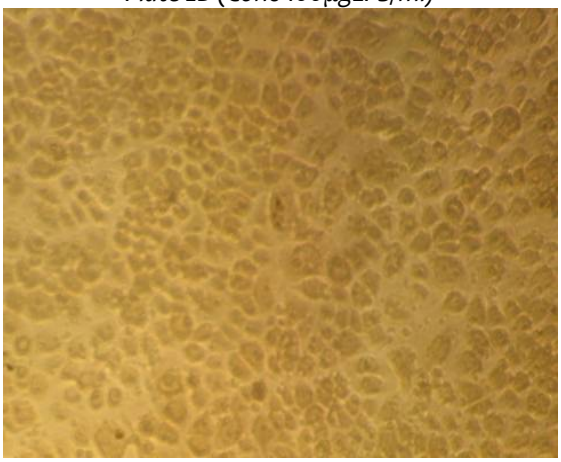

Plate 2C (Conc-150 µgLPS/ml)

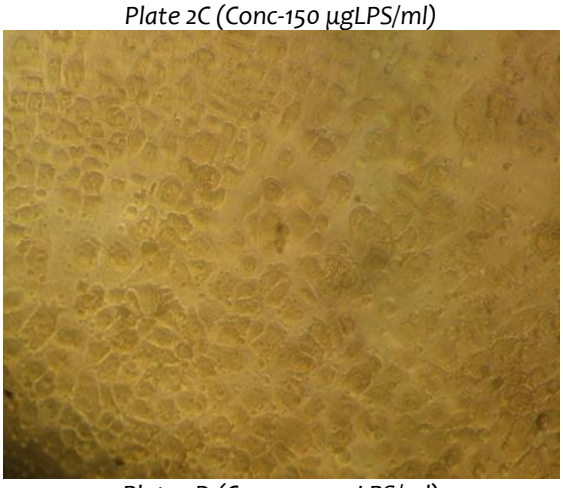

Plate 2D (Conc-250 µgLPS/ml)

Plate 2: MTT Assay of Lps
On Human Lung Cancer Cell Line-NCI-H69

The stability of membrane system was also found to be decreased resulting in hemolytic of erythrocytes. The cytokine-inducing capacity of LPS and its Lipid A from Coxilla burnetti strain Priscilla on human Mono Nuclear Cells (MNC) was analyzed.

The human MNCs were stimulated with Lipid A and TNF α production of the cells was determined in the supernatant by sandwich-ELISA using monoclonal anti-human TNF α antibody Toman et al. [20]

CONCLUSIONS

LPS was found to exhibit cytotoxicity on the lung cancer cell line NCI-H69. Of the various concentrations used, percentage cell viability was found to be less at 250µg. Cytotoxicity was determined by MTT assay. Thus on increasing the concentration of the LPS extract, the percentage cell viability was found to be reduced. Thus from the present study it is revealed that the LPS of Serratia rubidaea has cytotoxic properties. These properties of LPS can be used in the development of drugs for cancers. The study may be further extended to identify the pathways of LPS that have given rise to anti tumor activity.

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