Antifungal Macrodiode Production By *Streptomyces albidoflavus*-143: Fermentation, Purification and Biological Activities

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Abstract: This work was carried out for the biosynthesis of antifungal substance that demonstrated inhibitory effects against pathogenic fungi from *Streptomyces albidoflavus*, 143. The active metabolite was extracted using ethyl acetate (1:1, v/v) at pH 7.0. The separation of the active ingredient of the antifungal 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, spectroscopic characteristics and chemical reactions have been investigated. This analysis indicates a suggested imperical formula of $C_{22}H_{36}O_6$. The minimum inhibition concentrations "MICs" of the purified antifungal agent were also determined. The purified antifungal agent was suggestive of being belonging to Macrodiode antibiotic produced by *Streptomyces albidoflavus,* 143.
[Houssam M. Atta, El-Sehrawi M.H. and Bahobail A.S. Antifungal Macrodiode Production By *Streptomyces albidoflavus*-143: Fermentation, Purification and Biological Activities. Journal of American Science 2011;7(3):13-22]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Antifungal Macrodiode; *Streptomyces albidoflavus*; Fermentation; Purification; Biological Activities

1. Introduction

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Joo, 2005). The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (Fguira *et al.*, 2005) and extracellular hydrolytic enzymes (Taechowisan *et al.*, 2005). Chitinase and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls, as for example, cell walls of *Fusarium oxysporum*, *Sclerotinia minor*, and *S. rolfsii* (Mukherjee and Sen, 2006)

The macrotetrolides are a family of cyclic polyethers produced by a number of *Streptomyces* species (Birch and Robinson, 1995 and Fleming and Ghosh, 1996). Nonactin (NON), the smallest homolog and a symmetric member of the family, was first isolated in 1955 (Corbaz *et al.*, 1955). Its structure was initially deduced from spectroscopic analysis and was later confirmed by X-ray crystallography (Dobler, 1972, Kilbourn *et al.*, 1967), revealing that the intriguing molecular topology of NON consists of the (+)(-)(+)(-)-ester linkage of the enantiomeric nonactic acid (NA) building blocks. NA-type building blocks have also been identified in several macrodiolides (Jois *et al.*, 1986), including the pamamycins (Natsume *et al.*, 1991). The macrotetrolides exert a broad spectrum of biological activities (Zizka, 1998), ranging from antifungal, antitumor (Borrel *et al.*, 1994), antiprotozoan, antiparasitic, and insecticidal activities to immunosuppressive activities (Callewaert *et al.*, 1988). In fact, comparative studies on the immunosuppressive activities of tetranactin and cyclosporin, the latter being the most widely used immunosuppressant agent, showed that these two compounds were approximately equally effective and that tetranactin has the advantage of low toxicity (Teunissen *et al.*, 1992). The biological activities of the macrotetrolides are generally traced to their ionophoric properties (Marrone and Merz, 1992.), and the potencies of these activities appear to parallel the size of the alkyl substituent's of the macrotetrolides: tetranactin is often the most potent member of the family, while NON is generally inactive. The biosynthesis of NON has been extensively studied by in vivo feeding experiments with ^{13}C-, ^{2}H-, and ^{18}O-labeled precursors and biosynthetic intermediates (Ashworth *et al.*, 1989) and by isolation of both enantiomers of NA and the dimeric NA (Fleck *et al.*, 1996). These results established unambiguously the polyketide origin of NON, the assembly of which from one molecule each of propionate and succinate and two molecules of acetate must have invoked (i) the rare use of succinate as an intact four-carbon fragment (C-3 to C-6) and (ii) the derivation of a three-carbon unit (C-7 to C-9) from two molecules of acetate (one of which is activated in the form of malonate). Feeding experiments with ^{13}C- and ^{18}O-doubly-labeled

precursors indicated that the C-3-O bond is formed during closure of the tetrahydrofuran ring, presumably by an intramolecular Michael addition of the 6-hydroxy group onto the enone moiety of 2-methyl-6,8-dihydroxynon-2*E*-enoic acid (NEA) (Ashworth and Robinson, 1988). The involvement of the latter step in NON biosynthesis was further substantiated by the efficient and enantiospecific incorporation of both (6*R*,8*R*)-NEA into NON (Spavold and Robinson, 1988) and by the drastic reduction of NON production upon the addition of an NEA analog into the fermentation medium, which presumably acts as a suicide inhibitor for this enzymatic step (Priestley and Earle, 1997).

The Macrodiode has molecular weight 396 and empirical formula $C_{22}H_{36}O_6$, and (U.V) strong end absorption spectrum (Jois and Gurusiddaiah, 1986).

In the present study, the productio of the bioactive substances that demonstrated inhibitory affects against microbial pathogenic, from *Streptomyces albidoflavus,* 143 were reported, along with some physico-chemical properties of secondary metabolites with high biological activities.

2. Material and Methods

2.1. Test organisms

2.1.1. Unicellular fungi

Saccharomyces cerevisiae, ATCC 9763, *Candida albicans* IMRU 3669.

2.1.2.-Filamentous fungi

Aspergillus niger, IMI 31276.*; Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424; *Aspergillus terreus; Fusarium solani; Fusarium oxysporum, Fusarium moniliform, Alternaria alternata, Botrytis cinerea, Penicillium chrysogenum* and *Rhizoctonia solani*.

2.2. Fermentation

A loopful of the, *Streptomyces albidoflavus,* 143 from the 5-day culture age was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of liquid starch nitrate 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 *etal.,* 2001).

2.3. Extraction

The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator (Atta, 2010).

2.4. 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 antifungal activities (Atta *et al.,* 2009).

2.5. Separation

Separation of the antifungal agent(s) into its individual components was conducted by thin layer chromatography using chloroform and methanol (24:1, v/v) as a solvent system (Atta *et al.,* 2009).

2.6. Purification

The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform and Methanol 9**:**1 (v/v), was used as an eluting solvent. The column was left for overnight until the silica gel (Prolabo) 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).

2.7. Physico-chemical properties of the antifungal agent

2.7.1. Elemental analysis

The elemental analysis C, H, O, N, and S was carried out at the micro analytical center, Cairo University, Egypt.

2.7.2. Spectroscopic analysis

The IR, UV, Mass spectrum, and NMR spectrum were determined at the micro analytical center of Cairo University, Egypt.

2.7.3. Reaction of the antifungal agent with certain chemical test

For this purpose, the following reactions were carried out: Molish's, Fehling, Sakaguchi, Ninhydrin, Ehrlish, Nitroprusside, Ferric chloride, and Mayer reactions (Atta *et al.,* 2011).

2.7.4. Biological activity

The minimum inhibitory concentration (MIC) could be determined by the cup assay method (Kavanagh, 1972).

2.7.5. Characterization of the antifungal agent

The antifungal agent produced by *Streptomyces albidoflavus*, 143 was identified according to the recommended international references of (Umezawa, 1977; Berdy, 1974; Berdy, 1980a b & c; Jois and Gurusiddaiah, 1986).

3. Results

3.1. Fermentation and Separation of the antifungal agent

The fermentation process was carried out for three 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 Ethyl acetate 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 antifungal activities. The antifungal agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 5000 r.p.m for 15 minute where a yellowish brown oil precipitate could be obtained. Separation of the antifungal agent(s) into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Among three bands developed, only one band at R_f 0.9 showed antifungal activity. The purification process through column chromatography packed with silica gel indicated that the most active fractions against the tested organisms ranged between 20 to 31 Fig. (1).

3.2. Physicochemical characteristics of the antifungal agent

The purified antifungal agent produces characteristic odour, their melting point is 180°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 antifungal agent(s) revealed the following: **C**=66.17; H=9.70; N= 0.0, O = 25.3 and S=0.0. This analysis indicates a suggested imperical formula of $C_{22}H_{36}O_6$.

3.4. Spectroscopic characteristics

The infrared (IR) spectrum of the antifungal agent showed characteristic band corresponding to 17 peaks, 810.21, 1060.19, 1201.17, 1268.12, 1331.21, 1426.10, 1512.10, 1604.08, 1715.24 (lactone), 1998.20, 2880.21, 2940.05 (C-H stretching), 2320.12, 3278.23, 3601.11, 3624.07 and 3788.13 (Fig.2).The ultraviolet (UV) absorption spectrum of the antifungal agent recorded a maximum absorption peak region at 223.30, 260.7 and 271.58 (Fig. 3). The Mass spectrum revealed that the molecular weight is 396 (Fig. 4). The NMR-Spectrum exhibited the multiple at δ 5.0 to 4.85 was due to the methine proton-bearing ester bonded oxygen (R-CH-O-COR)., the doublet at δ 1.24 was due to the methylene group of homononactic acid moieties attached to a carbon-bearing ester-bonded oxygen (R-CH_2-CHOCOR). Multiplets at δ 4.1 to 3.95 and 3.95 to 3.75 are characteristics of tetrahydrofuran methine protons (-CH-O-CH-). Peaks at δ 2.1 to 1.7 were assigned to methylene protons of the tetrahydrofuran moiety (-CH_2-CH_2-) (Fig.5).

3.5. Biochemical reaction of the antimicrobial agent

The reactions revealed the detection of certain groups in the investigated agent. The antifungal agent exhibited positive results with ninhydrin, ferric chloride and Mayer tests and negative results with nitroprusside, Molish's, Fehling Sakaguchi, and Ehrlish reactions (Table 1).

3.6. Biological activities of the antifungal agent

Data of the antifungal agent spectrum indicated that the agent is active against unicellular and filamentous fungi (Table 2). The MIC of antifungal antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against unicellular fungi *Saccharomyces cervisiae* ATCC 9763 (31.25 µg/ ml) and *Candida albicans*, IMRU 3669 (25.25 µg/ ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger* IMI 31276 (46.9 µg/ ml), *Aspergillus flavus* (46.9), *Botrytis fabae* (46.9 µg/ ml), *Fusarium oxysporum* (52.7 µg/ ml), *Rhizoctonia solani* (52.7 µg/ ml), *Alternaria alternate* (62.5 µg/ ml), *Aspergillus fumigatus* ATCC 16424 (62.5 µg/ ml), and *Penicillium chrysogenium* (62.5 µg/ ml).

3.7. Identification of the antifungal 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 antifungal agent, it could be stated that the antifungal agent is suggestive of being belonging to Macrodiode antibiotic (Table 3).

Table 1. Summarizes the response of the antifungal agent to certain biochemical reactions.

Chemical test	Result	Remark
Molish's reaction	-	Absence of sugar moiety
Fehling test	-	Absence of free aldehyde or keto sugar
Ninhydrin test	+	Present of free-NH_2 group
Sakaguchi reaction	-	Arginin is Absence
Nitroprusside reaction	-	Absence of Sulfur
Ferric chloride reaction	+	Absent of Di-ketons group
Ehrlish rection	-	Absence of indolic acid
Mayer reaction	+	Presence of nitro group

Table 2. Biological activities (MIC) of the antifungal agent by paper method assay.

Test organisms	MIC (µg/ml) concentration
1-Unicellular fungi:	
Candida albicans, IMRU 3669	25.25
Saccharomyces cervisiae, ATCC 9763	31.25
2-Filamentous fungi:	
Aspergillus niger, IMI 31276	46.9
Aspergillus fumigatus, ATCC 16424	62.5
Aspergillus flavus, IMI 111023	46.9
Fusarium oxysporum	52.7
Rhizoctonia solani	52.7
Alternaria alternata	62.5
Botrytis fabae	46.9
Penicillium chrysogenium	62.5

Table 3. A comparative study of the characteristic properties of the antifungal agent in relation to reference antifungal Macrodiode

Characteristic	Purified antibiotic	Antifungal Macrodiode
1- Melting point	180°C	ND
2- Molecular weight	396	396
3- Chemical analysis:		
C	66.17	66.17
H	9.70	9.73
N	0.0	0.0
O	25.3	25.0
S	0.0	0.0
4- Ultra violet	240	240
5- Formula	$C_{22}H_{36}O_6$	$C_{22}H_{36}O_6$
6- Active against	Unicellular and filamentous fungi	Unicellular and filamentous fungi

ND=No data

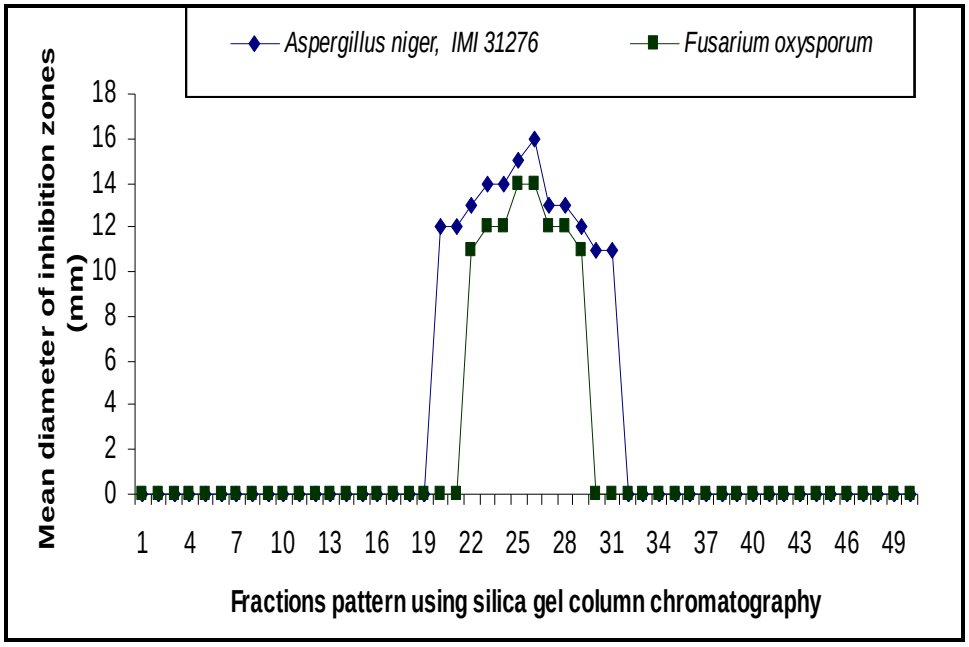

Figure 1. Antifungal activity of fractions obtained using silica gel column chromatography technique for antifungal agent produced by *Streptomyces albidoflavus*, 143.

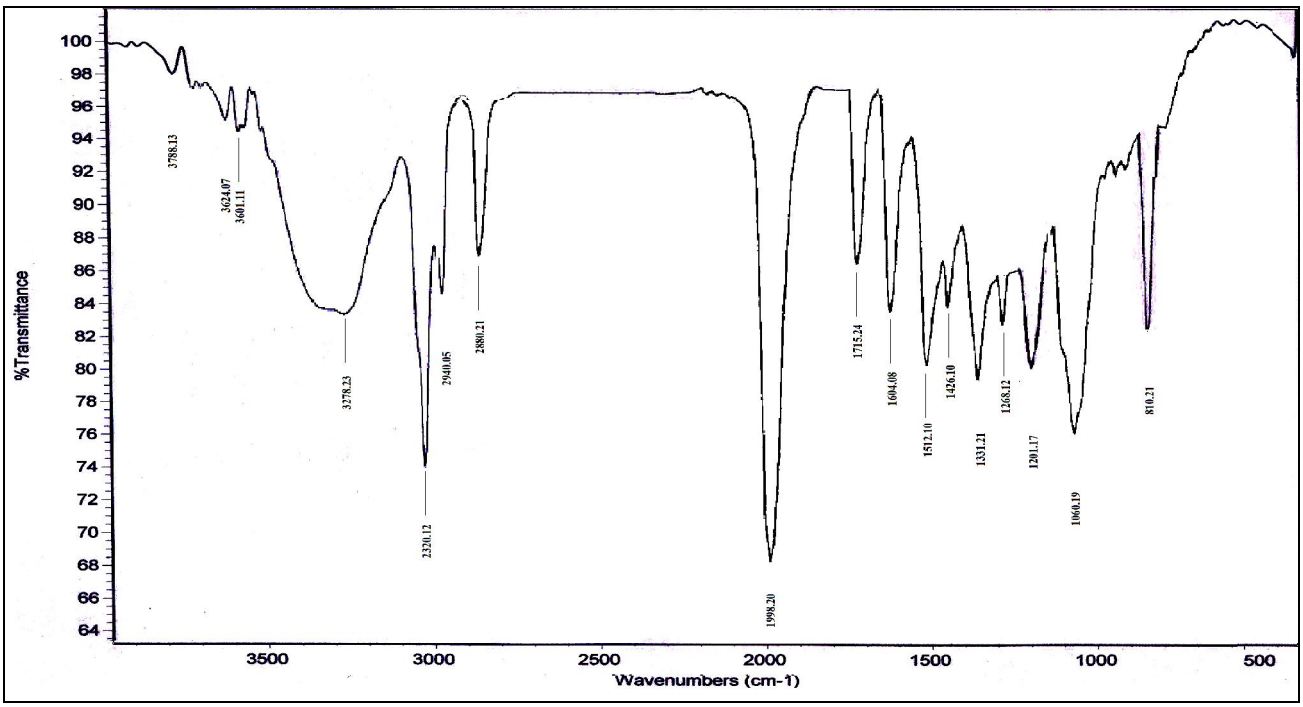

Figure 2. I.R spectrum of antifungal agent produced by *Streptomyces albidoflavus*, 143.

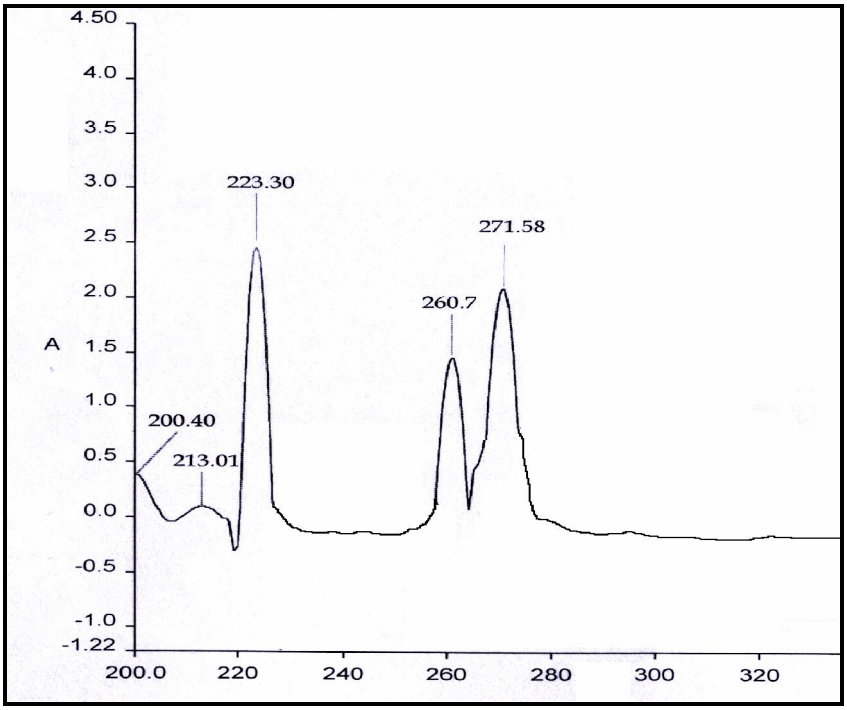

Figure 3. Ultraviolet absorbance of antifungal agent produced by *Streptomyces albidoflavus*, 143.

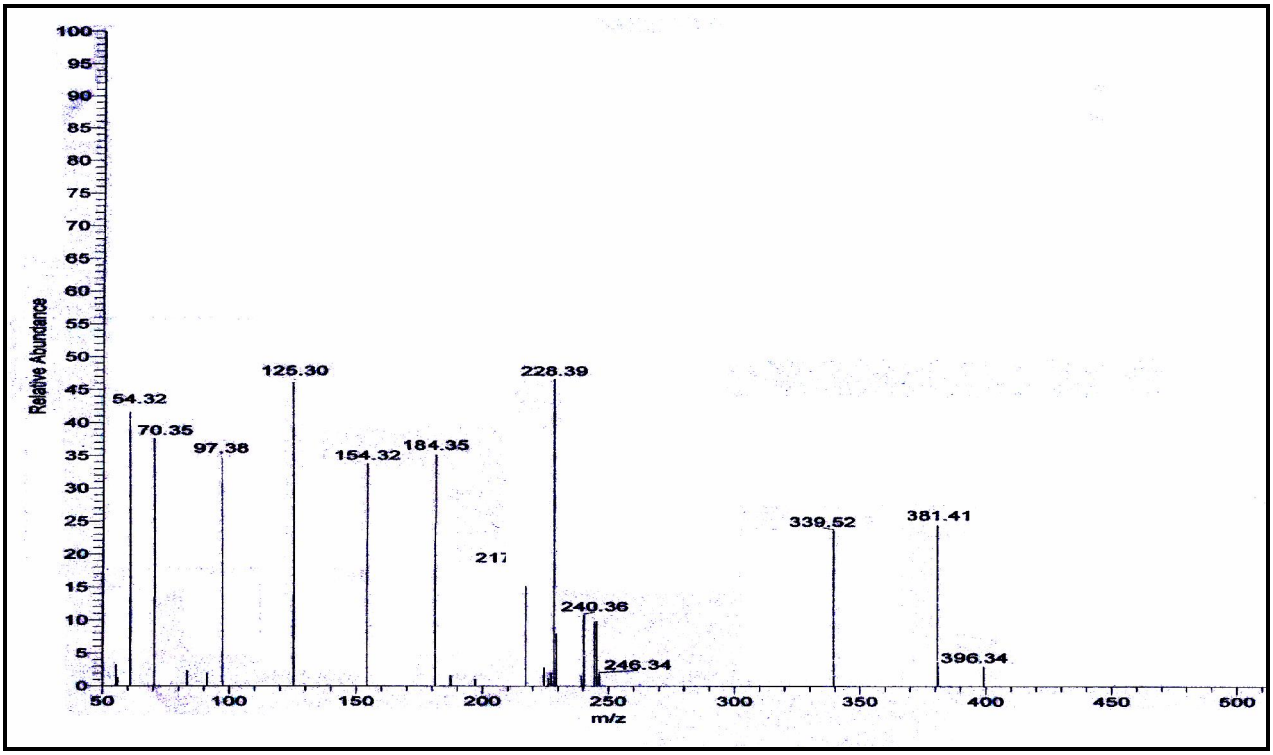

Figure 4. Mass-Spectrum of antifungal agent produced by *Streptomyces albidoflavus*, 143.

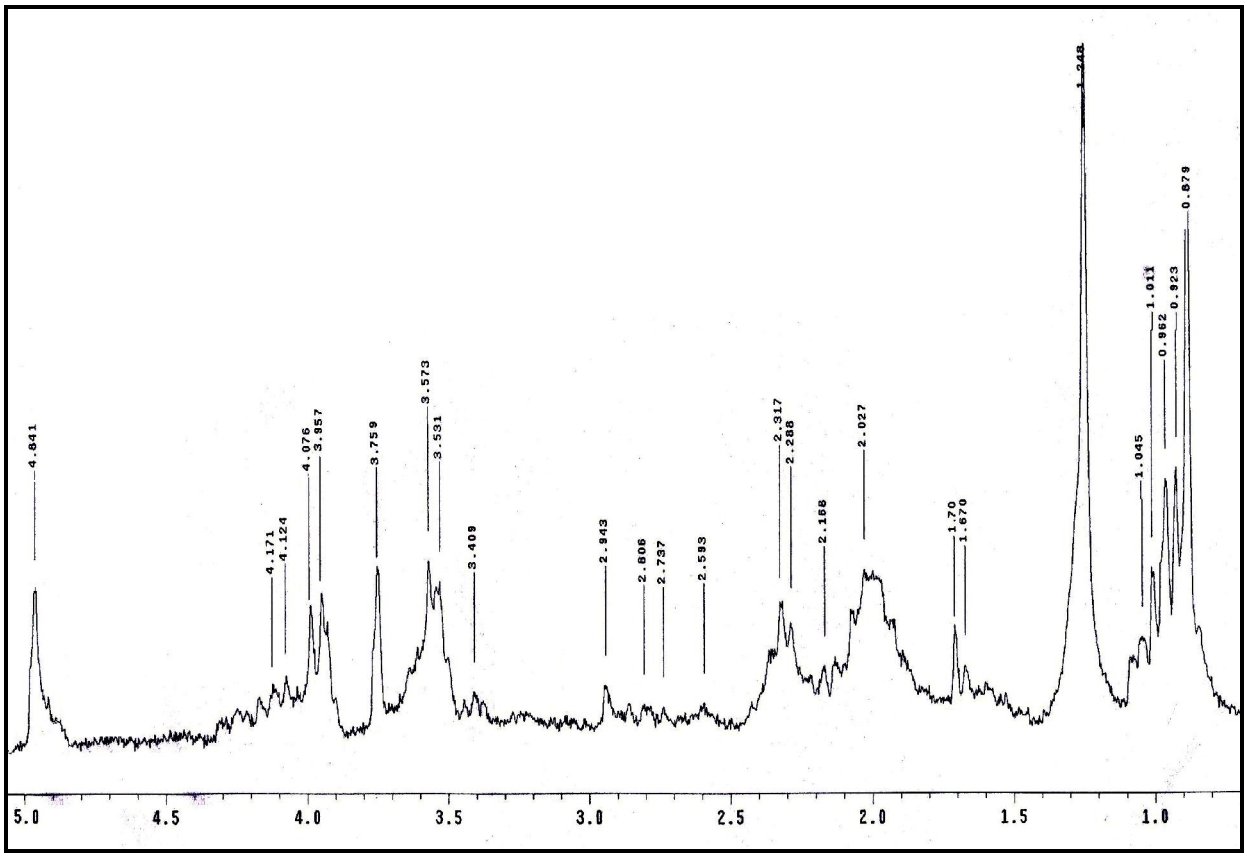

Figure 5. NMR-Spectrum of antifungal agent produced by *Streptomyces albidoflavus,* 143.

4. Discussions

The active metabolites were extracted by ethyl acetate 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 yellowish brown oil. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of chloroform and methanol (9:1, v/v), indicated that fractions activities was recorded from fraction Nos. 20 to 31. Many workers used a column chromatography packed with silica gel. Similar results were obtained by (Jois and Gurusiddaiah, 1986; 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 180°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 (Yanai, 2004; Yoram *et al.,* 2006 and Wenli *et al.,* 2008). A study of the elemental analysis of the antifungal agent C=66.17; H=9.70; N= 0.0, O = 25.3 and S=0.0 lead to an imperical formula of $C_{22}H_{36}O_6$. The spectroscopic characteristics of the antifungal agent under study revealed the presence of a maximum absorption peak in UV. at 223.30, 260.7 and 271.58 nm, infra-red absorption spectrum represented by 17 peaks, 810.21, 1060.19, 1201.17, 1268.12, 1331.21, 1426.10, 1512.10, 1604.08, 1715.24 (lactone), 1998.20, 2880.21, 2940.05 (C-H stretching), 2320.12, 3278.23, 3601.11, 3624.07 and 3788.13. The spectral characteristics of the hydrolysis product were as follows: IR spectrum 3278 (broad, OH), 2940 and 2880 (C-H stretching), 3601 to 2880 (broad nature of peak, COOH), 1715 (COOH), 1426, 1331, 1268, 1201 and 1060 cm^{-1}(Jois and Gurusiddaiah, 1986).

The Mass spectrum revealed that the molecular weight is 396 and NMR-spectrum exhibited the multiple at δ 5.0 to 4.85 was due to the methine proton-bearing ester bonded oxygen (R-CH-O-COR)., the doublet at δ 1.24 was due to the

methylene group of homononactic acid moieties attached to a carbon-bearing ester-bonded oxygen (R-CH_2-CHOCOR). Multiplets at δ 4.1 to 3.95 and 3.95 to 3.75 are characteristics of tetrahydrofuran methine protons (-CH-O-CH-). Peaks at δ 2.1 to 1.7 were assigned to methylene protons of the tetrahydrofuran moiety (-CH_2-CH_2-) (Kumar and Kannabiran, 2010). The biochemical tests of the antifungal agent gave positive reaction ninhydrin, ferric chloride and Mayer tests and negative results with nitroprusside, Molish's, Fehling Sakaguchi and Ehrlish reactions. Similar results were recorded by (Pamboukian and Facciotti, 2004 and Atta *et al*., 2011).

The MIC of antifungal antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against unicellular fungi *Saccharomyces cervisiae* ATCC 9763 (31.25 µg/ ml) and *Candida albicans*, IMRU 3669 (25.25 µg/ ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger* IMI 31276 (46.9 µg/ ml), *Aspergillus flavus* (46.9), *Botrytis fabae* (46.9 µg/ ml), *Fusarium oxysporum* (52.7 µg/ ml), *Rhizoctonia solani* (52.7 µg/ ml), *Alternaria alternate* (62.5 µg/ ml), *Aspergillus fumigatus* ATCC 16424 (62.5 µg/ ml), and *Penicillium chrysogenium* (62.5 µg/ ml), similar investigations and results were attained by (Kavitha and Vijayalakshmi, 2007 and Atta, 2010).

Identification of the antifungal agent according to recommended international keys indicated that the antibiotic is suggestive of being Macrodiolide antibiotic (macrotetrolide antibiotic) (Jois and Gurusiddaiah, 1986).

5. Conclusion

It could be concluded that: The Macrodiolide antibiotic produced by *Streptomyces albidoflavus*, 143 demonstrated obvious inhibitory affects against pathogenic fungi.

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1/21/2011