Zagazig J. Pharm. Sci., June 1997,
Vol. 6, No. 1, pp. 41 -47

ISSN 1110-5089

MICROBIAL SUSCEPTIBILITY AND BIODEGRADABILITY TO *TAXODIUM DISTICHUM* SEED OIL

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ABSTRACT

Isolates of two fungal species from rotted orange fruits as well as standard bacterial cultures were screened for their susceptibility and ability to metabolise *Taxodium Distichum* (bald cypress) seed oil over a twelve- day period by adding the oil to fungal spore or bacterial culture suspension. Under these conditions, all tested strains metabolized the oil in variable rates ranged from 8 to 100% . The proportions of the oil component degraded differed widely with the microorganism. The highest degradation was observed with *Aspergillus* and *Penicillium species* (100%), certain other standard cultures , notably of *Pseudomonas aeruginosa ; Klebsiella pneumoniae* and *Bacillus subtilis* metabolized , about 15-30%, of the oil. The in vitro antimicrobial activity of the oil revealed higher efficacy against *Candida albicans* but limited towards Gram - positive bacteria *(Staphylococcus aureus* and *B. subtilis).* No significiant activity was noted against Gram - negative bacteria and true fungi. Microbial degradability of *T. distichum* oil is found to be inversely proportional with the oil bio-activity complete resistance followed by complete degradation exhibited by true fungi to bald cypress oil. By the end of the biodegradation period, antimicrobial activity of the resulted products exhibited no significant difference than that of the substrate . Thus , cultures that were established within 12 days failed to yield significant oil biodegraders . The estimation of the oil was carried out by Gas chromatography (GC) and GC - mass spectrum (GC-MS).

INTRODUCTION

Oils are extracted, packed and stored for various purposes at different circumstances. Otherwise, these processes are performed under aseptic conditions of sterilization, fungal or bacterial contaminants will metabolize such oils inside the containers leading to biodegraded or biotransformed products of different nature.

Many microbes have the ability to metabolize these products. Crude extract containing volatile oil has traditionally been treated for storage but anaerobic biological processes have been shown to be biodegradable to the extract into methane and carbon dioxide in cases of microbial contamination [1,2].

The organism *Alcaligenes denitrificans* has been used in studies on the degradation of volatile fatty acids [3]. Kinetic models are of value in investigating both the capacity and stability of biological processes which utilize inhibitory substances. That biodegradation may offer both thermodynamic and kinetic disadvantages when mineralization involves syntrophic bacteria of methanogenic consortria.

Taxodium distichum belongs to family Taxodiaceae which includes two species. In Egypt the tree is growing successfully in the north west coast , Alexandria , Damietta also in Giza and Fayoum . In Mexico the bark extraction of *Taxodium mucronatum* was used to promote menstruation [4] .

Herein , this study was chosen to throw light on the effect of oil contamination on the activity of an oil-degrading consortuim . The main goals were to develop a wild microbial contamination convenient to take place under anaerobic conditions of package and storage and maintain the long - term survivability of cells within the oil extract and formulate a kinetic model which describes the rate of oil biodegradation by the contaminant .

The purpose of the experiments reported here was to establish, whether the ability to metabolize *Taxodium distichum* oil is widespread among environmental microorganisms and the extent to which it is associated with contamination.

MATERIALS AND METHODS

The study was conducted in the Department of Medicinal and Aromatic Plants Research (M & APR), Alexandria Horticultural Research Station, Sabahia and Microbiology Department, Faculty of Pharmacy, Zagazig University (1995-1997).

Oil extraction :

The seeds of *Taxodium distichum* tree. (Family Taxodiaceae) were the source of this essential oil which was extracted by water distillation method according to [5]. The same technique was also used to extract the oil from the whole culture of biodegradation after the incubation period.

Oil analysis :

Gas chromatography-Mass spectrum (GC - MS) and Gas Chromatography (GC) techniques were used to determine the principal components of the essential oil of bald cypress seeds. GC-MS conditions were: instrument (Hewlett Packard; HP); column (stainless steel capillary; 12m); stationary phases (SMG); flow rate (1.5ml /min), Temp. rate (5°C/min); injection temp.

(250°C); detector temp. (280°C) and recorder (HP) and for GC, the conditions differ in: instrument (Fisons 8000), stationary phases (MGE) and column temp. (30-180°C).

The principal compounds of the essential oil in *Taxodium distichum* oil were estimated from the measured peak area of the chromatogram according to previous method (6,7) . The principal components, which were used as reference for the determination in the essential oil by GC and GC -MS , were obtained from Buch Boake Allen Limited, London, England (Borneol) and Siba Giegi, N.Y. , USA. (Citral, Citronellol and Linalool).

Antimicrobial activity :

Cultures and Inocula Preparation : Wild isolates of *Aspergillus niger* and *Penicillium sp.* from spoiled orange fruits (stored on slopes of Dico Bacto malt extract agar at 1°C) as well as standard cultures of *Candida albicans* (ATCC 10231) *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 6538) ; *Micrococcus luteus* (ATCC 9341) , *E. coli* (ATCC 10536) , *K. pneumoniae* (ATCC 10031) and *P. aeruginosa* (ATCC 9037) were used. Suspensions were prepared by inoculating fresh stock cultures into separate broth tubes each containing 5 ml of Brain heart infusion broth and incubated at 37°C/24 hrs. for bacteria or Sabouraud dextrose agar and incubated at 25°C/3-7 days for sporulation (8) for fungi. Spore suspension was prepared in water (10^5-10^6 spores/ml) for inoculation.

Test solutions preparation: *Taxodium distichum* oil is water - insoluble so that two fold serial dilutions (1/1 to 1/128) were done with dimethyl formamide (DMFA) to obtain oil concentrations from 50 to 0.39%. Absolute oil (100%) and solvent (oil conc. 0%) were used as positive and negative controls.

Susceptibility testing :

Disc technique (9) was applied using sterile discs of whatman filter paper (6mm diameter) with a final charge 10µl/disc. The discs were placed onto the surface of inoculated agar plates (Brain heart infusion agar or Sabouraud maltose agar). The plates were incubated at 37°C /24-48 hrs (Bacteria) or 25°C/48-72hrs (Fungi). After incubation, the inhibition zones were recorded in (mm) diameter . Less than 6 mm indicates no effect .

Microbial biodegradation :

Attempt was made to establish semi- anaerobic cultures including substrate and inoculum simulated those taking place under storage and package of the oil. Because of *Taxodium distichum* oil water - insolubility, modified technique of that previously reported (10) was initiated by adding oil directly to the microbial suspension. According to **Perlman** (11), resting bacterial cell suspensions and previous reports (12,13) fungal spores were added to the substrate. A sterile plug consisting of a cork wrapped in aluminium foil was used and encircled with PTFE tape to prevent any loss of the volatile oil. The cultures were incubated at 28°C (fungi) or 37°C (bacteria) on a rotary shaker at 200 rpm. Cultures were established within 12 days.

RESULTS AND DISCUSSION

The results are recorded in the tables (1 & 2). A potent anticandidial effect was exhibited (14mm) while no antimycotic effect was noticed against both *Aspergillus niger* and *Penicillium species.* A slight antibacterial activity was noticed against *Bacillus subtilis* (8mm) but no effect was noticed against other standard bacterial cultures *(E. coli, S. aureus, P. aeruginosa,K. pneumoniae* and *M. luteus).*

The absolute solvent DMFA exhibited (per se) potent antibacterial (but not antifungal) activity against *P. aeruginosa* (16mm), *E. coli* (12mm) , *M. luteus* (12mm) and *K. pneumoniae* (11mm). This efficacy was gradually decreased along with the dilution by the oil up to the minimum zones (7 or 8 mm at double dilution).

Meanwhile, it seems that synergistic effect has taken place between the oil and the solvent against *S. aureus* and *B. subtilis* where large zones of inhibition (14 and 10mm, respectively) were noticed at first then gradually decreased on dilution (9 and 8 mm, respectively) though the non - recognized efficacy of the oil per se against *S. aureus* . The efficacy of the oil against both *B. subtilis* and *Candida albicans* is assured by the observation of the gradual decrease in the inhibition zones by dilution as well as the minimal zones exhibited by the absolute solvent (negative control).

The results also, showed that the antimicrobial activity of the end product after the biodegradation period, was similar to that of the substrate. This indicates that the produced byproducts or metabolites had no additional effect on the microorganisms or that the biodegraded portion was completely exhausted (perhaps to methane and water). Thus, cultures that were established within 12 days failed to yield oil degraders.

Generally, as shown in table (2) , the susceptibilities of the tested isolates to *Taxodium distichum* oil could be interpreted as : Gram negative bacteria (*E. coli* , *K. pneumoniae,* and *P. aeruginosa*) and *M. luteus* were affected only by the solvent ; Gram positive bacteria *(S. aureus* and *B. subtilis)* exhibited higher effect by the combination of the solvent and substrate than of each; yeast like fungus *(Candida albicans)* exhibited low, high and higher sensitivities by the solvent, substrate and their combination, respectively; while true fungi *(Aspergillus niger* and *Penicillium sp.)* exhibited resistance to any of them.

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Table (1) : Antimicrobial activities of *Taxodium distichum* oil before and after exposure to microbial degradation

Micro-organism	Absolute oil (+C)	Zone diameter inhibition (mm) / dilutions								Absolute solvent (-C)
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
S. aureus ATCC 6538	7	14	14	14	12	11	9	9	9	12
B. subtilis ATCC6633	8	10	10	10	10	9	8	9	8	8
M. luteus ATCC9341	0	7	9	9	10	11	12	12	12	12
E. coli ATCC10536	0	7	9	10	11	12	12	12	12	12
K. peumoniae ATCC10031	0	8	8	9	9	10	10	11	11	11
P. aeruginosa ATCC9087	0	7	11	13	13	14	13	15	17	16
C.albicans ATCC10231	14	16	16	16	14	10	9	8	8	7
Aspergillus niger (Wild)	0	0	0	0	0	0	0	0	0	0
Penicillium sp. (Wild)	0	0	0	0	0	0	0	0	0	0

(+C) : Positive control (-C) Negative control

These results might be explained by taking in consideration the differences in cell wall structures of these microbial groups. The special components of the cell wall are generally simple and few in Gram positive bacteria (teichoic acid and polysaccharide sugars) ; complex and large in Gram negative bacteria (lipoprotein, phospholipid bilayer and lipopolysaccharide) and more complicated in fungi.

Because the flasks were sealed after the addition of the microbial suspension, condition may not have remained aerobic for the whole of the subsequent days of the incubation . This experimental limitation was accepted to allow a large number of isolates to be screened with simple apparatus . The isolates were able to metabolize the oil (Tables 3 & 4 and Figure 2) and only few showed little activity. To determine whether the growth was at the expense of total degradation of *Taxodium distichum* oil, the extent of the physical loss of the oil was determined .

Table (2) : Sensitivity patterns of different microbial groups.

Microbial group	Sensitivity pattern with		
	Oil	Oil / Solvent	Solvent
Gram positive bacteria	+	++ → +	++
Gram negative bacteria	0	+ → ++	++
Yeast - like fungi	++	++ → +	+
True fungi	0	0	0

+ : Low effect , ++ : High effect and 0 : No effect.

At the end of the incubation period, there was a loss of the oil ranged from 100% with true fungi to about 8% with staphylococci. Thus, complete metabolism or consistently high conversion was observed with *Penicillium sp.* and *Aspergillus niger* while the amount of oil metabolized varied (ranged from 8 to 30%) with most of the other species .

The above results were obtained after exposure of the fungi or bacteria to the oil for a fixed period of 12 days . The disappearance of bald cypress oil and the appearance of degradation products were not monitored over time but the final products were recovered and analyzed. The extracts were analyzed by GC using a Fisons 8000 stainless steel capillary column operated at column temp. 30-180°C with the same gas chromatographic conditions and a Hewlett-Packard 5992 gas chromatograph - mass spectrometer (Fig. 1&2).

On the basis of the gas chromatographic measurements , the oil was completely biodegraded by fungal contamination but partially biodegraded by bacterial contamination within 12 days . No degradation or transformation of *Taxodium distichum* oil was detected in sterile controls, evidenced by the lack of both the disappearance of the of the substrate and the appearance of products, even when incubation times were extrended to 30 days.The relationship between microbial susceptibility and biodegradation of *Taxodium distichum* oil was highlightened by the compatible results obtained , as shown in tables (2& 4) .

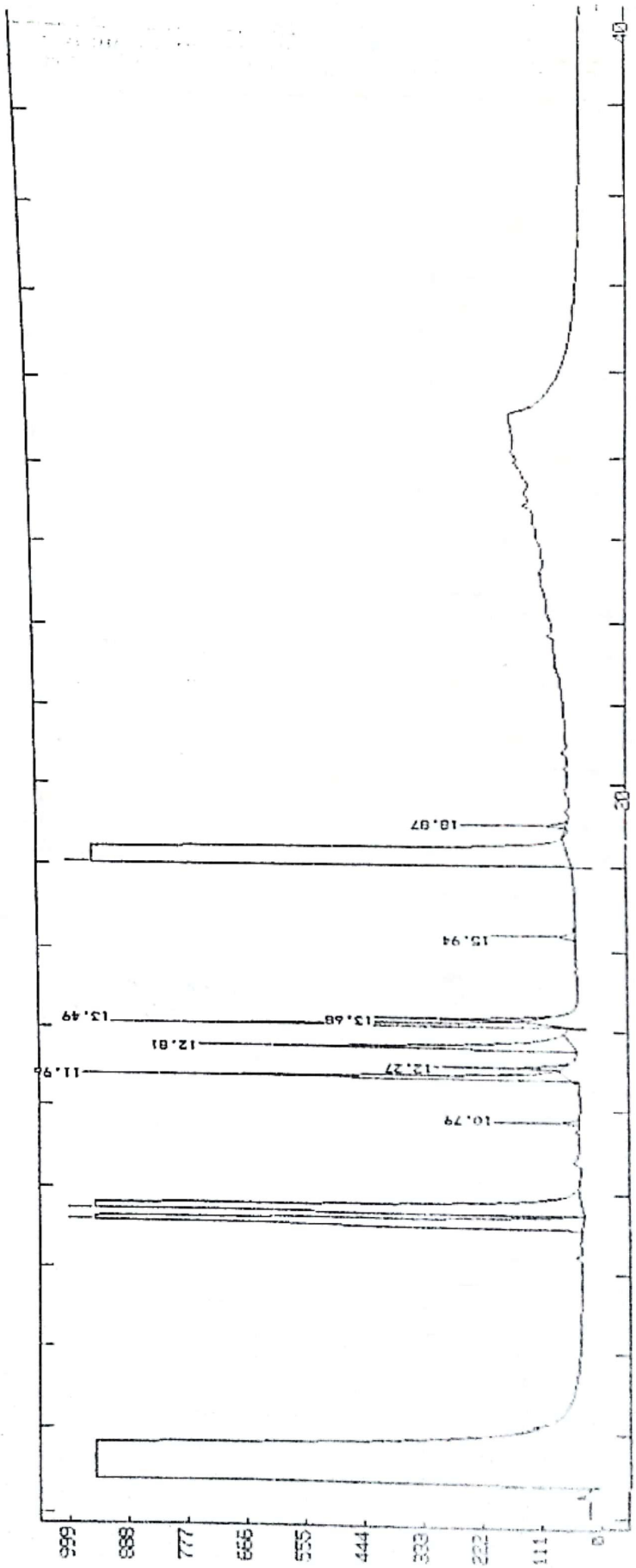

Fig. (1) Typical Chromatogram of *Taxodium distichum* oil by gas Chromatography (GC).

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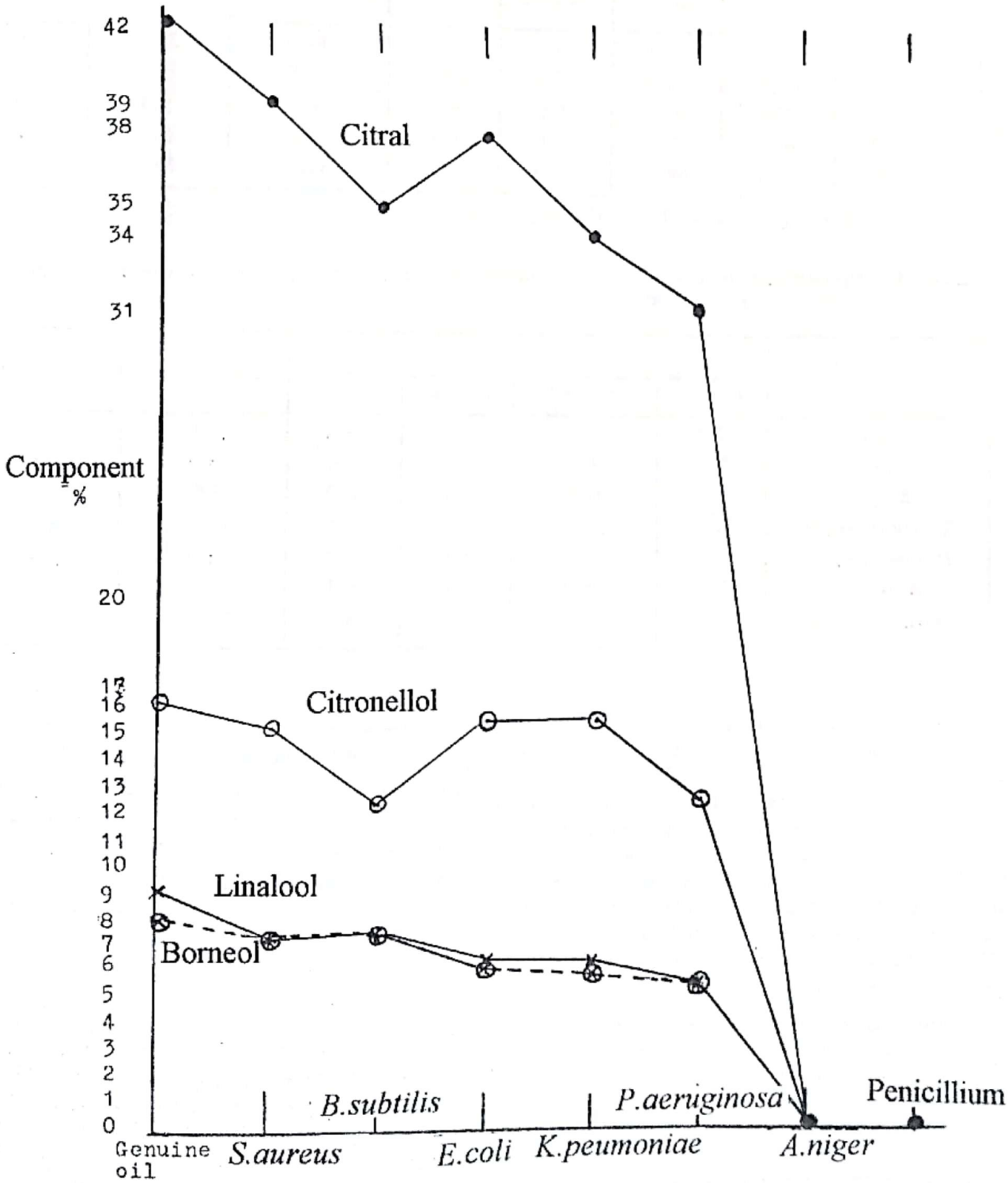

Fig. (2) Microbial biodegradation effect on *Taxodium distichum* oil main components.

Table (3) : Residual principal compounds of *Taxodium distichum* oil after microbial biodegradation.

Main component of the oil	Component %						
	Genuine	*S. aureus*	*B. subtilis*	*E. coli*	*K. pneumoniae*	*P. aeruginos*	*A. niger* or *Penicillium sp.*
Citral	42.1	39.8	35.1	38.0	34.0	31.2	0
Citronellol	16.3	15.2	12.2	15.5	15.6	12.2	0
Linalool	9.0	7.3	7.4	6.3	6.2	5.4	0
Borneol	8.1	7.2	7.3	6.1	5.9	5.4	0
Total	**75.5**	**69.5**	**62.0**	**65.9**	**61.7**	**54.2**	**0**

N. B. : True fungi exhausted the oil completely

Table (4) : Percentage microbial biodegradation of *Taxodium distichum* oil main components after 12 days incubation.

Degradable microbe	% degraded component				total degradable %	% degraded
	Citral	Citronellol	Linalool	Borneol		
S. aureus	2.3	1.1	1.7	0.9	6.0	7.94
B. subtilis	7.0	4.1	1.6	0.8	13.5	17.88
E. coli	4.1	0.8	2.7	2.0	9.6	12.72
K. preumoniae	8.1	0.7	2.8	2.1	13.7	18.15
P. aeruginosa	10.9	4.1	3.6	2.7	21.3	28.21
A. niger	42.1	16.3	9.0	8.1	75.5	100
Penicillium sp.	42.1	16.3	9.0	8.1	75.5	100

This illustrates the inverse proportionality between microbial sensitivity to the oil and microbial ability to biodegrade its components . The lower the susceptibility of the microbe to the effect of the extract, the higher the biodegradability exhibited to its components . Hundred percent resistance of the true fungi and Gram negative bacteria against the oil, resulted in 100 and ≅ 25% biodegradation of its components, respectively after 12 days incubation at 37°C . Moderate sensitivity of Gram positive bacteria to the oil yielded lower ability of biodegradation (≅ 15%).

The study introduced a form of mild contamination enough to maintain the syntrophic activity necessary for oil biodegradation with mininal exposure of cells to oxygen.

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Received : February 2, 1997
Accepted : March 10, 1997

التأثير الميكروبى والتحلل البيولوجى لزيت بذور نبات التكسوديوم ديستيكم ببعض الميكروبات

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فى هذا البحث تم إختبار فاعلية الزيت المستخرج من بذور شجر التكسوديوم على سلالات قياسية من البكتريا بالإضافة إلى بعض الفطريات المعزولة من ثمار البرتقال المتعفنة لمعرفة مدى التأثير المثبط وكذلك مقدرة تلك الميكروبات على تكسير أو تغيير طبيعة الزيت إذا مالوث بها أثناء التحضير أو التعبئة أو التخزين.

وقد أوضحت الدراسة أن جميع العزلات المذكورة لها القدرة على تحليل الزيت بقدرات متفاوته تراوحت بين ٨ إلى ١٠٠٪ فى خلال ١٢ يوم من التجربة وهذا التأثير التدميرى للزيت يتناسب عكسى مع مدى تأثر الميكروب وتثبيطه به وعليه فلم تتأثر الفطريات مطلقاً بالزيت بل نمت عليه وحللته كاملاً خلال المده المذكوره وكذلك لم تظهر فاعلية تذكر للزيت على البكتريا السالبة لصبغة جرام التى استهلكت معدلات متوسطة من الزيت تراوحت بين ١٥-٣٠٪.

أما الفاعلية التثبيطية للزيت فقد كانت واضحة على البكتريا الموجبة لصبغة جرام وكذلك فطر الكانديدا وقد كان ذلك واضحاً فى المعدلات المتدينه التى تمكنت تلك البتكريا من استهلاكها خلال مدة الإختبار.

والجدير بالذكر أن الأختفاء الكامل أوالجزئى للزيت بالفطريات أو البكتريا لم يظهر أى دلائل على وجود مواد تكسرية أو معدلة ذات تأثير ميكروبى بدليل الحصول على نفس النتائج الخاصة بالفاعلية للزيت أو الناتج المفصول بعد أنتهاء فترة التحضين وكذلك تحليل الـ GC, GC-MS الموضحة.