Antibiotic Production by *Streptomyces hygroscopicus*, M 121 Isolated from Kingdom of Saudi Arabia

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Abstract: Carriomycin is an polyether antibiotic that active against Gram-positive and unicellular and filamentous fungi. It is biosynthesized in this research by *Streptomyces hygroscopicus*, M 121was isolated from soil sample in Al-Khurmah governorate. It is active in *vitro* against some microbial pathogenic viz: *Staph. aureus*, NCTC 7447; *Micrococcus luteus*, ATCC 9341; *Bacillus subtilis*, NCTC 10400; *Bacillus pumilus* NCTC 8214; *S. cerevisiae* ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus fumigatus*, ATCC 16424; *Aspergillus flavus*, IMI 111023; *Aspergillus niger*, IMI 31276; *Fusarium oxysporum* ; *Botrytis fabae* ; *Rhizoctonia solani* and *P. chrysogenum*. The production media was optimized for maximum yield of secondary metabolites. The active metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation of the active ingredient of the antimicrobial agent and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis and spectroscopic characteristics have been investigated. This analysis indicates a suggested imperical formula of $C_{47}H_{80}O_{15}$. The chemical structural analysis with UV, IR, Mass and NMR spectra analyses confirmed that the compound produced by *Streptomyces hygroscopicus*, M 121 is Carriomycin antibiotic.
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Keywords: Carriomycin antibiotic; *Streptomyces hygroscopicus*; Fermentation; Purification and Biological Activities.

1. Introduction

The resistance of a large number of pathogenic bacteria and fungi to bioactive secondary metabolites in common use is presently an urgent focus of research, and new antifungal and antibacterial molecules are necessary to combat these pathogens [Fguiraa *et al.*, 2005 and Baltz, 2007]. The emergence of antibiotic resistance is an evolutionary process that is based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal (Cowen, 2008). Filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of a high number of bioactive natural products with biological activity, which are extensively used as pharmaceuticals and agrochemicals [Rogers, 2008]. These filamentous bacteria produce about 75 % of the commercially and medically useful antibiotics [Miyadoh, 1993], and approximately 60 % of antibiotics developed for agricultural use were isolated from *Streptomyces* species as well [Tanaka and Ômura 1993]. Actinomycetes especially streptomyces are the most economically and biotechnologically valuable prokaryotes [Sunaryanto *et al.*, 2010]. They are responsible for the production of about half of the discovered bioactive secondary metabolites [Berdy, 2005], notably antibiotics [Strohl, 2004], antitumor agents [Cragg *et al*., 2005] and immunosuppressive agents [Mann, 2001 and Pearson and Carol, 2008].

Carriomycin, a polyether antibiotic, was isolated from culture broth of *Streptomyces hygroscoprcus* strain T-42082. It is active against Gram-positive bacteria, several fungi, yeasts and mycoplasma [Selvameenal *et al.*, 2009]. Moreover, Imada *et al.*, (1977) reported that, the elemental analysis gave the following values: free acid, C 64.01, H 9.30, 0 26.04 (%); sodium salt, C 62.27, H 8.99, Na 2.50 (%); potassium salt, C 61.62, H 8.79, K '-.25 (%). The free acid of carriomycin occurs as colorless prisms having the molecular formula $C_{47}H_{80}O_{15}$ (M.W. 885.15), and m.p. 120-122^{0}C. It has no characteristic absorption maxima in the ultraviolet spectrum. Infrared absorption spectra of carriomycin as follows: free acid, 2940, 1700, 1462, 1383, 1085, 972 cm^{-1}; sodium salt, 2935, 1610, 1461, 1382, 1080, 972 cm^{-1}. Peaks at 1700 cm^{-1} and 1610 cm^{-1} correspond to COON and COO-, respectively.

In the present study, the production of the bioactive substances that demonstrated inhibitory affects against pathogenic microorganisms, from *Streptomyces hygroscoprcus* M 121 was reported. The objective of this research was production, purification and Biological activities of antibiotic produced by *Streptomyces hygroscoprcus*, M 121.

2. Material and Methods

2.1. Test organisms

2.1.1. Gram Positive: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *Bacillus pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341.

2.1.2. Unicellular fungi: *Saccharomyces cerevisiae*, ATCC 9763 and *Candida albicans* IMRU 3669.

2.1.3. Filamentous fungi: *Aspergillus fumigatus*, ATCC 16424; *Aspergillus flavus*, IMI 111023; *Aspergillus niger*, IMI 31276; *Fusarium oxysporum* ; *Botrytis fabae* ; *Rhizoctonia solani* and *P. chrysogenum*.

2.2. Media

The solid medium for slants and isolation was composed of (g/l): glucose, 10.0; malt extract, 3.0; yeast extract, 3.0; peptone 5.0; and agar, 20.0. The pH of the medium was adjusted to 7.2-7.4 before sterilization. The seed medium was composed of (g/l): glucose, 20.0; malt extract, 6.0; peptone, 6.0; and NaCl, 0.2. The pH of the medium was adjusted to 7.0-7.2 before sterilization. The antibiotic production medium was composed of (g/l): soluble starch, 20.0; KNO_3, 2.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.7; KCl, and $CaCO_3$, 0.5. The pH of the medium was adjusted to 7.4 before sterilization.

2.3. Fermentation

A loopful of the, *Streptomyces hygroscoprcus*. from the 5-day culture age was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of antibiotic production medium (seven flasks). The flasks were incubated on a rotary shaker (200 rpm) at 30 ^{0}C for 5 days.

Twenty-liter total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 r.p.m for 20 minutes. The clear filtrates were tested for their activities against the test organisms [Sathi *et al.,* 2001].

2.4. Extraction

The culture broth was centrifuged to remove the biomass. The cell-free supernatant was adjusted at different pH values (4 to 9) and extracted with an equal volume of organic solvent, 1:1 (v/v). Four extraction solvents were tested for effectiveness, including chloroform, ethyl acetate, dichloromethane and n-butanol. Each organic extract was concentrated to dryness under vacuum using a rotary evaporator [Atta, 2010]. The resulting dry extract was recuperated in 1 ml of DMSO and bioassayed against test organisms, by paper disk diffusion method [Kavanagh, 1972].

2.5. Precipitation

The precipitation process of the crude compound dissolved in the least amount of the solvent carried out using petroleum ether (b.p 60-80 °C) followed by centrifugation at 5000 r.p.m for 15 min. The precipitate was tested for its antimicrobial activities [Atta *et al.,* 2009].

2.6. Separation

Separation of the antimicrobial agent(s) into its individual components was conducted by thin layer chromatography. A dry crude extract, dissolved in DMSO, was spotted and developed in the solvent system n-butanol-acetic acid-water (3:1:1, v/v). The developed TLC plates were air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence) and the active spots detected by bioautography [Betina, 1973].

2.7. Purification

The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform-methanol-water (2:3:1, v/v/v), was used as an eluting solvent. The column was left for overnight until the silica gel (BDH – 60-120 mesh) was completely settled. One-ml crude precipitate to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities [Atta *et al.,* 2009].

2.8.Physico-chemical properties of the antimicrobial agent

2.8.1. Elemental analysis

The elemental analysis C, H, O, N, and S was carried out at the Microanalytical Center, Cairo University, Egypt.

2.8.2. Spectroscopic analysis

The IR, UV, Mass and NMR spectra were determined at the Microanalytical Center, Cairo University and National Research Centre, Dokki, Egypt.

2.8.3. Biological activity

The minimum inhibitory concentration (MIC) could be determined by the cup assay method [Kavanagh, 1972 and Zamanian *et al*., 2005].

2.8.4. Characterization of the antimicrobial agent

The antimicrobial agent produced by *Streptomyces hygroscopicus*, M 121 was identified according to the recommended international references of [Imada et *al.,* 1977; Umezawa, 1977 and Berdy, 1974; 1980a b & c].

3. Results

3.1. Fermentation and Separation of the antimicrobial agent

The fermentation process was carried out for five days at 30°C using liquid starch nitrate medium as production medium. Filtration was conducted followed by centrifugation at 5000 r.p.m. for 15 minutes. The clear filtrates containing the active metabolite (20 liters), was adjusted to pH 7.0 then the

extraction process was carried out using n-Butanol at the level of 1:1 (v/v). The organic phase was collected, and evaporated under reduced pressure using rotary evaporator. The residual material was dissolved in the least amount of DMSO and filtered. The filtrates were test for their antimicrobial activities. The antimicrobial agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 5000 r.p.m for 15 minute where a whitish yellow oil precipitate could be obtained. Separation of the antimicrobial agent(s) into individual components was carried out by thin-layer chromatography using a solvent system composed of n-butanol-acetic acid-water (3:1:1, v/v). Among three bands developed, only one band at R_f 0.8 showed antimicrobial activity. The purification process through column chromatography packed with silica gel indicated that the most active fractions against the tested organisms ranged 22 to 28.

3.2. Physicochemical characteristics of the antimicrobial agent

The purified antimicrobial agent produces characteristic odour, their melting point is 120°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in water, petroleum ether, hexane and benzene.

3.3. Elemental analysis

The elemental analytical data of the antimicrobial agent(s) revealed the following: C= 64.02%; H= 9.29%; 0 26.04%; N= 0.0 % and S= 0.0%. This analysis indicates a suggested empirical formula of $C_{47}H_{80}O_{15}$.

3.4. Spectroscopic characteristics

In the IR spectrum, the peaks at 975, 1082, 1300, 1386, 1465, 1703, 1812, 1983, 2080, 2465, 2527, 2945, 3410 & 3750cm^{-1}. Peak at 1703 cm^{-1} correspond to COON (Fig.2). The UV spectrum showed that the active compound dissolved in methanol-water the absorbance peak at wavelengths 278 nm (Fig. 3). The mass spectroscopy revealed that the molecular weight is 885 (Fig.4). The NMR-spectrum were also determined (Fig.5).

3.5. Biological activities of the antimicrobial agent

Data of the antimicrobial agent spectrum indicated that the agent is active against Gram-positive and unicellular and filamentous fungi (Table 1). The MIC of antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the antibiotic produced by *Streptomyces hygroscopicus*, M 121 against: *Staph. aureus*, NCTC 7447 and *Bacillus subtilis*, NCTC 10400 15.73 µg/ ml); *Micrococcus luteus*, ATCC 9341; *Bacillus pumilus*, NCTC 8214 and *S. cerevisiae* ATCC 9763 (31.25 µg/ ml); *Candida albicans*, IMRU 3669; *Fusarium oxysporum* and *Rhizoctonia solani* (46.9 µg/ ml); *Aspergillus niger* IMI 31276; *Asp. flavus*, IMI 111023 and *Botrytis fabae* (52.7 µg/ ml); *Aspergillus fumigatus*, ATCC 16424 (93.75 µg/ ml) and *P. chrysogenum* (> ١٠٠ µg/ ml).

3.6. Identification of the antimicrobial agent

On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antimicrobial agent, it could be stated that the antimicrobial agent is suggestive of being belonging to Carriomycin "polyether " antibiotic (Table 2).

Table 1. Biological activities (MIC) of the antimicrobial agent by paper method assay.

Test organism		Mean diameters of inhibition zone (mm)
A-Bacteria		
Gram Positive		
Staphylococcus aureus,	NCTC 7447	**15.73**
Micrococcus luteus,	ATCC 9341	**31.25**
Bacillus subtilis,	NCTC 1040	**15.73**
Bacillus pumilus,	NCTC 8214	**31.25**
B- Fungi		
1-Unicellular fungi		
Candida albicans,	IMRU 3669	**46.9**
Saccharomyces cervicea	ATCC 9763	**31.25**
2-Filamentous fungi		
Asp. niger,	IMI 31276	**52.7**
Asp. fumigatus,	ATCC 16424	**93.75**
Aspergillus flavus,	IMI 111023	**52.7**
Fusarium oxysporum		**46.9**
Botrytis fabae		**52.7**
Penicillium chrysogenum		> ١٠٠
Rhizoctonia solani		**46.9**

Table 2. A comparative study of the characteristic properties of the antimicrobial agent in relation to reference Carriomycin (polyether) antibiotic

Characteristic	Purified antibiotic	Carriomycin antibiotic
1- Melting point	120°C	120-122°C
2- Molecular weight	885	885.15
3- Chemical analysis (%):		
C	64.02	64.01
H	9.29	9.30
N	0.0	0.0
O	26.04	26.04
S	0.0	0.0
4- Ultra violet	278	ND
5- Formula	$C_{47}H_{80}O_{15}$	$C_{47}H_{80}O_{15}$
6- Active against	Gram-positive bacteria and several fungi and yeasts	Gram-positive bacteria and several fungi and yeasts

ND=No data

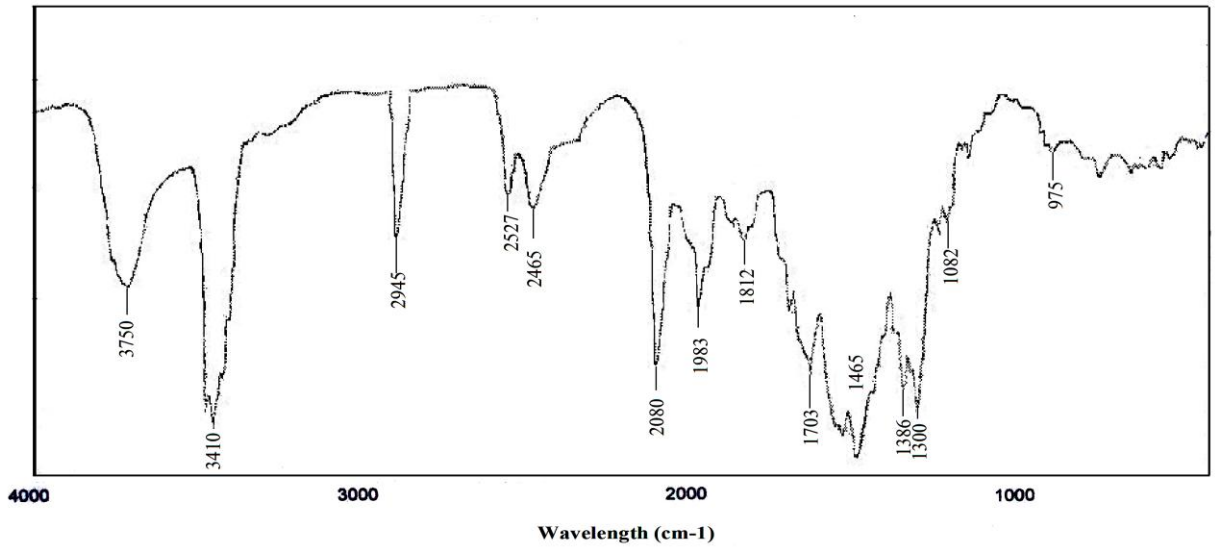

Figure 2. I.R spectrum of antimicrobial agent produced by *Streptomyces hygroscoprcus*.

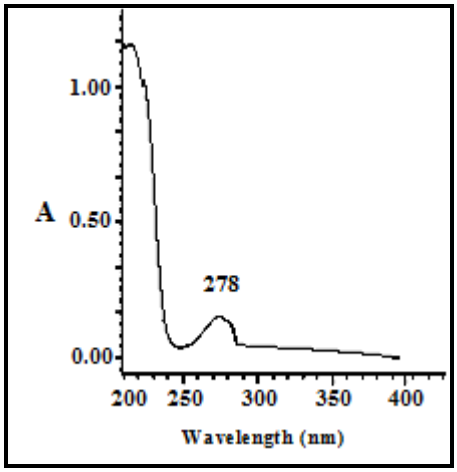

Figure 3. Ultraviolet absorbance of antimicrobial agent produced by *Streptomyces hygroscoprcus.*

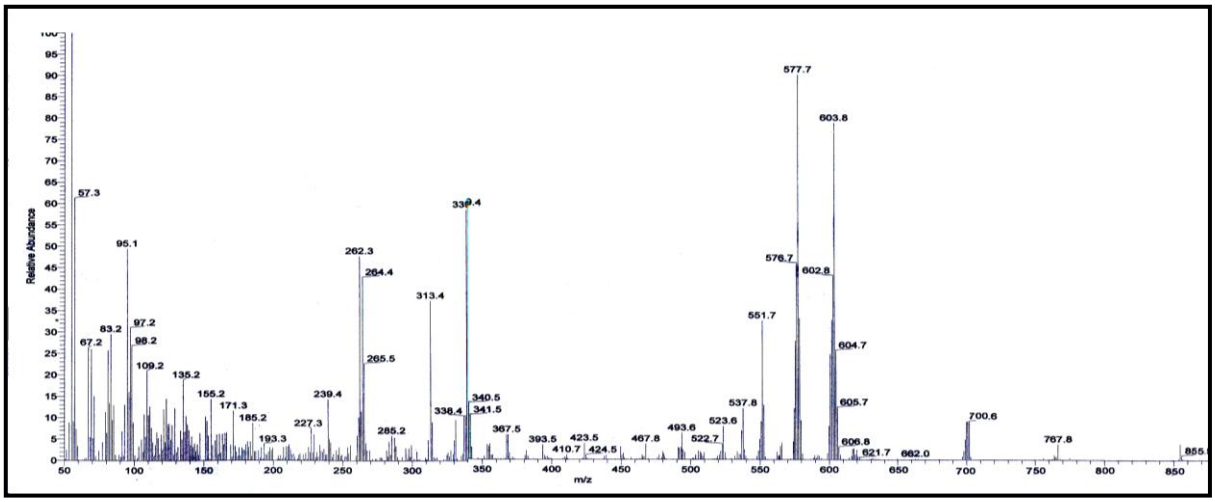

Figure 4. Mass-Spectrum of antimicrobial agent produced by *Streptomyces hygroscoprcus*.

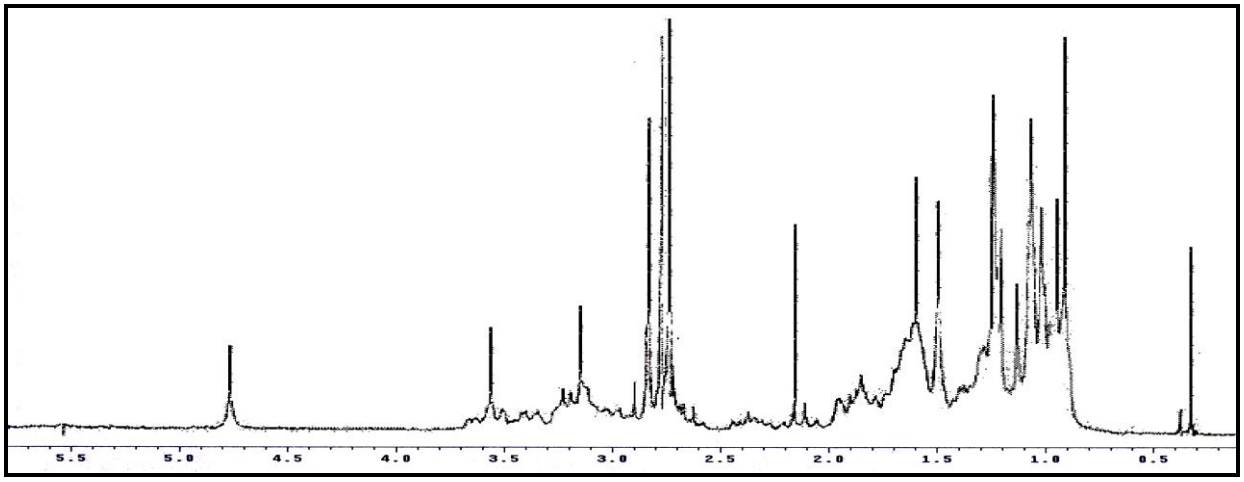

Figure 5. NMR-Spectrum of antimicrobial agent produced by *Streptomyces hygroscoprcus*.

4. Discussion

The active metabolites were extracted by n-Butanol at pH 7.0. Similar results were obtained by [Criswell *et al.*, 2006; Sekiguchi *et al.*, 2007 and Atta *et al.*, 2011]. The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process, where only one active fraction was obtained in the form of whitish yellow oil. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of Chloroform-methanol-water (2:3:1, v/v/v), indicated that fractions activities was recorded from fraction Nos. 22 to 28. Many workers used a column chromatography packed with silica gel. Similar results were obtained by [Hitchens and Kell, 2003; El-Naggar, 2007 and Atta *et al.*, 2009]. The physico-chemical characteristics of the purified antibiotic revealed that, their melting point is 120°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in water, petroleum ether, hexane and benzene. Similar results were recorded by [Ibrhaim H., 2006; Yoram *et al.*, 2006; Wenli *et al.*, 2008 and Houssam *et al.*, 2012]. A study of the elemental analysis of the antimicrobial agent C= 64.02%; H= 9.29%; 0 26.04%; N= 0.0 % and S= 0.0%. This analysis indicates a suggested empirical formula of $C_{47}H_{80}O_{15}$. The spectroscopic characteristics of the antimicrobial agent under study revealed the IR spectrum, 975, 1082, 1300, 1386, 1465, 1703, 1812, 1983, 2080, 2465, 2527, 2945, 3410 & 3750cm^{-1}. Peak at 1703 cm^{-1} correspond to COON. The UV spectrum showed that the active compound dissolved in methanol-water the absorbance peak at wavelengths 278 nm. The mass spectroscopy revealed that the molecular weight is 885. Similar results were recorded by [Imada *et al.*,

1977; Umezawa, 1977 and Berdy, 1974; 1980a b & c].

The MIC of antimicrobial antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against Gram-positive and unicellular and filamentous fungi viz:- *Staph. aureus*, NCTC 7447 and *Bacillus subtilis*, NCTC 10400 15.73 µg/ ml); *Micrococcus luteus*, ATCC 9341; *Bacillus pumilus*, NCTC 8214 and *S. cerevisiae* ATCC 9763 (31.25 µg/ ml); *Candida albicans*, IMRU 3669; *Fusarium oxysporum* and *Rhizoctonia solani* (46.9 µg/ ml); *Aspergillus niger* IMI 31276; *Asp. flavus*, IMI 111023 and *Botrytis fabae* (52.7 µg/ ml); *Aspergillus fumigatus*, ATCC 16424 (93.75 µg/ ml) and *P. chrysogenum* (> ١٠٠ µg/ ml), similar investigations and results were attained by [Charan *et al*., 2004; Laidi *et al.*, 2006; Kavitha and Vijayalakshmi, 2007; Valan *et al.*, 2008 and Atta, 2010&2011].

Identification of the antimicrobial agent according to recommended international keys indicated that the antibiotic is suggestive of being Carriomycin antibiotic (polyether antibiotic) [Imada *et al.*,1977; Umezawa, 1977 and Berdy, 1974; 1980a b & c].

5. Conclusion

It could be concluded that: Active compound produced by *Streptomyces hygroscoprcus* M 121 was detected as polyether antibiotic namely Carriomycin ($C_{47}H_{80}O_{15)}$, molecular weight 885 g/mol with UV visible maximum absorbance 278 nm. This active compound was produced by extracellular secretion and active to *Staph. aureus*, NCTC 7447; *Micrococcus luteus*, ATCC 9341; *Bacillus subtilis*, NCTC 10400; *Bacillus pumilus* NCTC 8214; *S. cerevisiae* ATCC 9763; *Candida albicans*, IMRU3669; *Aspergillus fumigatus*, ATCC 16424; *Aspergillus flavus*, IMI 111023; *Aspergillus niger*, IMI 31276; *Fusarium oxysporum*, *Botrytis fabae*, *Rhizoctonia solani* and *P. chrysogenum*. ATCC27853.

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