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BIOCHEMICAL AND BIOLOGICAL STUDIES ON PURSLANE AS A PROTECTIVE AGENT

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ABSTRACT

Purslane (*Portulaca oleracea* L) is considered a source of livestock fodder and source of vegetable crops for human consumption. Total soluble protein (TSP) and Purslane protein isolate (PPI) were prepared at pH 8.6 and 3.9, respectively..Lysine and threonine were the first and second limiting amino acids. Therefore, it must be added to any legumes protein to utilize a complementry balance for the essential amino acids. On the other hand, crude methanolic extract (A) and purified compound (B) had antioxidant activity (AOA) about 66.2 and 50.8 % of the total AOA of α-tocopherol with respecting control. Data also showed that AOA for A and B were more stable until 30 days of storage. In addition, the two extracts A and B from *P. oleracea* L. had inhibitory effect against *Clostridium botulism*. On the other side, three pathogenic fungi were isolated from infected tomato, apple fruits and sesame root rot plants, they were identified as *Alternari solani*, *Botrytis cinera* and *Fusarium oxysporium*, respectively. Purified compound (B) had a potent inhibitory effect on *Botrytis cinera* particularly at concentration ranged from 150 to 200µg/ml. Therefore, it can be concluded that antioxidant substances of *P. oleracea* L. are considered as protective agent against some pathogenic microorganisms.

Keywords: Crude methanolic extract (A), purified compound (B), antifungal agent,antioxidant activity (AOA) and protective source.

INTRODUCTION

The diffeciency of protein sources for meeting the nutritional requirements of the increasing world population has stimulated intensive researches into all pottential sources. One of these sources is the protein extracted from *Portulaca oleracea*. L. leaves. Leaf protein affords good poptential as a protein supplement (Kinsella, 1970). Biochemical studies on the extracted protein from purslane were studied by Carlsson, *et al.* (1984); Pirie (1984); Reddy and Joshi (1986) and Moussa, *et al.* (1990).

They investigated the extratability of purslane protein, chemical composition and its mitritive value as a food additive source. Moussa, *et al.* (1990) studied also the protein fraction of purslane leaf protein concentrate and they identified two major fractions with molecular weights of 42.5 and 32.0 KDa. They showed that purslane protein concentrate contained 60 to 70% of the total dried matter.

On the other hand, common purslane (*Portulaca oleracea* L.) was investigated for its identification, etymology, histology as a weed, distribution weedines, uses and poisonous properties by Mitich (1997). In greenhouse trials, *Portulaca oleracea*. L. plants were carried out by Grieve and Suarez (1997).

Although purslane has a high medicinal compound uses (Navas, *et al.*, 1998), but slightly litterature citted for the antioxidant compounds which play an important role as protective agent. However, purslane might be contributed phytotoxic substances to the soil with inhibitory effect on the seed germination of some field crops (Khalaf, *et al.*, 1998). Although *Portulaca oleracea* L. was severly damaged by leaf minor feeding, this injury did not insufficient biological weed content to reduce the need for use of other weed managament techniques (Norries, 1997). However, other investigation was reported by Navas, *et al.* (1998) who identified the important role of purslane against cucumber mosaic virus as a potent protective agent.

The aim of study to evaluate the extracted protein from *Portulaca oleracea*. L. and determining antioxidant activity in the methanolic extracts, as well as evaluate these antioxidants and their functions as a protective agent against some pathogenic microorganisms.

MATERIAL AND METHODS

Purslane (*Portulaca oleracea* L.) leaves and stems were obtained from the Central Agricultural Researches, Giza, Egypt in 2003. The leaves and stems were cut out and air dried then ground in a coffee grinder and stored at 4°C until used.

Biochemical studies:

The defatted matter was extracted in alkali-saline buffer (0.5 N NaCl + 0.1N NaOH, adjusted pH at 8.6) to obtain total soluble protein (TSP) according to the method described by Tella and Ojehomon(1980). Solubility was carried out at gradient pH ranged from 2 to 10 (El-Morsi, 1982). Purslane protein isolate (PPI) was prepared by precipitation at the isoelectric point (pH 3.9). Both of TSP and PPI were freeze dried.

Amino acid contents for the two extracted protein were determined for their acid hydrolyzates using automatic amino acid analyzar model Alfa plus 4151 LKB Biochrom at Central Laboratory, Fac. of Agric., Cairo University. Tryptophan was determined colorimetrically according to Smith and Agize (1951). Amino acid score were calculated reffering to the provisional amino acid scorring patterns of FAO/WHO (1985).

Extraction and assay of antioxidant substances:

The prepared dried matter of purslane (5.0 g) was extracted with 50 ml methanol/water (9:1), followed by filtration and evaporated to dryness under vacuum at 40°C. The final obtained powder was divided into two portions, the first represented the crude extract (A) and the second one was fractionated on the silica gel colum (60x2cm) using solvent (methanol/water/1N HCl, 90: 9 :1 by volumes). The major component was separated on thin layer chromatography (TLC). The major compound was eluted, then redissolved in 1 ml of the same solvent and separated on TLC to identify the purified compound (B).

Antioxidant activity (AOA) of the two prepared methanolic extracts (A and B) were stimulated using linoleic acid system (Osawa and Namiki, 1981). Each sample (200 µg) was added to solution mixture of linoleic acid (0.13 ml), 99% distilled ethanol (10 ml) and 50 mM phosphate buffer (pH 7.0,

10 ml). The volume was completed to 25 ml with distilled water, then incubated at 40°C to determine the oxidation degree according to the method described by Mitsuda,*et al.* (1966). The obtained colour yielded from adding $FeCl_2$ +ammonium thiocyanate was measured at 500 nm., alpha tocopherol (200µg) was performed as standard AOA. The control was conducted using the all solutions and substrate (linoleic acid) without antioxidants. Both two extract (A and B) were measured as AOA % by comparing with ∝ tocopherol with respecting of control as 100% lipid oxidation. All measurement were carried out in triplicates. The all used chemical were purchased from Sigma chemical Co. (Pool Dorset, U.K.).

Furthermore, the two extracts (A and B) were stored in the dark at 40°C for intervals periods (0, 15, 30, 45 and 60 days) to ilucidate the effect of storage periods on the antioxidant activity.

On the other hand, polyphenolic contents for both A and B were determined according to the procedure of Folin-Denis (Swain and Hills, 1959) to explain the relationship between polyphenolics and AOA at the same intervals periods as mentioned previously.

Inhibitory effect against some pathogenic bacteria and fungi:

Effect of antioxidant substances of A and B as inhibitor agent against some pathogenic bacteria (*Bacillus subtilus*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Clostridium botulism*) and some pathogenic fungi (*Alternari solani*, *Botrytis cinera* and *Fusarium oxysporium*) were carried out according to the method described by Jain and Kar (1971). All bacterial strains were obtained from Microbiology Department, Central Agricultural Researches, Giza and all fungi were isolated from infected fruits of tomato, apple and rot root of sesame plants, respectively in Department of plant pathology, Faculty of Agriculture, El-Minia University.

However, the width zones of inhibition was measured for each bacterium and fungus, individually with respecting of control. In addition, gradient concentrations from the purified compound (B) as follows: 50, 100. 150 and 200 µg/ml were bioassayed against the most susceptable strain of bacterium *C. botulism* and *B. cinera* to limit the optimal effective concentration.

Statistical analysis:

The mean values of three replicates were analyzed by the student's t-test one way ANOVA using Spass package. Data recorded with their ±SD and the differences were represented a significant at $p < 0.05$.

RESULTS AND DISCUSSION

Total soluble protein (TSP) was extracted at pH 8.6 and it contained 22.3% protein, while purslane protein isolate (PPI) was prepared at pH 3.9 and it contained 65.8% protein. The solubility curve (Fig. 1) showed about 82% protein was soluble at pH 8.6. Minimum solubility was arround at pH 3.9 (isoelectric point). Similar results were reported by Moussa *et al.* (1990) for the leaf protein concentrate of purslane. They indicated that optimal solubility of this protein at pH 9.0 and the isoelectric point at pH 4.0.

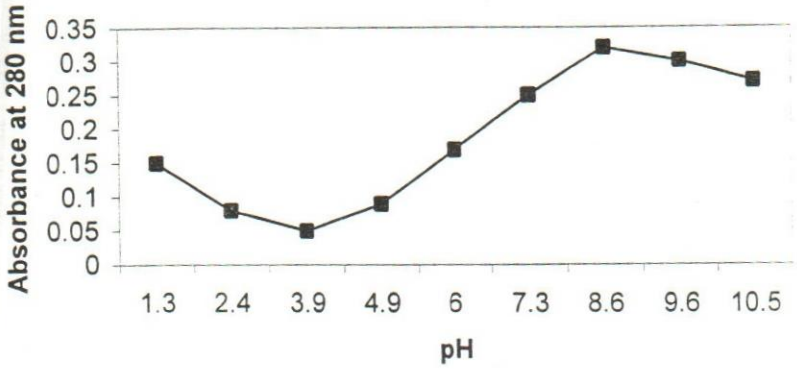

Fig (1) Solubility of the total extractable protein from P. *oleracea* L.

Amino acids content:

Data are shown in Table (1) indicated that total essential amino acids were 35.0 and 36.0 g/100g protein for TSP and PPI, respectively. The first limiting amino acid was lysine and the second one was threonine for both TSP and PPI. It is interst to notice that sulfar amino acids (cys.+Met.) were not limiting ones, therefore, purslane protein can be added to any common legume protein to utilize a complementry balance between the essential amino acids among purslane protein and other legume protein. This supplement improve the chemical score and increase the quantity of essential amino acids such as lysine, threonine, aromatic and sulfar amino acids (Hussein and El Shewey, 1999).

Table (1): Amino acids content of *P. oleracea* L.

Amino acids	Total soluble protein (TSP)		Purslane protein isolate (PPI)		FAO/ WHO (1985)
Essential A.A.	g/100g protein	Score %	G/100g protein	Score %	
Lysine	4.9	84.5	5.0	86.2	5.8
Leucine	8.1	122.7	8.7	131.8	6.6
Isoleucine	4.0	142.9	4.0	142.9	2.8
Met+cys	2.3	100.0	2.3	100.0	2.3
Valine	4.9	140.0	5.0	142.9	3.5
Phe.+Tyr.	6.5	103.2	6.9	109.5	6.3
Threonine	3.1	91.2	3.0	88.2	3.4
Tryptophan	1.2	109.1	1.1	100.0	1.1
Non essential A.A.					
Glutamic acid	14.0		13.9		
Aspartic acid	12.2		11.9		
Alanine	5.9		6.0		
Glycine	5.9		6.0		
Serine	4.3		4.3		
Arginine	9.6		9.6		
Histidine	3.7		3.8		
Proline	4.9		5.0		
Total essential A.A.	35.0		36.0		
Total amino acids	95.5		96.5		
E/T ratio♣	36.7		37.3		
First limiting A.A.	Lysine		Lysine		
Second limiting A.A.	Threonine		Threonine		

♣= Total essential amino acids /Total amino acids

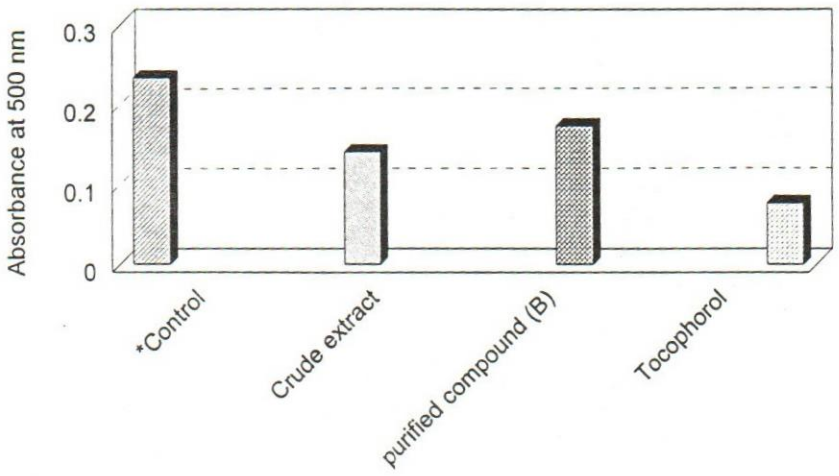

Fig (2) Antioxidant activity of crude methanolic extract (A) and purified compound (B) from P. oleracea L.,

*control was considered 100% lipid oxidation

Antioxidant activity of purslane:

Antioxidant compounds are principal ingradient that protect food quality be retarding oxidative breakdown of lipids (Modhavi and Solunke, 1995). Antioxidant activity (AOA) of crude methanolic extract (A) and purified compound (B) which were extracted from leaves and stems of *P. oleracea* L. in the linoleic acid system are shown in Fig. (2). The average values of three replicates were calculated. A control containing no antioxidant had an oxidation degree that can be considered 100% lipid oxidation. The AOA of both A and B are compared with standard antioxidant (∝ tocopherol). Data showed that crude methanolic extract (A) inhibited lipid oxidation about 47.8, while purified compound (B) inhibited the oxidation about 35.6% (Fig.2) when comparing to control. Whereas, the AOA of the two extracts were 66.2 and 50.8% of the total activity of ∝-tocopherol with respecting of control for A and B, respectively. The stability properties of two extracts (A and B) were conducted during different intervals periods in the dark at 4°C for 60 days. Data shown in Table (2) and Fig. (3) indicated that the presence of slightly changes for AOA during the first 30 days from 66.2 to 66.4% and from 50.8 to 47.3% for A and B, respectively. This is meaning that AOA was more stable during the first 30 days of storage for both A and B. In contrast, after 30 days at 45 and 60 days of storage, sharp decreasing was recorded for both extracts. These decreases were from 66.4 to 18.7 and 11.6% at the later period, as well as from 47.3 to 24.6 and 18.3% at the final periods for A and B, respectively (Table 2and Fig.3). Therefore, it can be concluded that AOA of *P. oleracea* L. methanolic extracts were more stable until 30 days of storage period, then sharp decreased at the later periods of storage (45 and 60 days). Similar results were reported by Hayes *et al.* (1977); Murcia *et al.* (1992); Schwarz and Ernst (1996); Yun *et al.* (2000); Ghazy *et al.* (2000) and Abdel-Galil and Latif (2003).

However, it is nice to observe that at the final period of storage, purified compound (B) was more relatively stable than that recorded for crude extract (A) for their AOA (Table 2 and Fig.3).

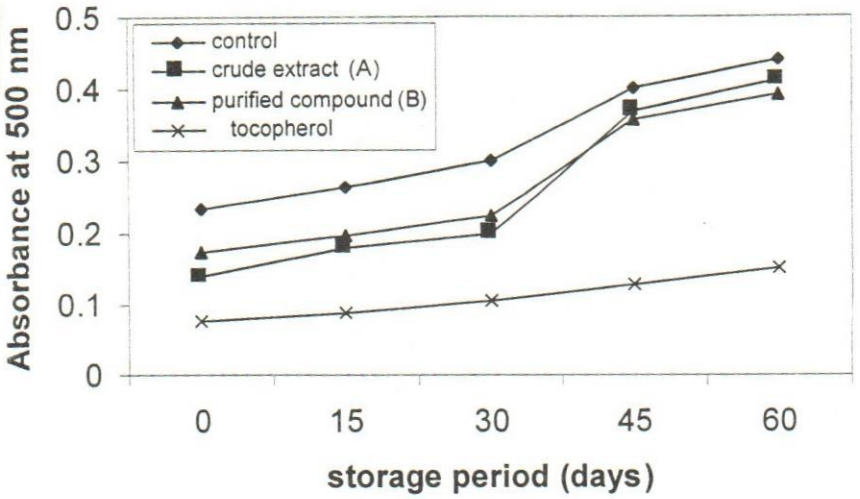

Fig (3) Effect of storage period (0-60 days) on AOA of crude extract (A) and purified compound (B) from P. *oleracea* L.

In discussing sources for AOA in the methanolic extracts (A and B), it can be suggested that the potent activity may be due to the presence of phenolic compounds (Chimi *et al.*,1991), as well as protein hydrolyzates, aromatic amins and sulphydryl groups which posses antioxidant activity (Hayes, *et al.*, 1977). Thus the relationship between AOA and polyphenolic substances must be discussed. Data are shown in Fig (4) indicated also that total phenolics were gradually decreased until the first 30 days of storage period, while sharply decreasing was occurred at the intervals periods of 45 and 60 days. The results indicated the presence of slightly decrease from 6.70 to 5.98 and from 5.75 to 5.38 mg/g for polyphenolics of A and B, respectively during the first 30 days of storage. In contrast, large decrease was occurred at the final periods (60 days)for polyphenolic contents (4.03 and 4.40 mg/g) for A and B, respectively. However, there is no controversy that a positive relationship between polyphenolic contents and antioxidant activity. El- sayed, *et al.* (1998) supported this finding for the positive responses of the AOA and polyphenolics in soybean.

Infection bioassays:

Isolation and identification of the causal fungi diseases for tomato, sesame and apple fruits exhibited symptoms of earliar blight, wilt & root rot and gray mold which revealed the presence of *A. solani*, *F. oxysporium* and *B. cinera*, respectively. Identification was carried out accordind to Barnett and Hunter (1972).

Table (2): Effect of storage periods on the antioxidant extracted from *P. oleracea L.*

Tested samples	Zero		15 days		30 days		45 days		60 days	
	A.500 nm	AOA %	A.500 nm	AOA %	A.500 nm	AOA %	A.500 nm	AOA %	A.500 nm	AOA %
Control	0.234		0.263		0.302		0.391		0.440	
Crude extract (A)	0.140	66.2	0.178	58.7	0.198	66.4	0.350	18.7	0.412	11.6
Purified compound (B)	0.172	50.8	0.195	42.7	0.225	47.3	0.357	24.6	0.391	18.3
∝-tocopherol	0.076		0.089		0.106		0.128		0.150	

Table (3): Inhibitory effect of the two extract from *P. oleracea* L. on some pathogenic microorganisms.

Methanolic extracts	Pathogenic bacteria						Pathogenic fungi							
	B. subtilus		*S. aureus*		*S. epidermidis*		*C. botulism*		*F. oxysporium*		*A. solani*		*B. cinera*	
	mm	I*	mm	I	mm	I	mm	I	mm	I	mm	I	mm	I
(A)	4	+	-	-	-	-	13	+++	-	-	-	-	14	+++
(B)	3	+	-	-	-	-	11	+++	-	-	-	-	13	+++

I* = Inhibitory effect as + slightly, ++ moderat and +++ strong inhibation.

Inhibitory effect of the methanolic extract:

Measurement of wildth zone (mm) of the inhibitor for some isolated pathogenic fungi such as *A. solani*, *F. oxysporium* and *B. cinera* as inhibited by antioxidant extracted from *P. oleracea* L.. Data are shown in Table (3) indicated that the most inhibitory effect by A or B extract was against *B. cinera*. Gradient concentrations from purified compound (B) were applied against the most susceptable fungus (*B. cinera*). Data are shown in Table (4) indicated that the most effective concentration was 150 and /or 200µg/ml.

On the other hand, the same study was carried out against some isolated pathogenic bacteria such as *B. subtilus*, *S. aureus*, *S. epidermidis* and *C. botulism*. Data are shown in Table (3) and (4) illustrated that the most effective against *C. botulism* paticularlyat high concentrations 150 and /or 200µg/ml., while slightly effect were recorded against *B. subtilus*, whereas other strains of bacteria were not affected (Table 3 and 4).

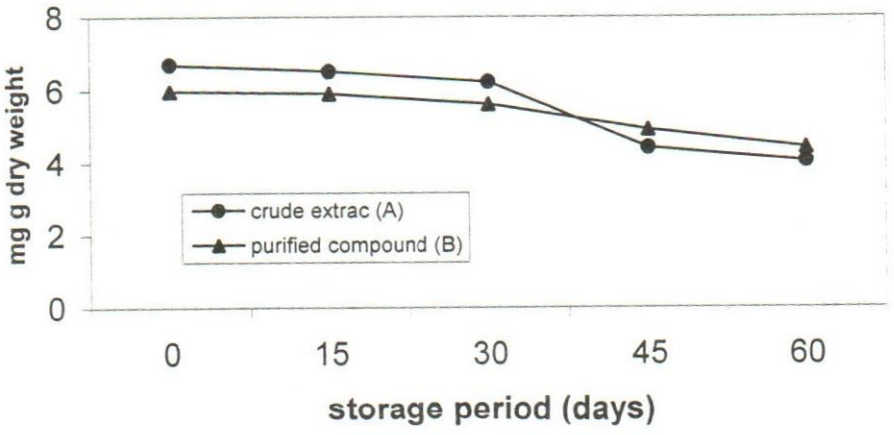

Fig. (4): Polyphenols content in the crude extract (A) and purified compound (B) from P. *oleracea* L.

Table (4): Inhibitory effect of different concentrations of purified compound extracted from *P. oleracea* L. against *B.cinera* and *C. botulism*

Concentrations (µg/ml)	Pathogenic microorganisms*	
	B.cinera I%**	*C. botulism* I%
Control (0.0)	0.0	0.0
50.0	21.8± 0.0 ^{c}	24.5± o.o3 ^{c}
100.0	53.0 ±0.5^{b}	55.1 ±1.1 ^{b}
150.0	76.7± 1.4$^{a/b}$	80.3 ±1.05 ^{a}
200.0	78.9 ±0.09^{a}	81.0 ±0.05 ^{a}

* = Mean values of three replicates ± SD, a,b and c indicated significant differences at p < 0.05.

** = Inhibition present.

However, it can be concluded that purified compound from *P. oleracea* L. had strong inhibitory effect against bacterium *C. botulism* which has toxic effect against canning foods, and fungus *B.cinera* particularly at concentrations of 150 and 200µg/ml. Similar results were reported by Agarwöl, *et al.* (1979); Al-Jassir (1992); Madhavi and Salunkhe (1995) and Abdel-Galil and Latif (2003) who investigated the antioxidant of *N.sativa* L. which had potent inhibitory effect against *R. stolonifer.* In addition, El Shewey *al.* (2003) discussed the function of the aqueous methanolic extracts from *P. harmala* against some pathogenic microorganisms.

In conclussion, purslane (*P. oleracea* L.) can considered a potent food additive source and the methanolic extracts had strong antioxidant activity, as well as at the same time it can be used at concentrations ranged from 150 and 200µg/ml as antibacterial and antifungal agent against *C. botulism* and *B.cinera*, respectively.

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دراسات كيميائية حيوية وبيولوجية على نبات الرجلة كعامل وقائى

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يعتبر نبات الرجلة كمصدر علف جيد للماشيه وأيضا مصدر محصول خضري للاستهلاك الآدمى . هذا وقد تم استخلاص البر وتينات الذائبة الكلية (TSP) على pH (٦ر٨) وفصـــل معزول بروتين الرجلة (PPI) عند نقطه الترسيب الكـــهربى (٩و٣) . كمـــا أجـــرى لكـــل مـــن المستخلصين تقدير الأحماض الأمينية والتى أكدت نفص حمض الليسين والثريونين كحمض محـــدد أول وثانى على التوالى وكانت نسبه الأحماض الكبريتية مرتفعه وبالتالى يجب اضافـــــه بروتيـــن الرجلة إلى بروتين أى من البقوليات حتى يتحقق تكامل الأحماض الأمينية الأساسية .

تم تقدير نشاط مضادات الأكسدة فى كل من المستخلص الميثانولى الخام (A) والمركـب الرئيسى بعد أجراء عمليات الفصل والتنقيه (B) وكانت النتائج حوالى ٢ر٦٦ / ٨ر٥٠ % مـــن النشـــــاط الكلى المضاد للأكسدة للمادة القياسية الفا توكوفيرول قياسا للكنترول لكل مـــن A/B على التوالى . هذا وقد أجريت عملية تخزين على فترات زمنية (صفر /١٥/٣٠/٤٥/٦٠ يـــوم) وكان ثبات نشـــــاط مضادات الأكسدة AOA حتى ٣٠ يوم من التخزين وفى نفس تلك المراحل الزمنية تم قياس الفينـــــولات الكلية وكان التغيير غير معنوى حتى ٣٠ يوم من التخزين ثم أعقبه انخفاض جاء فى الفترات الأخـــيرة ٤٥/٦٠ يوم وهذا يتماثل مع AOA مما يؤكد على وجـــود علاقة ايجابيه بين المركبات الفينوليـــــه وال AOA. هذا وقد تم عزل الفطريات وعمل العـــدوى بقسم أمراض النبات كلية الزراعة - جامعة المنيا .

تم تقدير النشاط المثبط لكلا المستخلصين (A/B) على عدة ميكروبات مرضيـــة وكـــان اشرها تأثرا سلبيا هو ميكروب (*Clostridium botulism*) والذي يسبب التسمم البيتولونـــى للأغذة المعلبـــــة وكان التركيز الأكثر فعالية فى تثبيط النمو الميكروبى هو ١٥٠ – ٢٠٠ ميكرو جرام /مل ، هذا بالأضافه لوجود تأثير مثبط واضح على نفس التركـــيز المذكـــور ضـــد فطـــر (*Botrytis cinera*) والذي يصيب ثمار التفاح.

من كل النتائج السابقة يتضح أهميه نبات الرجلة كقيمه غذائية وكعامل وقائى قوى ضـــد بعـــــض الكائنات الدقيقة الممرضة .