Bull. Fac. Agric., Cairo Univ., 56 (2005): 483-500.

ANTIOXIDATIVE AND ANTIFUNGAL ACTIVITY OF AZADIRACHTIN DERIVATIVE ON INFECTED TOMATO SEED GERMINATION

(Received: 16.2.2005)

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ABSTRACT

Neem (*Azadirachta indica*) has been known for ages as an insecticidal plant and recently is classified as a therapeutic plant. In this study, more investigation has been done on the plant evaluation. Neem seeds extract has been fractionated into methanol, chloroform, butanol and hexane fractions. The fractions have been investigated for the antioxidative activity using the thiocyanate method under storage in dark at 40°C up to 25 days. The methanol fraction showed antioxidative activity (11%) extended to 25 days, comparing to 21.5% for vitamin E. A pro-oxidative effect has been noticed from the butanol, chloroform and hexane fractions at the same period of time.

The methanol fraction has the highest reducing power. It showed (0.55) absorbance value at 700 nm. This value has been followed by the hexane and chloroform fractions values. On the other hand, the butanol fraction showed no reducing power in the experiment. The potential of the neem methanol fraction might be due to the content of the phenols. The methanol fraction had the highest phenol content (0.147 g/100g as tannic acid or 5.78 g/100g as gallic acid) followed by the chloroform, butanol and hexane fractions, respectively.

Further fractionation between methanol : distilled water showed that 30:70 v/v fraction, had the highest inhibition

percentage against some pathogenic fungi. The inhibition (%) was 52.5 and 37.5 against *Rhizoctonia solani* and *Fusarium solani*, respectively. A potent compound has been separated from this fraction using TLC. The 300 ppm of this compound was the ideal concentration to inhibit *R. solani* (70.3 %) and *F. solani* (57.9 %). The storage of this concentration in dark at 40°C was also followed in the pathogenic fungi inhibition up to 15 days. This concentration showed inhibition of 70, 50 % against *R. solani* and *F. solani*, respectively at the first day of storage. Between 7- 9 storage days, the inhibition % was decreased dramatically, correlated to the decrease in the antioxidative effect of the methanol fraction. In addition, the methanol fraction and the effective compound enhance the germination (%) of infected tomato seeds by *R. solani* or *F. solani*.

The potent compound was identified using the MS technique. The compound was suggested to be; Trihydroxy-tetra acetyl-Azadirachtin. The hydroxyl groups, double bonds and the heterocyclic rings in the structure might be responsible for the variable potential of the compound.

Key words: *antifungal, antioxidant, Azadirachta indica, azadirachtin, neem, phenol, reducing power.*

1. INTRODUCTION

Neem (*Azadirachta indica*) is a subtropical tree native to the dry areas. Neem is planted in Pakistan, India, Indonesia, Thailand, Burma, Sri Lanka, Malaysia and East Africa. The traditional medicinal and old knowledge plant has now led to several therapeutic and industrial useful preparations, which generates enough encouragement among the scientists in exploring more information about the plant. Many valuable compounds have been extracted –in the last few years- from all the neem plant parts; the flowers, fruits, seeds, bark and leaf of the neem tree. They are known as insect feeding deterrents for ages, and recently as aflatoxin inhibitors (Hampden *et al.*, 1990), for treatment of major protozoal diseases (Phillipson and O'Neill, 1989) and blocking lipid peroxidation and scavenging hydroxyl radicals (Nychas, 1995 and Bandyopadhyay *et al.*, 2002). Neem leaf extracts have an aflatoxin inhibiting factor (Hampden *et al.*,

1990). Phillipson and O'Neill (1989) studied the effect of the terpenes and limonoids from the neem tree for the treatment of major protozoal diseases (malaria, amoebiasis, leishmaniasis and trypanosomiasis) in the third world, with special reference to traditional medicinal plants. Gupta *et al.* (1989) stated that neem leaves are good source of neutral- and acid- detergent fibers, and are rich sources of major and trace elements.

Many valuable compounds have been identified from the neem through the last few years. Siddiqui *et al.* (1988) isolated new diterpenoids from the acidic ethanolic extract from the park of *Azadirachta indica*; nimbionone (12-hydroxy-13-methoxypodocarpa-8,11,13-triene-3,7-dione), and nimbionol (3,12-dihydroxy-13-methoxypodocarpa-8,11,13-triene-7-one).

They have antibacterial activity against the Gram positive; *Bacillus subtilis*, *Staphylococcus epidermidis* and *S. aureus*, and the Gram negative bacterium *Klebsiella ozaenae*. On the other side, Ara *et al.* (1988) isolated new tricyclic diterpenoids from the stem bark; nimosone, nimbosone, methyl nimbiol and methyl nimbionone. They also found the known phenol diterpene sugiol. Kadir *et al.* (1998) determined the phonological characteristics for the neem trees (*Azadirachta indica*) in Peninsular Malaysia. Quantitative analysis of azadirachtin (potent insect antifeedant and growth-regulating agent) content has been analyzed in the neem seed kernel extract using high performance liquid chromatography (HPLC).

Rengasamy and Parmar (1994) evaluated the azadirachtin A concentration in the fresh flowers and fruits of 10 year old neem tree in India. Siddiqui *et al.* (2003) studied the chemical constituents of the flowers of *Azadirachta indica*. They isolated two new flavanones, flowerine (=5-hydroxy-7,4'-dimethoxy-8-(3-methylbut-2-enyl)flavan-4-one); and flowerone (=5,7,8,4'-tetrahydroxy-3'-(3-methylbut-3-enyl)flavan-4-one). They also isolated two new triterpenoids, O-methylazadironolide, diepoxyazadirol and triterpenoid trichilenone acetate, flavanones, and 4-(2-hydroxyethyl) phenol.

In a valuable study, Bandyopadhyay *et al.* (2002) studied the aqueous neem extract as an inhibitor of pylorus ligation and mercapto-methylimidazole induced acid secretion. The bark extract was as potent as ranitidine and more potent than omeprazole. Using the high pressure liquid chromatography, they

identified the major bioactive compound as a phenolic glycoside, isolated from the extract. They attributed to this compound the pharmacological effects of the bark extract.

The aim of the present study was to test the neem fractions as antioxidant factors among the therapeutic functions investigated by researchers. The study included their phenolic content and the reducing power for the different fractions from the neem seeds. The effectiveness of the most potent fraction has further fractionated and tested as antifungal factors. Identification and analyzing the potent effective fraction has been done using the MS technique.

2. MATERIALS AND METHODS

2.1. Preparation of the main fractions

Neem (*Azadirachta indica*) seeds were collected from Aswan Governorate in the summer of 2003. The seeds were washed with tap water and air dried. About 10 g of ground dried seeds were extracted with 100 ml methanol and well stirred for 2 h. The methanol fraction was filtered, evaporated under vacuum and further fractionated according to the method of Alkofahi *et al.* (1996). Chloroform (20 ml) was added to the methanolic extract, and partitioned into two layers. After that, 20 ml of butanol and 20 ml of hexane were added to the lower and upper layers, respectively and further partitioned. The fractions were evaporated under vacuum at 40°C to about 5 ml. The concentrated fractions were kept in the refrigerator to further measurements.

2.2. Antioxidative activity

Half ml from each of methanol, chloroform, butanol and hexane fractions was tested for linoleic acid according to the method of Mitsuda *et al.* (1966). The total volume (5ml) contains 2.5 ml linoleic emulsion, 2 ml phosphate buffer pH 7. Common antioxidant such as α–tocopherol (0.5 ml, 500 ppm) was used instead of the neem fraction. Control without using any additives was also used in the method. The absorbance at 500 nm was recorded at interval time after storage in dark at 40°C up to 25 days using ferrous chloride and ammonium thiocyanate method. All values are the average of three replicate samples.

Antioxidative activity was expressed as % inhibition relative to the control (Ogata *et al.*, 1997) using:

AA= [(degradation rate of control – degradation rate of sample) / degredation rate of control] 100

2.3.Reducing power

The reducing power of neem fractions was determined according to the method of Oyaizu (1986). Neem fractions (0.05 ml) were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion of trichloroacetic acid (2.5 ml, 10%) was added to the mixture, which was then centrifuged at 300 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water and $FeCl_3$ (0.5 ml, 0.1 %), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increase in reducing power.

2.4. Determination of phenolic substances

Phenolic compound contents were determined from 1.0 g fresh weight by refluxing with 50 ml of methanol containing 1% HCl for 4 h. Polyphenols were determined as tannic acid and gallic acid equivalent (Taga *et al.*, 1984). The phenolic contents of the methanol, hexane, chloroform and butanol fractions (0.05 ml) were determined as gram percentages of tannic or gallic acid equivalents (per 100 g fresh weight).

2.5. Antifungal activity of the neem seed methanol fraction and further fractions

The most potent neem seed fraction (methanol) was further fractionated in silica gel column using ingredient methanol:distilled water (90:10, 70:30, 50:50, 30:70, 10:90). The fractions were evaporated to dryness and their antifungal activity was tested against some pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum*, and *Botrytus cinerea*). These pathogenic fungi infect pepper, cowpea, sesame and apple fruits, respectively. The antifungal bioassay was carried out in the Microorganisms Dept., National Research Center, Giza.

The inhibitory effect for each fraction (100µg) was tested in Czapek's agar medium containing individual grown fungi using

the linear growth procedure (Jain and Kar, 1971). The bioeffective compound was separated from the most potent fraction using TLC with hexane:methanol (3:1, v/v).

This compound was tested against *R. solani*, and *F. solani* individually at different concentrations (100, 150, 200, 250, 300, 350 and 400 ppm). The lower effective concentration of the compound was tested at different storage periods up to 15 days in dark at 40°C against *R. solani*, and *F. solani* individually.

The inhibition % for the neem seed extracts was calculated as follows;

I % = [(growth fungus of control – linear growth fungus of sample) / growth fungus of control] 100

2.6. Effect of methanol fraction on the germination of tomato seeds

The fungi (*Rhizoctonia solani* and *Fusarium solani*) were individually isolated from the infected tomato plants (Shihata and Gad El Hak, 1989). The fungi were grown and maintained on Czapek's agar medium and divided to three groups. Each group of the study contained 100 tomato seeds; (1) control of the unaffected seeds, (2) the infected seeds with *R. solani* or *F. solani*. The infected seed groups were treated (3) with methanol fraction or the separated compound.

2.7. Identification of the potent compound in the methanolic fraction using MS technique

Characterization of the potent compound in the methanol fraction was carried out at the Micro-Analytical Center, Faculty of Science, Cairo University. Identification of the mass spectra was done using a HP 6989 Mass spectrometer, electron energy 70eV and final temperature 60°C. Comparing with MS at the commercial library (Wiley 138K, Mass Spectral Database, Wiley 1990) was used, beside the previous data of published research for the identification of the chemical structure of the compound.

2.8.Statistical analysis

The mean values of three replicates were analyzed by student t-test one way ANOVA using Spass package. Data of the

mean values ± SD and the differences were considered significant at $P < 0.05$ (Gomez and Gomez, 1984).

3. RESULTS AND DISCUSSION

3.1. The antioxidative activity for neem fractions

The hexane and chloroform fractions showed antioxidative activity close to that for the natural antioxidant vitamin E up to 10 days storage at 40°C (Figure 1). The antioxidative activity of hexane and chloroform fractions after 10 days storage was 85.1, and 78.3%; respectively, comparing to that for vitamin E (88.3%). The butanol fraction -as well- showed a moderate activity until the seventh day. On the other hand, the butanol, chloroform and hexane fractions showed pro-oxidative activity on 19 days storage. Only the methanol fraction showed a reasonable activity until the fourth day, then showed weak antioxidative activity in the rest of the storage periods up to 25 days. The methanol neem fraction activity was 11%, comparing to 21.5% for vitamin E at 25 days storage. Generally, the antioxidative activity for vitamin E lasted more than the neem fractions up to 19 days, and then decreased dramatically.

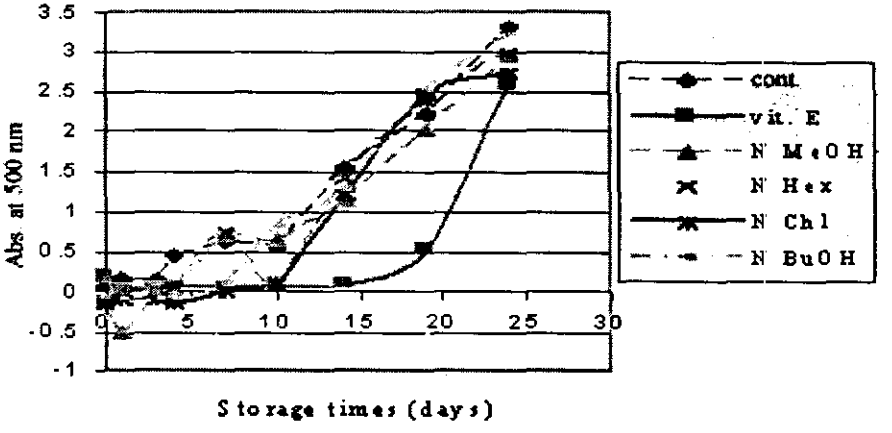

Fig. (1): The antioxidative activity for the neem fractions for 25 day storage at 40°C in dark.

3.2. The reducing power activity for neem fractions

The methanol fraction has the highest reducing power activity (0.55 absorbance) at 700 nm (Figure 2). These results correlate with the antioxidative activity for the methanol fraction (Figure 1). The hexane and chloroform fractions showed absorbance (0.17 and 0.04), respectively. On the other hand, the butanol fraction showed no reducing power activity (Figure 2). The reducing power may be to the electron donor activity for the compounds methanol fraction. The reducing power -or the antioxidative activity- is due to the ability to react with the free radicals to convert them to more stable products and terminate radical chain reaction (Yen and Chen, 1995).

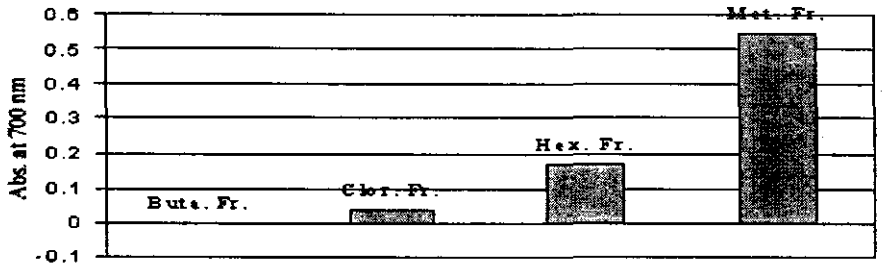

Fig. (2): The reducing power for the neem fractions measured at 700 nm.

Recently, Bandyopadhyay *et al.* (2002) showed that aqueous neem bark extract prevented oxidative damage of the gastric mucosa by significantly blocking lipid peroxidation and by scavenging the hydroxyl radical (.OH) *in vitro*. In agreement to present results, they proved that bark extract was more effective than melatonin, vitamin E, desferrioxamine, and alpha-phenyl N-tert butylnitrone, the known antioxidants having antiulcer effect.

3.3. The phenolic content in neem fractions

Many factors could affect the antioxidative activity or the reducing power potential. One of these important factors is the

phenolic content. The methanol fraction has phenolic content which correlates with the previous reducing power results (Figures 1 and 2) showing the highest content measuring as tannic 0.147 g/100g and as gallic 5.78 g/100g acids (Figure 3).

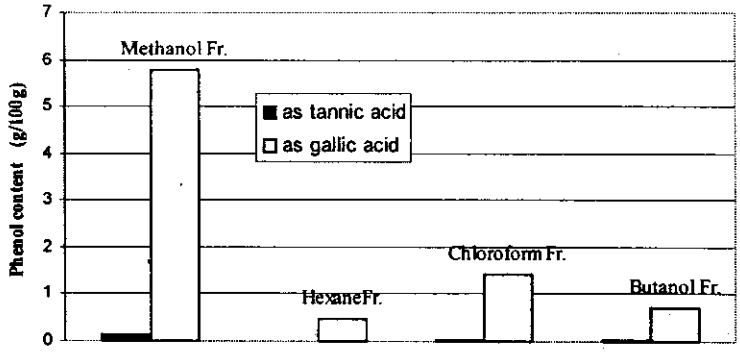

Fig. (3): The phenolic content for the neem fractions measured gm/100 gm fresh weight as tannic and gallic acids.

The chloroform fraction contains phenol as high as (0.037 g as tannic and 1.44 g as gallic acid); followed by the butanol fraction (0.018 g as tannic and 0.717 g as gallic acid) and hexane fraction (0.012 g as tannic and 0.472 g as gallic) fractions which have a moderate phenolic content. The antioxidative results for the methanol fraction correlate with the reducing power and the phenolic content of the fraction. In agreement with this correlation, Proteggente *et al.* (2002) found that ferric reducing ability (FRA) and oxygen radical absorbance capacity (ORAC) values were well-correlated with the total phenolic and vitamin C contents.

3.4. The antifungal activity for neem seed methanol fractions

Methanolic fraction (MeOH:water, 30:70, v/v) was the most effective against *R. solani*, and *F. solani* among the other fractions (Table 1). On the other hand, the other two tested fungi were not affected. Similar results have been found against *Fusarium spp.* (Arnoldi *et al.*, 1989) and *Botrytus cinerea* (Elad, 1992).

The unknown compound in the most active fraction was separated and bioassayed against *R. solani*, and *F. solani* (Table 2). The ideal concentration for the active compound was 300 ppm for the high inhibition % among the other concentrations. In agreement, Galal and Abdou (1996) revealed the increase of the inhibitory effect of antioxidant against some *Fusarium* species with increasing concentration.

Table (1): Effect of different fractions of methanol/water of neem seeds against some pathogenic fungi.

Methanol:water fractions	Inhibition %			
	R. solani	*F. solani*	*F. oxysporum*	*B. cinerea*
90:10	0.0	0.0	0.0	0.0
70:30	0.0	0.0	5.3±0.3	0.0
50:50	2.6±0.1	5.1±0.1	0.0	0.0
30:70	52.5±0.2	37.5±0.05	0.0	0.0
10:90	7.7±0.2	5.3±0.3	0.0	0.0

Data are mean of three replicates ± SD.

Table (2): Effect of different concentrations of the unknown compound against some pathogenic fungi.

Potent compound concentration (ppm)	Inhibition %	
	R. solani	*F. solani*
100	52.5±0.05	37.6±0.1
150	60.0±0.02	45.3±0.25
200	62.5±0.0	50.6±0.15
250	65.0±0.2	55.3±0.25
300	70.3±0.05	57.9±0.03
350	70.3±0.15	57.9±0.0
400	70.3±0.05	60.1±0.33

Data are mean of three replicates ± SD.

The effects of the leaf extracts of *Azadirachta indica* against the rice blast pathogen *Pyricularia grisea* as antifungal properties have been proved. Pre- and post-inoculation of *A. indica* exhibited a greater reduction in the percentage disease incidence than the control with decreasing efficacy as time increased (Kamalakannan *et al.*, 2001).

The oxidation increased dramatically (Figure 4) with decreasing the antioxidative effect of the methanol fraction (Figure 1) between the storage days 7 and 9. In the same storage

time, the potent compound in the methanol fraction showed dramatic decrease in the inhibition %. Similar findings were found between the antioxidative activity and the inhibitory effect against some pathogenic fungi (Elad, 1992; Galal and Abdou, 1996). The antifungal activity had decreased until reached 21, 14% at storage day 15 against *R. solani*, and *F. solani*, respectively. The dramatic decrease may be due to compound degradation.

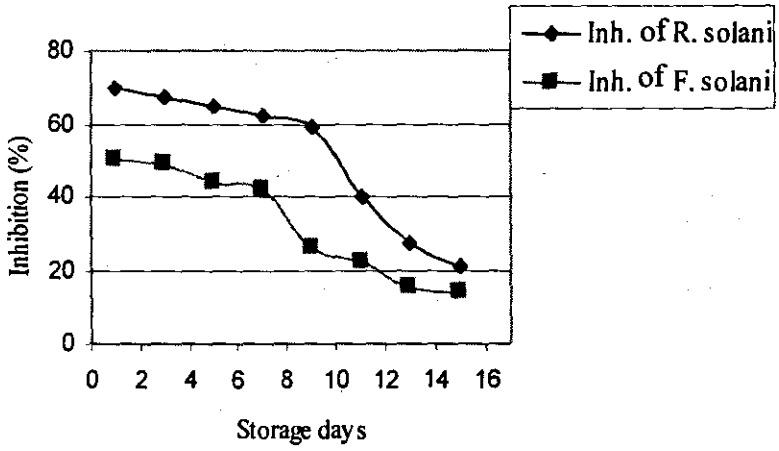

Fig. (4): The inhibition (%) of the active purified compound (300 ppm) extracted from neem seeds methanolic fraction in different storage periods in dark at 40°C.

3.5. Effect of the methanol fraction on the germination of tomato seeds

The data shown in Table 3 indicate that no significant differences were noticed between the treated infected seeds of methanol fraction or the effective compound. Both of the treated infected seed groups have significant higher germination percentage than that for the nontreated infected seed group. Generally speaking, the control (not infected) showed the highest germination percentage.

Table(3): Effect of methanol neem seed fraction and the effective compound on the germination of the infected tomato seeds.

Group	Germination %	
	R. solani	*F. solani*
Control seeds	**94.8±0.84**	
Infected seeds (I.s.)	**44.0±2.51**	**50.0±1.33**
Methanol fr. + I.s.	**75.3±3.33**	**73.3±3.0**
Effective compound + I.s.	**74.3±2.85**	**72.6±2.95**

Data are means of three replicates ± SD.

3.6. Identification of the potent compound in methanolic fraction using Mass Spectrum technique

The potent compound has mp. 155-8°C, and the MS analysis showed mass/charge 67, 195, 294, 564, 728, and 947 (Figure 5). The most abundant fragments were 67, followed by 294 then 195 (100%). The compound fragment m/z 728 was suggested to be Hydroxy-Azadirachtin C_{35} H_{36} O_{17} (Figure 6). The compound m/z 947 was suggested to be Trihydroxy-tetra acetyl-Azadirachtin C_{43} H_{47} O_{24}. It is worth to say, that the structure of Azadirachtin had been studied (Schaaf *et al.*, 2000) using HPLC-MS and identified as very active insecticide and systemic growth disruptor.

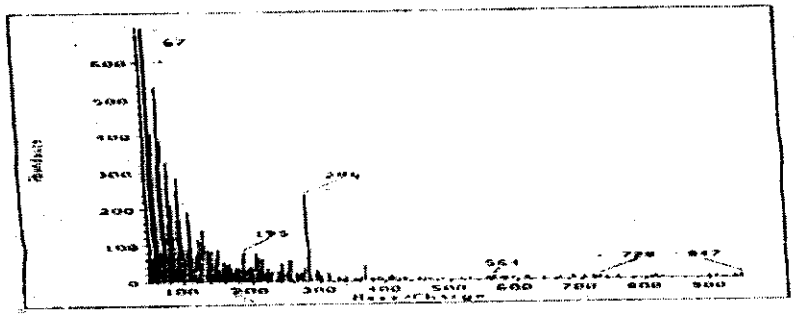

Fig. (5): The MS analysis of the potent antioxidant and antifungi compound extracted from the neem seed methanol fraction.

The simplest fragments (Figure 6) could be iso-pentene (m/z 67) and it was the most abundant fragment. The following three fragments could be mono ring (m/z 195), bi-rings (m/z 294), and tri-rings (m/z 364) respectively. Two oxine (five membered heterocyclic rings) could exist in the non-stable fragment (m/z 564).

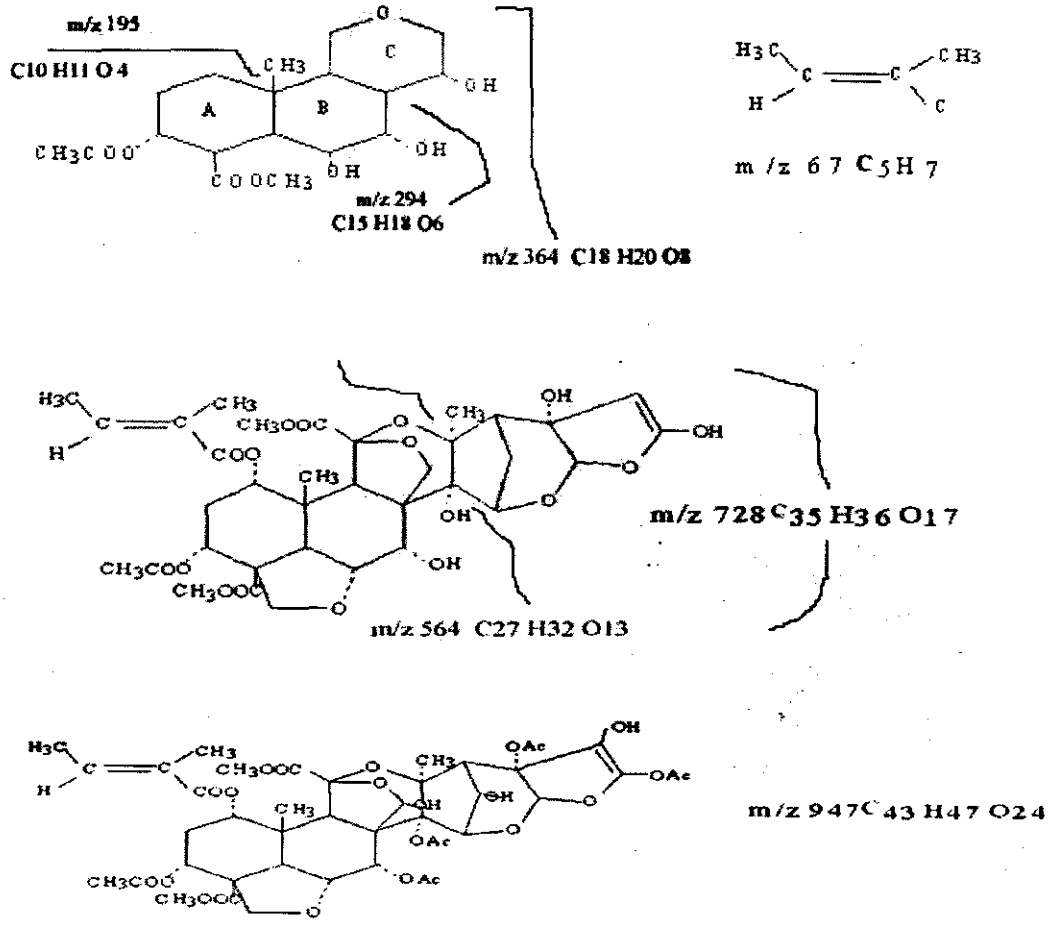

Fig. (6): The suggested fragments appeared in MS of the effective compound extracted from the neem seed methanol fraction.

The structure of the potent identified compound has polyhydroxyl groups, double bonds, and heterocyclic rings. For that, these groups may be responsible for the antioxidative, reducing power, antifungal activities and high phenolic content in this study. Some of the valuable compounds have been recognized and identified recently in the seed neem extracts. Dai *et al.* (2001) determined azadirachtin, limonoids and terpenoids in the neem seed extracts. Similarly, Kadir *et al.* (1998) identified the azadirachtin in the neem seed extract using high performance liquid chromatography HPLC. An extensive research should be undertaken for the different parts of the neem plant and its compound molecules for a better economic and therapeutic utilization.

Acknowledgment

The authors would like to thank Prof. M. N. Mahmoud, Microorganisms Dept., National Research Center, Giza, for his assistance in the antifungal activity method.

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التأثير المضاد للأكسدة والمثبط للفطريات لمشتق الأزاديراكتين علي إنبـــات بذور الطماطم المصابة

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ملخـــص

لقد عرف نبات النيم لعدة سنوات كمقاوم للحشـرات و حـديثا أثبتـت الأبحاث الأهمية الطبية لهذا النبات. تم فى هـذه الدراسـة تقيـيم و فصـل مستخلص بذور النيم و تقدير النشاط المضاد للأكسدة تحت تخزين لفترة ٢٥ يوما و القوة الأختزالية و محتوى الفينولات و كذلك نسبة تثبـيط الفطريـات للمستخلصات المختلفة و كانت النتائج كالتالى:

كان المستخلص الميثانولى الأكثر نشاطا كمضاد للأكسـدة (١١%) بعد فترة تخزين ٢٥ يوما فى الظلام عند ٤٠ °م مقارنة بفيتـامين E (٢١,٥ %) و أما مستخلصات البيوتانول و الكلوروفورم و الهكسان فلقـد أظهـرت نشاطا مساعدا للأكسدة بعد نفس هذه الفترة.

كانت أعلى قوة اختزاليـة و أعلـى محتـوى للفينـولات (٠,١٤٧ جم/١٠٠ جم مقدرة كحمض تانييك و ٥,٧٨ جم/١٠٠ جم مقدرة كحمـض جالييك) من نصيب المستخلص الميثانولى. بينمـا لـم يظهـر المسـتخلص البيوتانولى أى قوة أختزالية فى التجربة.

فى تجربة أخرى أظهر المستخلص ميثانول:مـاء (٧٠:٣٠) أعلـى نسبة لتثبيط *Rhizoctonia solani* (٥٢,٥ %) و *Fusarium solani* (٣٧,٥ %). و لقد تم فصل المركب الفعال من هذا المستخلص و كان التركيز الأمثل لهذا المركب هو ٣٠٠ جزء فى المليون. وفى ظروف تخزين هذا التركيـز من المركب فى الظلام عند درجة ٤٠ °م لفترة ١٥ يومـا (نفـس ظـروف تخزين تجربة مضادات الأكسدة) بدأ النشاط التثبيطى يقل فجأة ما بين اليومين السابع و التاسع متواكبا مع نقص النشاط المضاد للأكسدة فـى هـذه الفتـرة للمستخلص الميثانولى.

أظهر كلا من المستخلص الميثانولى و المركب الفعال السابق فصله تأثيرا فى زيادة نسبة الإنبات لبذور الطماطم المصابة بـأى مـن الفطـرين *R. solani* أو *F. solani*.

تم التعرف على التركيب الكيميائى لهذا المركب الفعـــال باســـتخدام مطياف الكتلة و يرجح أن يكون هو: ثلاثى هيدروكسى – رباعى الأســـيتيل أزادير اكتين.

المجلة العلمـــية لكلية الزراعة –جامعة القاهرة– المجلد (٥٦) العدد الثالـــث (يوليو ٢٠٠٥) ٤٨٣-٥٠٠.