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Isolation, Purification and Characterization of an Actinomycete Isolate Having the Ability to Produce an Antimicrobial Metabolite

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IN THE COURSE of screening for new antimicrobial agents, an actinomycete isolate was isolated from a sand soil collected from Gabal Mokattam, Cairo, Egypt. The isolate was found to produce an antibacterial agent(s).This isolate is characterized by white, brown to light gray aerial mycelia and brown, yellow and red substrate mycelia on different ISP media, as well as on other morphological, physiological and biochemical characteristics of the isolate could be detected. From the taxonomic features of the isolate it was found that its characters matches with *Streptomyces hygroscopicus*, so it was given the generic name *Streptomyces hygroscopicus* GM-8. The active metabolite was extracted by chloroform. The separation, purification and characterization of the active metabolite(s) was performed using thin layer chromatography. The physicochemical studies of the purified active metabolite(s) including solubility, elemental analyses, spectroscopic characteristics and chemical reactions have been investigated. The biological activities of the purified active metabolite(s), *i.e.* MICs values were also determined.

Keywords: *Streptomyces* sp., Antibiotics .

A lot of secondary metabolites have been produced by *Streptomyces* sp., for example, colabomycins an antibiotic produced by *Streptomyces griseoflavus,* (Grote and Zeech, 1988), glutarimide antibiotic produced by *Streptomyces hygroscopicus*, (Urakawa *et al.*, 1993), gremmiycin an antibiotic produced by *Streptomyces* sp., (Igarashi *et al.*, 1998), cerdarmycin A and B new antibiotics produced by *Streptomyces* sp. TFA0456, (Sasaki *et al.*, 2001), new type II mannmycins produced by *Streptomyces nodosus* sp. *asukaensis* (Hu and Floss, 2001) and novel geldanamycin analogues produced by *Streptomyces hygroscopicus* (Hu *et al.*, 2004).

This article deals with isolation, purification and characterization of an actinomycete isolate which has the ability to produce an antimicrobial agent. This was attained through the course of screening for new antibacterial agent and studies the physico-chemical characters of this metabolite.

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Material and Methods

Collection of samples

Ten samples used in this study were collected from different areas at Gabal Mokattam, Cairo, Egypt, during March 2002. The actinomycete isolates were isolated from soil samples by dilute plating using starch nitrate agar medium (El-Nakeeb and Lechevalier, 1963) and then incubated at 30°C for four days. All isolates were purified by repeated streaking on starch nitrate agar medium.

Screening of actinomycete isolates

Screening of actinomycete isolates was carried out according to their antimicrobial activity against six different test organisms including *Staphylococcus aureus* NCTC 7447, *Bacillus subtilis* ACTCC 7972, *Escherichia coli* NCTC 19416, *Pseudomonas aeruginosa* ATCC 10415, *Candida albicons* IMRU 3669 and *Aspergillus niger* ATCC 6275.

Identification of a selected isolate

The cultural, morphological and physiological characteristics of a selected isolate were studied using all media and methods of International Streptomyces Project (ISP) as described by Shirling and Gottlieb (1966, 1968 and 1969). Identification was carried out by using the keys suggested by Buchanan and Gibbson's (1974), Williams (1989) and Hensyl (1994).

Fermentation procedure

A slant culture of *Streptomyces* isolate grown on starch nitrate agar (ISP No.) was used inoculated a 250 ml Erlenmeyer flask containing 100 ml of the same medium in its liquid form. The cultivated flasks were incubated at 30°C for 4 days on a rotary shaker (230 rpm) to give a first seed culture. Approximately, 2 ml portions of the seed culture were inoculated into each of twenty 250 ml conical flasks containing 100 ml of the above liquid medium and incubated at 30°C on a rotary shaker (230 rpm) for 10 days. The samples were taken daily for determination of antimicrobial activity. Antimicrobial activity was determined using the classical diffusion method of Cooper (1963 and 1972).

Analytical procedure

The content of fermentation flasks was collected at the end of incubation period after which it was extracted using chloroform. The lower layer was collected and subjected for evaporation under vacuum. The purification process was monitored by thin layer chromatography (Silica gel fluka thickness 0.2 mm) using n-butanol : acetic acid : water (4 : 1 : 5) as mobile phase.

Determintion of MICs value

The minimal inhibitory concentration (MIC) of the active substance was determined by the conventional paper disk diffusion method (Cooper, 1963 and 1972) by using paper disk (266812 W. Germany 12.7 mm in diameters). Bacteria were grown on nutrient agar medium, and fungi and yeast were grown on sobourand agar medium. The active substance was dissolved in chloroform and a

paper disk containing each of the sample (0-2-4-6-8-10-12-14-16-20-40-60-80-100 µg) was placed on the agar plates. Growth inhibition was examined after 24 hr incubation at 37°C for bacteria and after 48 hr incubation at 27°C for fungi and yeast. The antimicrobial activity was estimated by measuring the diameter of inhibitory zone.

Physiochemical properties

Ultra violet (UV) spectra of the purified metabolite was recorded using Unicom SP 1570 ultra violet spectrophotometer. Infrared (IR) spectra were determined in potassium bromide (kBr) disk using Infrared spectrophotometer model PYE Unicom SP 1100. Mass-spectrum, NMR and elemental analysis (C, H, O_2 and N) were determined at the Micro Analytical Centre of Cairo University, Egypt. Different colour reaction was carried out to detect the presence of certain chemical groups in the metabolite molecule using the method of Hawk *et al.* (1954), Kohn (1961) and Smith (1969).

Results

Antagonistic activities of the isolates

Twenty actinomycetes isolates differ in their cultural, morphological and biochemical characters were isolated in pure form from sand soil from Gabal El-Mokattam, Cairo, Egypt, using starch nitrate agar medium. Among these isolates it was found that, the isolate No.(8) exhibited antimicrobial activity against the tested organisms as illustrated in Table 1. The isolate No. (8) showed high antagonistic activity against the four tested organisms. Therefore, it was subjected to further studies.

TABLE 1. The antagonistic activities of the actinomycete cultures isolated using starch-nitrate medium.

Isolate No.	*Bacillus subtilis* ATCC 7972	*Staphylococcus aureus* NCTC 7447	*E. coli* NCTC 10416	*Pseudomons aeuroginosa* ATCC 10415	*Candida albicans* IMRU 3669	*Aspergillus nigar* ATCC 627
1	--	--	--	--	--	--
2	--	--	--	--	--	--
3	+ve	+ve	--	--	--	--
4	++ve	++ve	--	--	--	--
5	--	--	--	--	--	--
6	+ve	--	--	--	--	--
7	--	--	--	--	--	--
8	+++ve	+++ve	+++ve	+++ve	--ve	--
9	--	--	--	--	--	--
10	--	--	--	--	--	--
11	+ve	+ve	--	--	--	--
12	--	--	--	--	--	--
13	+ve	--	--	--	--	--
14	--	--	--	--	--	--
15	--	--	--	--	--	--
16	--	--	--	--	--	--
17	+ve	+ve	--	--	--	--
18	+ve	+ve	+ve	+ve	+ve	--
19	--	--	--	--	--	--
20	--	--	--	--	--	--

Morphological and cultural characteristics of the selected isolate

The isolate GM-8 spore chain spiral and spore surface smooth in Fig.1 and grows well on various standard media including the ISP media which are recommended by Shirling & Gottlieb (1966, 1968, 1969 and 1972), Waksman (1961), Buchanan & Gibbson (1974), Williams (1989) and Hensyl (1994).

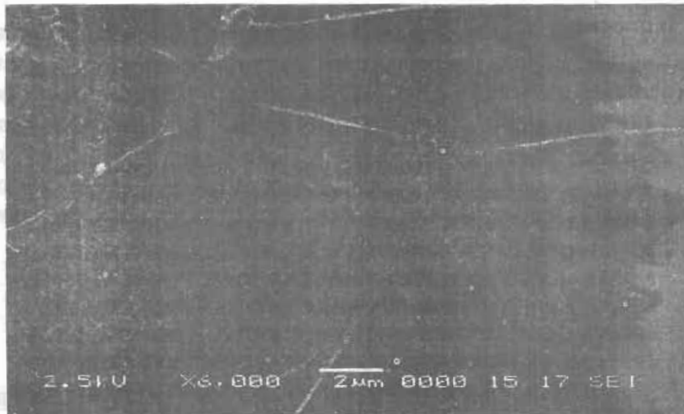

Fig. 1. Photoelectromicrograph of *Streptomyces hygroscopicus* GM-8.

Table 2 shows the results regarding the rate of growth, spore mass colour, reverse side growth colour and production of soluble pigment(s) by the isolated organism on 9 different nutrient media. The aerial mycelia are white, brown to light gray, anywhere the reverse side colour is brown, yellow to black. Formation of melanoid pigments is positive on ISP No. (1), ISP No. (2) and ISP No. (6) media.

TABLE 2. Cultural characteristics of the actinomycete isolate number 8.

No	Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1	Tryptone-yeast extract broth medium (ISP_1)	Good	263 white	Brown	Dark brown
2	Yeast extract-Malt extract agar medium (ISP_2)	Good	263 white	72 deep brown	Dark brown
3	Oat meal agar medium (ISP_3)	Good	263 white	89 p. yellow	No
4	Inorganic salts-starch agar medium (ISP_4)	Good	263 white	90 gy. Yellow	No
5	Glycerol-asparagine agar medium (ISP_5)	Good	263 white	17 deep red	No
6	Peptone yeast extract iron agar medium (ISP_6)	Good	Brown	Deep brown	276 black
7	Tyrosine agar medium (ISP_7)	Good	White	Brown	No
8	Starch-nitrate agar medium	Good	264 L. Grey	46 Grayish reddish brown	Brown
9	Dox agar medium	Good	61 brown	59 brown	No

Physiological characteristics of the selected actinomycete isolate

Physiological properties of the isolate under study are summarized in Table 3. This isolate could hydrolyze starch, protein and casein but not lipid, DNA or H_2O_2. Whole cell hydrolyzates of the isolate contained LL-diminopimilic acid. The isolate was found to be able to utilize, L-arabinose, D-xylose, D-ribose, D-mannose, D-glucose, D-fructose, D-glactose, mannitol, m-inositol, sucrose, raffinose and maltose as carbon sources.

Identification of the selected isolate

Identification of isolate investigated under study to the species level was carried out using working key of Kuster (1972), Nonomura (1974), Szabo *et al.* (1975) and Williams (1989) and characterization of type cultures of *Streptomyces* of the ISP (Shirling and Gottlieb, 1968a, 1969 and 1972). The isolate GM-8 is felt well on the bases of morphological, cultural and physiological characters to be *Streptomyces hygroscopicus* although it differs from the reference strain on some characters. For this reason it is likely to assume that, it is a variety of *Streptomyces hygroscopicus* and given the suggested name *Streptomyces hygroscopicus* GM-8.

Extraction and purification of the active metabolites from Streptomyces hygroscopicus GM-8

In this part of study, attempts were carried out to extract the metabolite(s) secreted by *Streptomyces hygroscopicus* GM-8. In this respect, 8 solvents were tested to explore the most favorable one for extraction of such metabolite(s).

The most suitable solvent for extraction of the studies metabolite(s) was found to be chloroform so it was used for the extraction of the metabolite(s) from the broth of a culture of the choice organism. The chloroform extract was concentrated and subjected to thin layer chromatography by using eluting solvent (n-butanol: acetic acid: water 4 : 1 : 5). The metabolite was eluted in the form of eight bands which fluctuate in their colour between red, organe to yellow. Only the fraction number eight was found to exhibit antimicrobial activity (the results are recorded in Table 4) .

Results indicated that the active metabolite under investigation was found to be soluble in methyl and ethyl alcohols, dimethylformamide and acetone and insoluble in water and n-hexane. It showed positive responses to ninhydrin , glycosidic test, Meyer test and negative response to Molish and Fehling reagents.

UV absorption spectra of the purified active metabolite exhibit maximum absorption peak at wave length of 246 nm in ethyl acetate as illustrated in Fig. 2. The Infrared absorption spectrum (IR) of the active metabolite in KBr is shown in Fig. 3. It shows bands at 3380.7, 2994.7, 2910, 2880.5, 2700, 2500.4, 2450, 2400, 2260, 2240.6, 1920, 1680, 1540, 1460, 1450, 1400, 1392.8, 1300, 1270, 1060, 1050, 900 and 820.4 cm^{-1}.

TABLE 3. Morphological and physiological data of isolate No. 8.

Character	Isolate No. 8
DAP type	L-DAP
Spore chain	Compact Spirals
Spore mass colour	White
Diffusible pigment	+ve
Melanoid formation on (ISP-7) medium	-ve
Spore surface	Smooth
Carbon utilization	
D-glucose	+ve
D-xylose	+ve
L-arabinose	+ve
D-fructose	+ve
D-galactose	+ve
D-mannitol	+ve
M-inositol	+ve
Sucrose	+ve
L-rhamnose	+ve
Raffinose	+ve
Protein hydrolysis	+ve
Starch hydrolysis	+ve
Lipid hydrolysis	-ve
Hydrogen peroxide hydrolysis	-ve
Esculin hydrolysis	-ve
Xanthine hydrolysis	-ve
Oxidase	-ve
H_2S production	-ve
Indole production	-ve
Nitrate reduction	-ve
Phenyl alanine deamination	-ve
Growth on:	
Sodium azide 0.01%	-ve
Thallus acetate 0.001%	+ve
Sodium tellurite 0.001%	-ve
Growth at temperature (°C)	
10	-ve
20	+ve
30	+ve
40	+ve
45	+ve
Growth in mineral salts	Week
Sodium chloride 15%	+ve
Utilization of nitrogen sources:	
Glycine and L-cysteine	+ve
L-alanine and L-Phenylalanine	+ve
L-serine and L-aspartic acid	+ve
Valine and L-tryptophane	+ve
L-Lysine and L-histidine	+ve
L-methionine	+ve
L-arginine	+ve
L-Threonine	+ve

-ve = negative +ve = positive

TABLE 4. In vitro antimicrobial activities of the purified metabolite produced by *Streptomyces hygroscopicus* GM-8.

	Microorganisms	MIC (μg/ml) concentration reference streptomycin	MIC (μg/ml) concentration
1	*Bacillus subtilis* ATCC 7972	7	5
2	*Streptococcus pyoyenes* ATCC 5309	10	8
3	*Staphylococcus aureus* NCTC 7447	6	5
4	*Escherichia coli* NCTC	11	10
5	*Proteus mirabilis* ATCC 21100	11	11
6	*Proteus vulgaris* IMRU 70	13	12
7	*Klebsiella pneumonia* NCTC 9111	13	12
8	*Pseudomonas aeruginosa* ATCC 10415	14	15
9	*Candida albicans* IMR 43669	---	>100.0
10	*Aspergillus niger* ATCC6275	---	>100.0

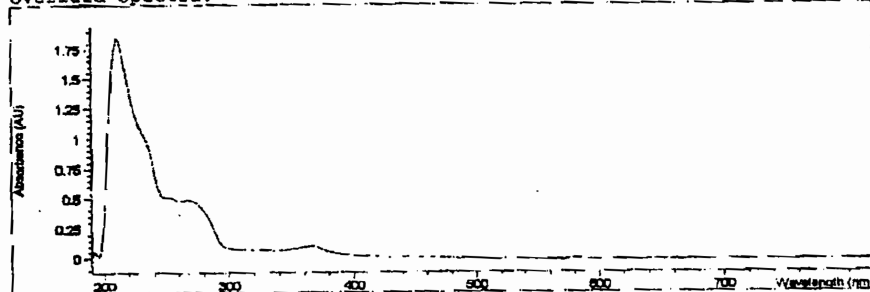

Fig. 2. UV. Absorption spectra of active substance produced by *Streptomyces hygroscopicus* GM-8.

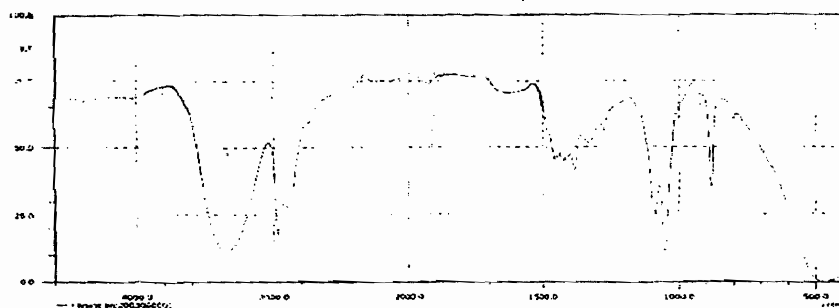

Fig. 3. Infrared spectra of active substance produced by *Streptomyces hygroscopicus* GM-8.

The NMR spectrum in DMSO is indicated as in Fig. 4a from which the following chemical shifts in 6 scales singlet at 0.883, 1.263, 2.5156, 3.3629,

6.9933 and 8.3294 ppm could be detected. The NMR spectrum in DMSO and D_2O (Fig. 4b) showed chemical shifts singlet at 0.0113, 1.296, 2.5783, 2.5854 and 3.9381. The Mass-spectrum as in showed the following fragments: at m/e 69 (100), 129 (51.41), 236 (20), 313 (32), 388 (10) and 578 (1.47) gave molecular weight of 578 atomic mass unit. The elemental analyses : C = 60.24 %, H=7.25 %, O= 27.68% and N = 4.84 % from which the following imperical formula could be calculated $C_{29}H_{42}O_{10}N_2$. From the fragmentation pattern of the MS, NMR, IR, UV, elemental analyses and chemical reaction it was found that, the active purified metabolite is closely related to geldanamycin. The results recorded in Table 4 revealed that the metabolite under investigation has promising antibacterial activity aginst both (Gram-positive and Gram-negative bacteria) but has low activity againt *Candida* and *Aspergillus*.

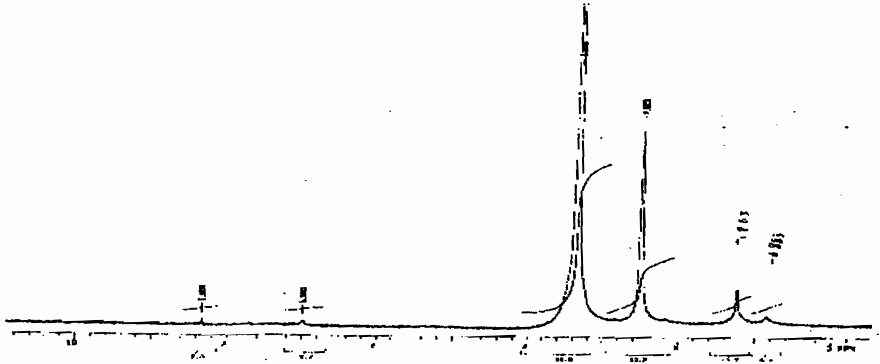

Fig. 4a. NMR spectrum in DMSO of active substance produced by *Streptomyces hygroscopicus* GM-8.

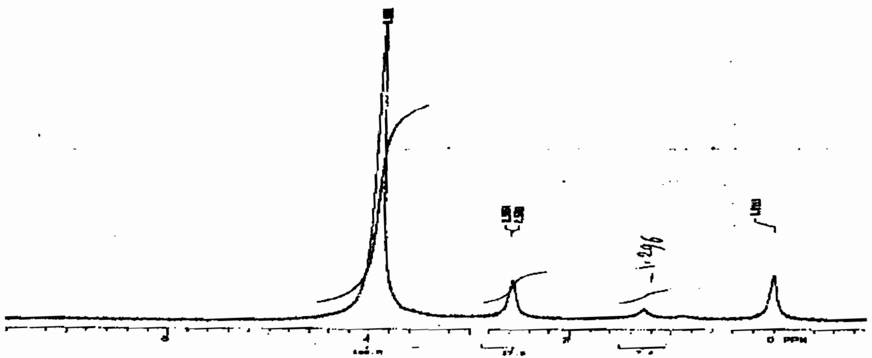

Fig. 4b. NMR spectrum in DMSO and D_2O_2 of active substance produced by *Streptomyces hygroscopicus* GM-8.

Discussion

Selmem Waksman, a soil microbiologist, firstly began a systematic search antibiotics from soil microorganisms after discover penicillin. Since the soil is the natural store of all microorganisms. This eventually leads to the discovery of Streptomycin, from *Streptomyces grisens.* The Streptomycin isolated by Waksman was used for the treatment of a number of bacterial diseases such as tubercubsis , pneumonia, typhoid and other bacterial diseases. Thus, he began his research to discover of new antibiotics from actinomycetes. The genus *Streptomyces* was considered to be the most antibiotic producing genus . For this reason an attempt to isolate *streptomyces* species having the capacity for producing active agents from natural soils habitat of microorganisms was achieved. The *streptomyces* isolate was subjected for a trial to identification using International keys (Shirling & Gottlieb, 1966, 1968a, 1969 & 1972; Buchanan, Gibbson, 1974; Williams, 1989 and Hensyl, 1994). From above mentioned keys the *Streptomyces* isolate was found closely related to *Streptomyces hygroscopicus* and was given the suggested name *Streptomyces hygroscopicus* GM-8 as a variety of this species. The *Streptomyces hygroscopicus* isolated from soil by Waksman (1961), Okanishi *et al.* (1962); Szabo (1975) and Haydock (2004) is similar to the isolate sporophores and it is monopodially branched with narrow compact sinistrorse spirals. The growth on ISP medium give an aerial mycelium colour fluctuate between, white, brown to light gray while substrate mycelium give yellow colour, brown to red colour on most ISP media .

Streptomyces hygroscopicus produce numerous antibiotics, angustmycin (A, B and C), glebomycin, azolomyins, carbomycin, endomycins, hygromycin, oxytetracycline glatarimide and geldanamycin (Yuntsen *et al.*, 1956; Eble *et al.*, 1959; Waksman, 1962; Urakawa, 1993; Hossain *et al.*, 2004 and Hu *et al.*, 2004). The use of antibiotics emerges in resistant populations of microorganisms within animals (both food producing and companion) and humans treated with those products. Development of resistance to antibiotics represented challenges and increasing problem in veterinary and human medicine . This required development of antibiotics and search of a new antibiotics used for treatment of resistant strains. (Goodyear and Threlfall, 2004). The active metabolite isolated in this study (produced by *Streptomyces hygroscopicus* sp). like geldonamycin and novel geldonamycin analogues as reported by Deboer *et al.* (1970), Deborer & Dietz (1976) and Hu *et al.* (2004). Geldanamycin is a benzoquinone ansamycin antibiotic produced by *Streptomyces hygroscopicus* as an antibacterial , antifungal and antiviral agent (Andreas *et al.*, 2003). From the fragmentation pattern of the MS, NMR, IR, UV, elemental analyses and chemical reaction it was found that the active purified metabolite is consider as a novel geldonamycin analogue closely related to geldanamycin and novel geldonamycin analogues reported by Deboer *et al.* (1970), Deborer & Dietz (1976) and Hu *et al.* (2004).

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عزل وتنقية ودراسة صفات بعض العزلات الأكتينوميسيتية التي تنتج بعض المواد الأيضية ذات النشاط ضد ميكروبي

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تم عزل وتنقية بعض سلالات الأكتينوميسيتية من عينات التربة جمعت من جبل المقطم بالقاهرة ، مصر. وقد أظهرت بعض تلك العزلات نشاطا أحيائيا ضد الكائنات الدقيقة تحت الأختبار، حيث تم اختيار العزلة رقم (٨) التي أظهرت نشاطا أحيائيا ملحوظا أقوى من باقي العزلات تحت الدراسة. وقد تم تعريف العزلة باستخدام الصفات الظاهرية والفسيولوجية والبيوكيميائية باستخدام مفاتيح الموصى بها عالميا لهذا الغرض حيث تبين أنها تنتمي إلى جنس *ستربتوميسيز ميجروسكوبكس*. كما تم عزل المادة الفعالة من الوسط الغذائي باستخدام الكلورفورم وتم تنقيتها باستخدام الفصل الكروماتجرافى على الصفائح الرقيقة والمذيبات العضوية. ولمزيد من الدراسة تم فحص الصفات الفزيائية والكيميائية للمادة الفعالة النقية من حيث الذوبان ، التحليل الكيميائي للعناصر والخواص الطيفية والتفاعلات الكيميائية. كما تم دراسة الخواص الاحيائية للمادة الفعالة النقية وتحديد أقل تركيز يعطي نشاط ضد الكائنات الدقيقة المستخدمة .