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Separation and Identification of a New Glycolipoprotein Produced by *Bacillus Psychrosaccharolyticus* Against *Sclerotium Rolfsii*

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Abstract: *Sclerotium rolfsii* is a devastating plant pathogenic fungus infecting over 500 plants species in 100 families. Out of 217 bacterial isolates isolated from Egyptian soil, *Bacillus psychrosaccharolyticus* showed maximum inhibition effect against *S. rolfsii* in both Petri dishes and pots experiments. The bacterial culture filtrate of *B. psychrosaccharolyticus* was evaporated to dryness under vacuum and the remaining residue was extracted by different solvents with increasing polarity. The methanol extract only showed inhibition effect against *S. rolfsii*. HPLC separation was optimized to separate the methanol extract components. Two columns (C18 and CN) were tested with different mobile phases. The optimum separation conditions were C18 column; methanol mobile phase; flow rate 0.5-ml/min. and detection wavelength 220nm. HPLC analysis of this extract showed presence of 11 compounds. The residue remaining after evaporation of methanol was extracted by different mixtures of methanol and chloroform starting from 5: 95 to 100: 0. The maximum inhibition effect was obtained from the methanol: chloroform 20: 80 extract. The residue remaining after evaporation of this extract was dissolved in methanol. Chloroform was added drop-wise to gradually precipitate the dissolved compounds. Three layers were obtained in which the active layer was the upper sticky layer. The nature of this layer was identified as a protein. Therefore, the ammonium sulfate precipitate method was used to separate the components of this layer. Six precipitates were obtained, in which the first precipitate (at 51.9% saturation) was the most active against *S. rolfsii*. HPLC analysis of this precipitate showed three compounds in which the most abundant was that having Rt. 4.1 min. This compound was separated by water extraction of the ammonium sulfate precipitate. The inhibition test showed that this was the most active compound. Electrophoresis analysis of the pure compound confirmed its purity and showed that the molecular weight was 11.9 KD. The compound was identified as glycolipoprotein. It consists of 7.6 % total carbohydrates and 24.4% total fatty acids. GC mass analysis showed that the sugars were Glucose, galactose and ribonic acid. The amino acids were proline, hydroxy proline, phenylalanine and leucine. The fatty acids were tetradecanoic acid (C14); pentadecanoic acid (C15); 14-methyl-pentadecanoic acid; palmitic acid (C16); 9-hexadecenoic acid (C16:1); heptadecanoic acid (C17), heptadecamonoenoic acid (C17:1); and stearic acid.

Key words: *S. rolfsii, B. psychrosaccharolyticus*, glycolipoprotein.

INTRODUCTION

In biological control, the reduction of number of harmful organisms (insects, weeds or plant pathogens) is carried out by using their natural enemy. The biological control mechanisms include competition for nutrient resources or infection sites, antibiosis, hyperparasitism and induced host resistance (Cook and Baker, 1983). A number of biological control mechanisms can be involved in the control of soil-borne plant pathogens by microbial antagonists (El-Tarabily *et al.* 2000). Several studies were carried out to separate and identify the antagonistic compounds from the producing microorganisms (Brigitte *et al,* 2008; Masami *et al,* 2008; Masayuki et al, 2008; Md. Zakir *et al,* 2008; Naoya *et al,* 2008; Sloan *et al,* 2008 and Stephen 2008). The nature of these antagonistic compounds is varied. It may be volatile compounds, antibiotics, siderophores or enzymes (Mahmoud 2000).

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Sclerotium rolfsii is a devastating plant pathogen infecting over 500 plants species in 100 families (Punja, 1985, Punja 1988, Sarma *et al*., 2002 and Azhar *et al*., 2003). It attacks diverse phases of the development of his hosts, from the seed to the agricultural products. It is able to survive and thrive within a wide range of environmental conditions. Growth is possible within a broad pH range (5-8). The mycelium growth occurs between 20 and 35 °C (Azhar *et al*., 2003). This fungus is able to produce sclerotia, which can survive for several years. In a previous study (Abdel Azeiz *et al,* 2003) we selected a bacterial isolate identified as *Bacillus psychrosaccharolyticus* out of 217 bacterial isolates, isolated from Egyptian soil, showed the maximum inhibition effect against *S. rolfsii* in both Petri dishes and pots experiments.

The aim of the present study was to separate and identify the antagonistic compound which is produced by *Bacillus psychrosaccharolyticus* against *Sclerotium rolfsii*.

MATERIALS AND METHODS

Materials:

- All microbial media (Potato Dextrose Agar (PDA), Nutrient Agar and Nutrient Broth) were obtained from International Diagnostics Group. - Silica gel 60 was obtained from Merck co.
- Neutral alumina was obtained from Sigma.
- The pathogenic fungus *Sclerotium rolfsii* was obtained from Plant Disease Division, Faculty of Agriculture, Ain Shams University. It was maintained on potatoes dextrose agar medium. -- The antagonistic bacterium *Bacillus psychrosaccharolyticus* was isolated from the rhizosphere of cucumber plant, obtained from El-Mansheyh village, El-Aiatt center, El-Giza governorate. It was maintained on nutrient agar.

Methods:

Production of the Antagonistic Compounds:

The antagonistic bacterium *B. psychrosaccharolyticus* was grown in nutrient broth medium for 2 days in shacking incubator at 28 °C and 150 rpm. Ten ml of the culture filtrate was taken for inhibition test to know if this volume contains a considerable amount of the antagonistic compounds or not.

Extraction of the Antagonistic Compounds from Bacterial Culture Filtrate:

1) 50 ml of the bacterial culture was centrifuged at 300 rpm. The culture filtrate was extracted three times by the same volume of either ethyl acetate or chloroform.
2) One liter of the culture filtrate was evaporated to dryness under vacuum using rotary evaporator at 50 °C. The remaining residue was extracted sequentially three times by different solvents with increasing polarity. These solvents were: acetone, methanol, ethanol, acetonitrile and water. Each solvent extract was evaporated to dryness using rotary evaporator at 50 °C and the remaining residue was used for inhibition effect test.

The Inhibition Test:

A weight of each residue -corresponding to 10 ml medium- is dissolved in 10 ml of distilled water and 0.4 g of PDA powder was added. The media were sterilized in autoclave at 121°C for 10 min. and poured into 5-cm diameter Petri dish. After solidification, it was inoculated with a disk of *Sclerotium rolfsii* taken from 3-day-old PDA culture of *S. rolfsii*. The dishes were incubated at 28 °C for five days. The fungal growth was measured and compared with control growth.

Uv-visible Wavelength Scanning:

The methanol extract of the culture filtrate residue (the active fraction) was scanned using Beckman DU 640 spectrophotometer. This was carried out to determine the optimum detection wavelength for HPLC analysis.

- HPLC Analysis:

It was carried out using Hewlett Packard HPLC 1100, equipped with G1322A degasser, G1311 quaternary pump, G1313A auto sampler, G1316A column thermostat and G 1314A variable wavelength detector. The optimization of the HPLC separation method was carried out using two types of columns: C18 and CN columns 250mm X 4.6 X 5um. Different mobile phases were tested with both columns. It was methanol, acetonitrile, tetrahydrofuran, methanol: tetrahydrofuran (75: 25 and 50: 50) and methanol: dichloromethane (75: 25 and 50: 50). Different detection wavelengths were used: 220, 254 and 310nm. as showed from the spectrophotometer scanning.

Biuret Test:

This test was used to identify the protein nature of the active residue. It was carried out as sited in Furniss *et al,* (1978).

Purification of the Methanol Extract:

The purification was carried out through three steps:

1- Liquid Solid Extraction:

The methanol extract was evaporated under vacuum and the remaining residue was extracted by different mixtures of methanol and chloroform. Starting with methanol: chloroform 5: 95, followed by 10: 90, 20: 80, 30: 70, 40: 60, 50: 50, 60: 40, 80: 20 and finally with 100% methanol. Each extract was evaporated to dryness under vacuum and the remaining residue was used in inhibition test against *S. rolfsii.*

2- Fractional Precipitation by Chloroform:

In order to purify the active fraction (methanol: chloroform 20: 80 extract); chloroform was added gradually (drop-wise) until formation of the first precipitate. The precipitate was separated by centrifugation. Each fraction was tested in inhibition test.

3- Fractional Precipitation by Ammonium Sulfate:

The ammonium sulfate was grind in a glass mortar to be a fine powder that easily to dissolve. This powder was added gradually to the antagonistic solution (~0.02g each time). Each amount was not added until the previous amount was completely dissolved. The solution was shacked using a vortex. The formed precipitate was separated by centrifugation. The amount of ammonium sulfate was noted for each precipitate and calculated as saturation percent. Each precipitate was dissolved in water and re-precipitated again by ammonium sulfate as a purification step. (Shawn 1991) Each precipitate was dissolved in methanol and centrifuged to remove any residue of ammonium sulfate. This step was repeated several times until the removing all amounts of ammonium sulfate. Each precipitate was used for inhibition test and analyzed by HPLC.

Chemical Analysis of the Pure Active Compound:

1- Determination of Total Carbohydrates:

Total carbohydrates were determined by phenol sulfuric method as described by Dubois *et al.* (1956).

2- Determination of Total Fatty Acids Content:

Three ml of alcoholic 2N KOH solution was added to a known weight of the pure compound in a test tube. The tubes was covered and left overnight for Saponification. The saponified sample is then neutralized by adding four ml of 2N sulfuric acid. The tube was shacked well. The free fatty acids were extracted by diethyl ether several times. The combined ether extracts were washed with water several times, and then evaporated to dryness under a stream of nitrogen at 40°C. The remaining fatty acids were then weight and its percentage was calculated as described by Hamilton 1992.

3- GC/MS Analysis:

This instrument was used for determination of amino acids, sugars and fatty acids in the pure antifungal glycolipoprotein. The GC/Mass instrument was Trace GC 2000 produced by Thermo.; mass spectrometer model SSQ 7000 produced by Finningan. The separation conditions were: DB-5 Column 30m X 0.25 mm X 0.25 µm. Mobile phase helium at flow rate1.5 ml/min. Injection chamber temperature 220 ˚C. Oven temperature starts at 80 ˚C for 1 min., to 10˚C at a rate of 10˚C/ min., and finally to 300˚C at a rate of 6˚C/min. The ionization mode of the mass detector at 70 ev.

a- Amino Acids Analysis:

A known weight of the pure antifungal glycolipoprotein was put into a 10 ml screw cap glass tube with a Teflon cap. Two ml of 6 N HCl were added and the tube was filled with nitrogen gas and tightly capped. The mixture was hydrolyzed at 110 ˚C for 20 hr. and then evaporated to dryness at 50˚C under stream of nitrogen. This residue of amino acids was derivatized by adding one ml of methanolic HCl solution (prepared by mixing methanol and acetyl chloride in a 20:1 ratio immediately before use) in a 15-ml screw glass tube with a teflon cap and mixed gently. The reaction was allowed to proceed in an oven at 70˚C for 30 min. After cooling the excess reagent was removed by evaporating to dryness at 40 ˚C under stream of nitrogen. One ml of methylene chloride was added, in order to remove traces of water, swirled gently and evaporated to dryness

at 40 ˚C under stream of nitrogen. Trifluoroacetic anhydride (0.5 ml) was added, and the tube was capped, and set in an oven at 140 ˚C for 10 min. The excess reagent was removed by evaporating to dryness at 40 ˚C under stream of nitrogen. The final product was dissolved in 100 µl of methanol. Two µl. Of this solution was injected into the GC/MS. Gary 1990.

b- Sugars Analysis:

One ml of the pure compound methanolic solution was added into a 1ml screw-topped vial and evaporated under a stream of nitrogen at 40 °C to dryness. Two ml of 1N HCl were added and the solution was hydrilyzed at 100°C for 6hr in an oven. The solution was evaporated to dryness at 40°C under stream of nitrogen. After that, 0.5 ml of isopropanol (HPLC grade) was added to remove any residue of water, shacked gently, and evaporated to dryness under a stream of nitrogen at 40 °C. 250 µl of oximation solution (2.5 percent of hydroxyl amine hydrochloride in anhydrous pyridine) was added and put in an oven at 80 °C for half an hour. After cooling, 0.5 ml of silylation reagent (trimethylchlorosilane (TMS): N,N-O bis-(trimethylsilyl) acetamide ,1:5 by volume) was added and put in an oven at 80 ˚C for half-hour. Ronald and Ronald 1991.

4- Electrophoresis Analysis:

Both native and denaturing electrophoresis analysis were carried out using Bio-Rad mini-gel electrophoresis apparatus. The analysis was carried out to determine the purity and the molecular weight of the glycolipoprotein compound by the method sited in Daniel and Stuart 1991.

RESULTS AND DISSCUSION

Inhibition Test by the Bacterial Culture Filtrate:

The inhibition test by using 10 ml of the bacterial culture filtrate showed a positive result. This indicates the presence of a considerable amount of the antagonistic compounds in this volume of culture filtrate. It also indicated that, the nature of the antagonistic compounds is not either enzymes or volatile compounds because it still active after sterilization.

Extraction of the Antagonistic Compounds:

Both chloroform and ethyl acetate extracts of the culture filtrate didn't show any inhibition effect. This reflects the polar fashion of the antagonistic compounds. Therefore, the culture filtrate was evaporated to dryness under vacuum and the remaining residue was extracted by different solvents with increasing polarity. The residue remaining after evaporation of methanol extract only showed inhibition effect against *S. rolfsii.* The spectrophotometer scanning for the methanol extract showed two wavelengths at 254 nm and 220 nm. that have the maximum absorbance. These two wavelengths were used as detection wavelengths in HPLC analysis of this extract.

HPLC Analysis:

The optimum HPLC separation was obtained by using ODS (C18) column. Mobile phase: methanol. Flow rate: 0.5-ml/ min. and detection wavelength 220 nm. The HPLC for the methanol extract showed presence of 11 compounds. Their retention times are 4.3, 4.5, 4.7, 4.9, 5.3, 5.5, 5.9, 6.4, 6.6, 6.8, and 7.1 which is the major compound as appeared from the peaks areas (figure 1).

Purification of the Methanol Extract:

- Liquid -Solid Extraction:

The methanol extract was evaporated under vacuum and the remaining residue was extracted by different mixtures of methanol and chloroform. Starting with methanol: chloroform 5: 95, followed by 10: 90, 20: 80, 30: 70, 40: 60, 50: 50, 60: 40, 80: 20 and finally with 100% methanol. The first four extracts only showed inhibition effect, but the extract No. 3 was the strongest. Therefore, it was suggested that, the antagonistic compounds could be extracted from the culture filtrate residue by methanol: chloroform 20: 80. This extract was exposed to wavelength scanning. The maximum absorbance was obtained at 310 nm and 410 nm. The HPLC analysis of this extract was carried out using these two detection wavelengths. HPLC analysis showed that, 310 nm was the best detection wavelength. The extract contains only two major compounds that having retention times 3.9 and 4.03 min. Their percentages were 35.3% and 36.4% respectively (figure 2).

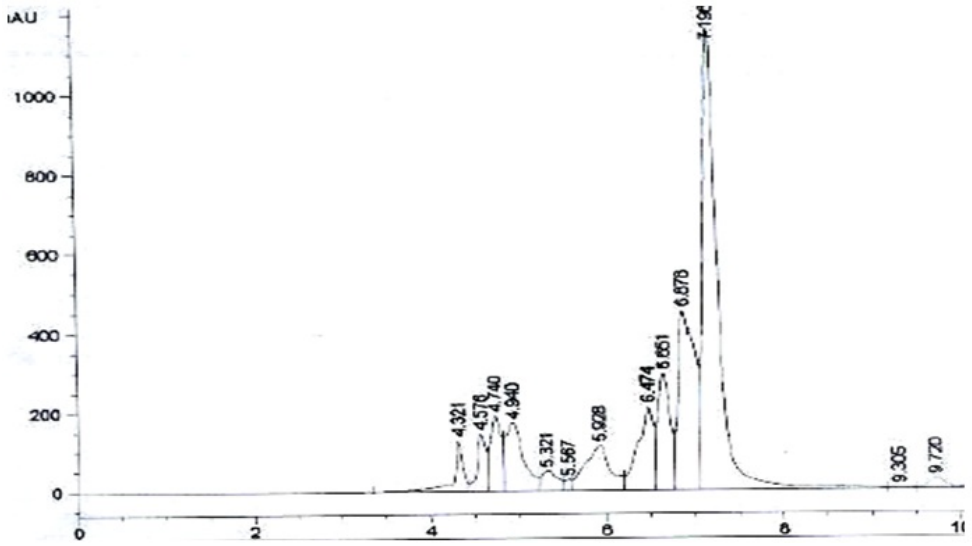

Fig. 1: The HPLC chromatogram of the methanol extract of the culture filtrate residue. The separation conditions are: Column: C18, Mobile phase: methanol, Flow rate: 0.5ml/ min. and detection wavelength: 220 nm.

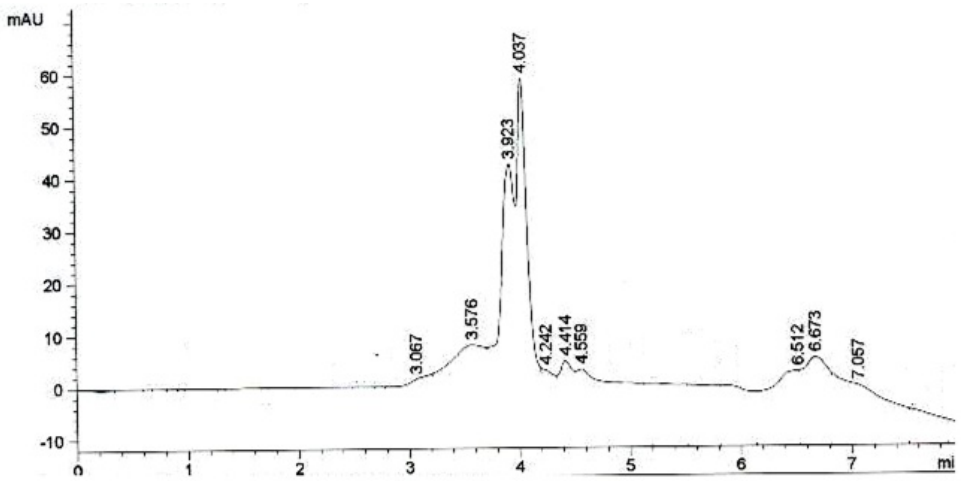

Fig. 2: HPLC chromatogram of the methanol: chloroform 20: 80 extract of the methanol residue. The separation conditions are: Column C18, Mobile phase: methanol, Flow rate: 0.5ml/ min. and detection wavelength 310 nm.

Fractional Precipitation by Chloroform:

Purification of the active fraction of the previous test (methanol: chloroform 20: 80 extract) was carried out by adding chloroform gradually (drop-wise) until forming of a precipitate. The precipitate was separated by centrifugation. Three layers were formed. The upper was sticky, the middle was a powder and the lower was the chloroform solution. The upper layer only showed inhibition effect against *S. rolfsii.* HPLC analysis showed that the major two compounds were stile that having Rt 3.9 and 4.02min.

Fractional Precipitation by Ammonium Sulfate:

Biuret test described the nature of this sticky layer as a protein or protein containing compound. Therefore, was suggested to purify this layer by ammonium sulfate precipitation method. Six precipitates were obtained at ammonium sulfate saturation percentages 51.9%, 79.4%, 81.8%, 83.6%, 98.7% and 100% respectively. This was in addition to the compounds presented in the solution after saturation. All of these seven fractions were analyzed by HPLC and used in inhibition test against *S. rolfsii*. The first precipitate only showed the maximum

inhibition percentage (50%), while the inhibition percentages for the other precipitates ranged from 4 to 5% only. HPLC analysis of the first precipitate showed the presence of three compounds. Their retention times were 3.4, 3.5 and 4.1 min. which was the most abundant.

The compound (Rt. 4.1) was separated from this mixture by water extraction because it has high water solubility than the other two components. The water extract was analyzed by HPLC to confirm the purity of this compound (figure 3).

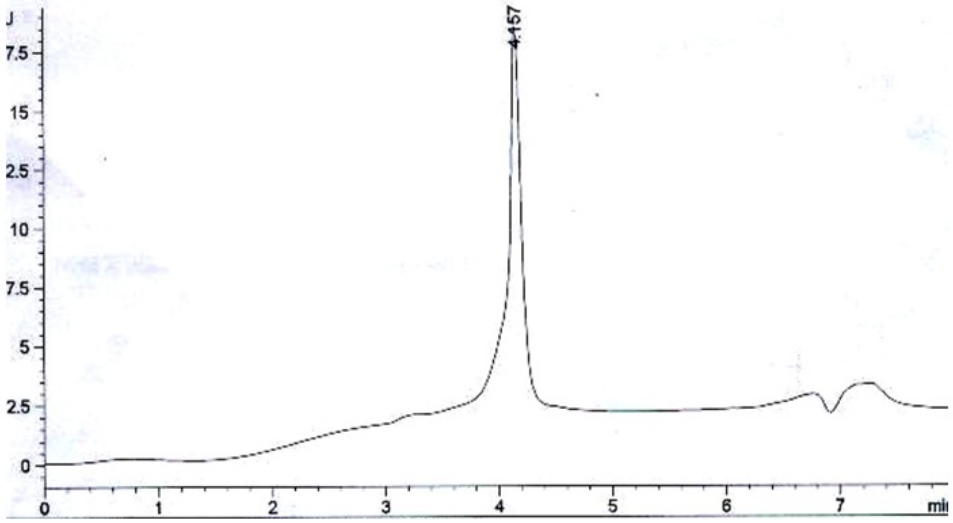

Fig. 3: HPLC chromatogram of the active pure compound that has Rt. 4.1 min. The separation conditions are: Column C18, Mobile phase: methanol, Flow rate: 0.5ml/ min. and detection wavelength 310 nm.

The inhibition test was carried out using the pure compound Rt. 4.1 and the other two compounds. The compound Rt. 4.1 showed 52% inhibition percentage while the mixture of the other two compounds showed 32% inhibition. Therefore, this compound was chosen for studding its chemical composition in detail.

Electrophoresis Analysis:

The electrophoretic pattern shown in figure (4), confirmed the purity of this compound since one band only was obtained. The molecular weight was 11.9 K D approximately. This compound, after digestion with mercaptoethanol and SDS, during sample preparation for denaturing electrophoresis analysis, showed one band only but it has lower molecular weight ~ 6.6 KD. This may be due to the loss of a non-protein moiety in the presence of the detergent SDS. GC/MS analysis was carried out to identify the nature of the additional components.

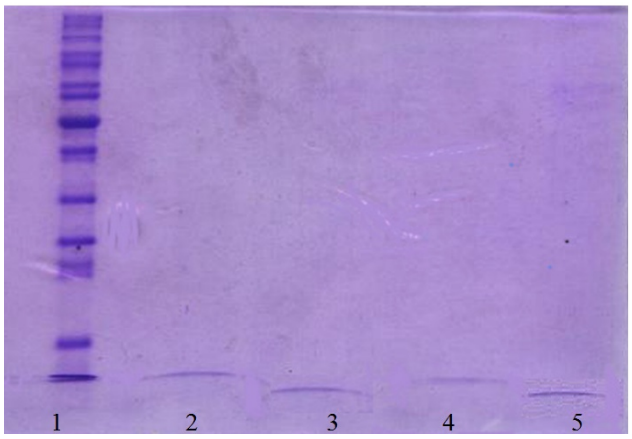

Fig. 4: The electrophoresis gel of the active compound (Rt. 4.1). (1) Protein standard molecular weights. (2) and (4) are the undigested compound. (3) and (5) are the digested compound.

GC/MS Analysis:

GC analysis showed presence of three sugar types (Glucose, galactose and ribonic acid). The most abundant of them was Glucose followed by galactose and ribonic acid respectively. The GC/MS analysis of amino acids content showed presence of four amino acids (proline, hydroxy proline, phenylalanine and leucine.

The most abundant of them was proline followed by hydroxy proline, phenylalanine and leucine. GC/MS analysis of fatty acids content showed presence of 8 fatty acids. It were tetradecanoic acid (C14); pentadecanoic acid (C15); 14-methyl-pentadecanoic acid; palmitic acid (C16); 9-hexadecenoic acid (C16:1); heptadecanoic acid (C17), heptadecamonoenoic acid (C17:1); and stearic acid. The most abundant of them were C15 followed by C16 respectively, as noticed from the peaks area.

This active glycolipoprotein (Rt. 4.1) was a sticky compound. It has a light brown color and has a maximum absorbance at 230 nm when dissolved in methanol. It consists of 7.6% total sugars and 24.4% total fatty acids.

The glycolipoprotein nature of microbial products were found by other researchers such as Leila *et al.,* (1981) on *Pseudomonas aeruginosa* which is a human pathogen. The extracellular slime glycolipoprotein (GLP) produced by this strain induces a tumor necroses factor (TNF-α) production by human monocytes. The analysis of this GLP showed that it consists of Hexose 12%, Hexosamine 20%, Uronic acid 4%, Protein 18% and Lipid 30%. While the work obtained by Anastassiou *et. al.,* (1987) about the slime material from *Pseudomonas aeruginosa* strain isolated from a patient with bacteremia, was found to contain 16% uronic acids, 48.5% carbohydrates, 11% protein, and 2% lipids. The sugars contents were galactose, glucose, and ribose, with trace amounts of rhamnose and mannose. Lathigra *et al.,* (1996) reported that the pathogenic bacterium *Mycobacterium tuberculosis* produces a 19-kDa glycolipoproteins. George *et. al.,* (2003) found that the slime glycolipoproteins of *Pseudomonas aeruginosa* consists of: neutral sugars 22.33%, Hexosamines 11.4%, Uronic acid 6.5%, Protein 15.8%. The sugars contents (g/100g total sugars) were glucose 36%, mannose 22%, galactose 25%, rhaminose 8.4%, fucose 5% and xylose 4%.

Several researchers separated and identified new protein in nature antimicrobial compounds. Naoya *et al,* (2008) separated and identified two new peptide antibiotics, unnarmicins C (1) and A (2), from the fermentation broth of a marine bacterium, *Photobacterium sp.* Both compounds selectively inhibited the growth of two strains belonging to the genus *Pseudovibrio spp.* Brigitte *et al,* (2008) separated two new secondary metabolites, named pedein A and B, from the cell mass of the myxobacterium *Chondromyces pediculatus*. Their planar structures were elucidated by spectroscopic methods, in particular 2D NMR as 24-membered cyclic hexapeptides composed of a variable tryptophan residue, glycine, sarcosine and three unusual hydroxy β- and γ-amino acids. Pedein A, strongly inhibited the growth of yeasts and fungi, induced hemolysis of erythrocytes. Masayuki *et al,* (2008) Separated a novel cyclic peptide antibiotic, pargamicin A, from the culture broth of an actinomycete strain. Pargamicin A showed potent antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis*/*faecium* strains.

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