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IN VITRO ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *ABRUS PRECATORIUS* LINN

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ABSTRACT

Natural products would be safe and effective alternatives of synthetic pesticides. The present study was carried out to investigate the antibacterial effects of the methanolic and ethyl acetate-ethanol extracts and di-(2-ethylhexyl)phthalate of seeds of *Abrus precatorius* Linn against *Pectobacterium carotovorum* subsp *carotovorum* (Pcc), *Ralstonia solanacearum* (Rs), and *Streptomyces scabies* (Ss) using the paper disc plate technique. The methanolic extract was more potent against Pcc compared to Rs and Ss as revealed by greater inhibition zone. The phytochemical analysis of the plant extract revealed the presence of flavonoids, alkaloids, resins, phenols, glycosides, sterols and triterpenes, which might help in exerting the antibacterial effect. The ethyl acetate-ethanol mix (80 : 20) extract showed potent effects against Sc compared to Pcc and Rs. Moreover, the GC-MS analysis showed that the main component of *A. precatorius* seed extract was di-(2-ethylhexyl)phthalate (DEHP). The DEHP was found to be more effective as antibacterial agent against Ss bacteria compared to Rs and Pcc. The DEHP had antibacterial activity against the tested bacteria in a concentration-dependent manner.

Keywords: GC-MS, Natural Products, Seed Extract of Jequirity, Antibacterial Activity

INTRODUCTION

Medicinal plants have been used for years in daily life to treat diseases all over the world. Moreover, there is enormous diversity of phytochemicals that were derived from those plants. Extracts of traditional medicinal plants have been tested to identify sources of therapeutics (Parekh and Chanda, 2007). *Abrus precatorius* Linn is commonly known as jequirity, crab's eye, and rosary pea. It is medically important plant from family Fabaceae. The plant native is Indonesia and grows in tropical and subtropical areas of the world (Mistry *et al*., 2010). The most common compound in the seed is the toxic protein abrin, an analogous to ricin (Harborne, 1996). In herbal medicine, the seed paste is applied locally against skin

diseases and leaves are used as substitutes for licorice, which is considered to be useful against biliousness, leukoderma, itching, and other skin diseases. Roots were used as diuretic.

Plant pathogenic bacteria are major problem in production of vegetable crops. Bacterial diseases cause loss in quality and quantity of vegetable crops every year. The bacteria *Pectobacterium carotovorum* subsp *carotovorum* (Pcc) (syn. *Erwinia carotovora* subsp. *carotovora*) is the cause of soft rot disease in potato (Perombelon and Kelman, 1980; NCBI, 2014). It's a Gram-negative, non-sporing, facultative anaerobes, and characterized by the production of large quantities of extracellular pectic enzymes, which cause the development of the soft rot disease (Collmer and Keen, 1986). Also, the bacterial wilt caused by *Ralstonia solanacearum* was reported on potato, tobacco, tomato, and many other host plants worldwide (Buddenhagen *et al*., 1962). Common scab caused by *Streptomyces scabies* (Waksman and Henrici, 1948) is a major disease of vegetables. In order to manage the damage caused by those pathogenic species, thousands of tons of fungicides and bactericides have been applied worldwide every year.

Therefore, the aim of current study was to identify natural products as possible alternatives of fungicides and bactericides to be incorporated in the pest management program of those bacterial diseases. The methanolic extract of seeds of *Abrus precatorius* L. was prepared and examined for their antibacterial activity against *Pectobacterium carotovorum* subsp *carotovorum*, *Ralstonia solanacearum*, and *Streptomyces scabies.* Moreover, the phytochemical content of *A. precatorius* was analyzed using spectrophotometric and gas chromatography – mass spectrometry (GC-MS) techniques.

MATERIALS AND METHODS

1-Phytochemical Analysis of the Tested Plants:

According to the methods adopted by Peach and Tracey (1955), Niazi (1972), Balbaa *et al*. (1976) and Abu-Shaweish (1981) the extracts of each powder were subjected to the following tests:

1.1-Test for Sterols and/or Triterpenes:

About 10 ml of the extract was evaporated to dryness and the residues were re-dissolved in 20 ml of chloroform ($CHCl_3$) and filtered through Whatman no 1 filter paper. Then the filtrate was subjected to the following tests to monitor the presence or absence of sterols and/or triterpenes

1.2-Tests of Alkaloids:
Ten grams of the seed powder was extracted with 50 ml of dilute HCl. The acidic extract was filtered, rendered alkaline with dilute NH_4OH (0.1 N), and then extracted with $CHCl_3$ (3 x 20 ml). The combined chloroform extracts were evaporated to dryness and the residue was re-dissolved in 1 ml of dilute HC1. If the color of the solute change into white turbidity with Mayer's reagent (Potassium Mercuric Iodide), brown precipitate with Wagner's reagent (iodine-potassium iodide solution) or orange color with Dragendorffs reagent (potassium bismuth iodide), this indicates the presence of alkaloids.

1.3-Test of Flavonoids:
Dried seed powder (5 g) were macerated overnight in 150 ml of cold 1% HCl and filtered. Ten milliliters of the filtrate were alkalinized with NaOH. The formation of a yellow color indicates the presence of flavonoid compounds.

1.4-Tests of Glycosides:
The reduction of the plant extract with Fehling reagent before and after the hydrolysis with mineral acid was compared as the following a) 1 g of *A. precatorius* seed powder was mixed with 10 ml of ethanol for 5 minutes, then filtered through cotton pads and the alcohol was evaporated to near dryness on a water bath. The residue was re-dissolve in 5 ml of hot water and filter through filter paper and divided into 2 equal portions. To the first portion, 0.5 ml of NaOH and 1ml of Fehling's reagent were added and the mixture was heated on a water bath; the occurrence of the reduction reaction was monitored. To the second portion, 1 ml of dilute HCl was added and the mixture was heated for 10 min on a boiling water bath, and then neutralized with NaOH (1N). The two test tubes were compared to test before and after hydrolysis, if the second tube shows excessive reduction than the first, therefore the plant may contain glycosides. To the aqueous filtrate of the extract, 0.5 ml NaOH (1N) and excess Fehling's solution were added and heated on a water bath at 100º C for 30 minutes, then filtered and if the filtrate showed a blue color of Fehling's solution, the solution was acidified with dilute H_2SO_4, the solution was heated on a water bath for 10 minutes. The presence of a red precipitate or even change of the color of the Fehling's solution to green indicates that the plant extract contains glycosides.

1.5-Test of Saponins:

Sample was dissolved in normal saline solution. An aliquot (5.0 ml) of the solution was added to 10 ml of 1: 40 suspension of red blood corpuscles in normal saline solution and the solution was then added for 5 minutes to detect the presence of saponins through the observation of hemolysis.

1.6-Test of Tannins:
Two milliliters of bromine water were added to 5.0 ml of the ethanolic extract of *A*. *precatorius* seed. The development of colored precipitate was an indication of the presence of tannins.

1.7-Test of Resins:
In a porcelain dish, 1 g of seed was mixed with 10.0 ml acetic acid by the aid of gentle heat, then cooled and a drop of concentrated sulfuric acid was added carefully. A bright purplish red color was produced, which was changed to violet and then to brown in the presence of resins.

1.8-Test of Phenols:
The Folin-Denis reagent was used to detect the phenolic compounds. Sample was dissolved in distilled water then 1 ml of Folin-Denis reagent was added. The development of a blue color indicates the presence of phenols.

2-Preparation of Plant Extracts:
Seeds of *Abrus precatorius* (Linn.) were washed with tap water and air-dried at room temperature and then in the oven at 50°C until complete dryness. Seed samples were ground in an electric blender to a fine powder. About 90 g of the plant powder was extracted 3 times successively with 180 ml methyl alcohol (95%) in Ultrasonic apparatus for 40 min each. Extracts were combined and dried over sodium sulfate (anhydrous) and the solvent was then evaporated under reduced pressure in a rotary evaporator (Unipan vacuum rotary evaporator type 350P, Poland). Then, the residue was re-dissolved in 95% methyl alcohol and used for the bioassay and identification assays.

3-Antibacterial Assay:
Concentrations of 1000, 2000, 3000, 4000, 5000 and 6000 mg/L of crude extract of *A. precatorius* and bactericides (oxolinic acid and streptrol at concentration 100 mg/L) were tested against the three bacterial species using the paper disc plate method (Loo *et al*., 1945; Thornberry, 1950). Petri plates with nutrient dextrose agar

media were inoculated with each isolate of the bacteria by spreading 0.1 ml of the bacterial suspension (ca. 108 cfu/ml) of 24 h-old broth culture using L-shaped spatula. Filter paper discs were impregnated in different concentrations of seed extract and placed onto the surface of inoculated petri plates. Plates were incubated at 28°C for 48 h. Five discs were used as replicates for each extract concentration. Filter paper discs were immersed in sterilized water and placed on the surface of inoculated plates as control. Inhibition zone around the disc was measured.

4-Isolation and Identification of Di-(2-ethylhexyl)phthalate:

The seeds of *Abrus precatorius* were extracted with methanol. The solvent was completely removed by rotary vacuum evaporator. Then, the methanolic extractives were applied onto a top of column chromatography loaded with silica gel (60~ 120 mesh) and eluted using solvent systems containing a gradient of ethyl acetate: absolute ethyl alcohol (5, 20, 40, 80, and 95%). The 20% fraction of ethyl acetate: absolute alcohol was more active against the bacteria than the other fractions. This fraction was further purified on silica gel and its 12 separated fractions were collected. Among these fractions, the 5^{th} to the 8^{th} fractions have same compound, were eluted onto a preparative thin layer chromatography (TLC) with a mobile phase of ethyl acetate and absolute ethyl alcohol to collect the pure compound, di-(2-ethylhexyl) phthalate. The purity of the isolated compound was checked on TLC plates and confirmed by GC-MS and tested against the selected bacteria.

5-GC-MS Analysis:

The GC-MS analysis was accomplished using an Agilent 6890 gas chromatography system equipped with an Agilent mass spectrometric detector, with a direct capillary interface and fused silica capillary column (30 m x 0.32 mm x 0.25 µm film thickness). Samples were injected under the following conditions: helium was used as carrier gas at approximately 1 ml/min pulsed splitless mode. The solvent delay was 3 min and the injection volume was 1.0 µl. The mass spectrophotometric detector was operated in an electron impact ionization mode, ion energy of 70 e.v., the scan was from 50 - 500 m/z, and the ion source temperature was 230º C. The electron multiplier voltage (EM voltage) was maintained at 1050 v above the auto tune. The instrument was manually tuned using perfluorotributylamine (PFTBA). The GC temperature program was 60º C for 3 min and then elevated to 280º and 250º C, respectively. Chromatographic fragments (Figure 1) (separated peaks) were

identified on Wiley and Nist 05 mass spectral database.

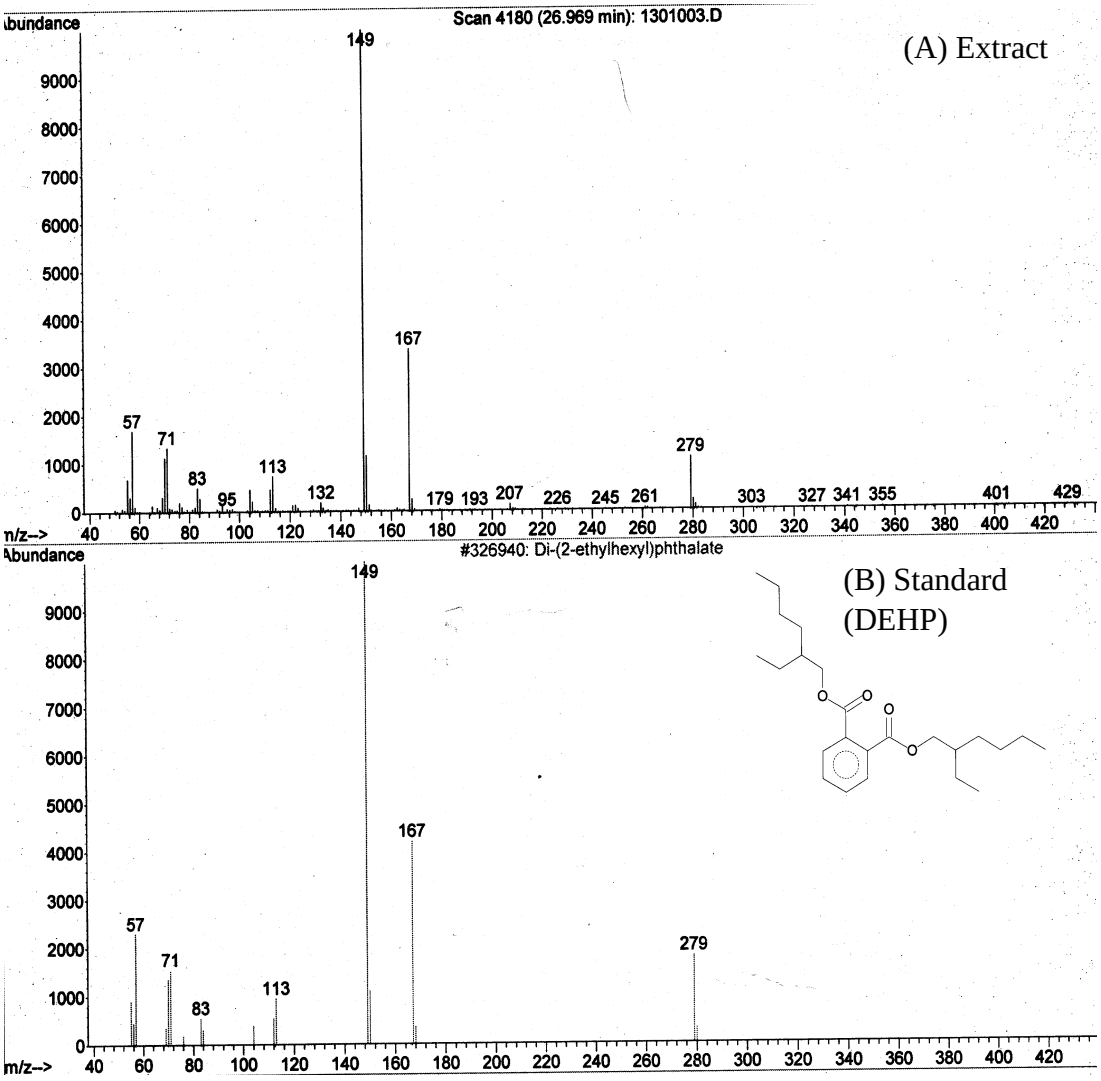

Figure 1. Di-(2-ethylhexyl)phthalate (DEHP) fragments identification using the Agilent 6890 gas chromatography system that was equipped with an Agilent mass spectrometric detector.

6-Statistical Analysis

Data were statistically analyzed as completely randomized design (CRD). Data were tested using the general linear model (GLM) procedure of the statistical analysis system (SAS) (version 9.3) and means were compared using the least significant difference (LSD) at $P<0.05$.

RESULTS

1- Phytochemical Analysis of *A. precatorius* Seed Extract

Data in Table 1 showed the presence of sterols or triterpenes, flavonoids, alkaloids, resins, phenols and glycosides compounds in

the methanolic extract.

Table 1. Preliminary phytochemical investigation of the tested plant.

Test	*Abrus precatorius*
Sterols or Triterpenes	+
Alkaloids	+
Glycosides	+
Phenols	+
Tannins	-
Saponins	-
Resins	+

+ = present, - =absent

Table 2. The bactericidal activity of methanolic crude extract of *Abrus precatorius* L and two standard bactericides against tested bacteria strains after three days of treatment (mean ±SD).

Treatment	Conc. (mg/L)	A verage inhibition zone (cm)*		
		P.c.c.	*R.s.*	*S.s.*
Control	0	0.00	0.00	0.00
Oxolinic Acid	100	3.63±0.13	3.63±0.09	3.91±0.13
Streptrol	100	3.51±0.12	3.84±0.17	4.12±0.20
A. precatorius	1000	1.47±0.14	1.57±0.15	1.67±0.10
	2000	1.57±0.11	1.64±0.10	1.80±0.11
	3000	1.98±0.11	2.20±0.19	2.25±0.12
	4000	2.37±0.15	2.55±0.11	2.85±0.13
	5000	2.82±0.13	2.89±0.12	3.11±0.14
	6000	3.21±0.14	3.31±0.15	3.62±0.16
	$LSD_{0.05}$	0.041	0.037	0.039

P.c.c.: *Pectobacterium carotovora* subsp *carotovora*, *R.s.*: *Ralstonia solanacearum*, *S.s.*: *Streptomyces scabies,* $LSD_{0.05}$: least significant difference was calculated at $P < 0.05$.

2- Antibacterial Effects of Crude Methanolic Extract of *A. precatorius*

Data presented in Table 2 summarize the results of the inhibition zone (cm) of the bacterial growth and the effects of methanolic extract of seeds of *Abrus precatorius L.* The results indicated that the methanolic extract exhibited varying levels of antibacterial activity against the bacterial strains. The methanolic extract at 1000 to 6000 mg/L gave inhibition zones from 1.47 to 3.62 cm against the tested bacteria. *Streptomyces scabies* was more sensitive than

Pectobacterium carotovorum subsp *carotovorum* and *Ralstonia solanacearum*. The reference bactericides were more effective in the inhibition of Pcc, Rs, and Sc compared to the methanolic extract.

Table 3. Mean inhibition zones (cm) of the bactericidal activities of the ethyl acetate fractions of seed extracts of *A. precatorius* against *S. scabies*, *R. solanacearum*, and *P.c. carotovorum*.

Conc. mg/L	Average of inhibition zone (cm)*														
	S. scabies					***R. solanacearum***					***P. c. carotovorum***				
	Ethyl acetate-absolute alcohol					Ethyl acetate-absolute alcohol					Ethyl acetate-absolute alcohol				
	5%	20%	40%	80%	95%	5%	20%	40%	80%	95%	5%	20%	40%	80%	95%
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
500	2.03	2.27	2.13	1.57	1.63	1.50	2.03	1.87	1.33	1.03	1.37	1.83	1.53	1.20	0.93
1000	2.27	2.50	2.33	1.70	1.70	1.57	2.17	2.00	1.40	1.23	1.47	1.97	1.73	1.33	1.03
2000	2.43	2.77	2.50	2.23	2.07	1.80	2.60	2.23	1.43	1.47	1.53	2.20	1.90	1.40	1.17
3000	2.70	3.17	2.97	2.40	2.40	2.23	2.87	2.47	1.77	1.63	1.67	2.33	2.20	1.53	1.30
4000	2.87	3.57	3.10	2.60	2.53	2.53	3.13	2.80	1.97	1.77	2.00	2.67	2.40	1.77	1.40
5000	3.23	3.87	3.40	2.63	2.63	2.80	3.40	3.03	2.30	1.80	2.20	3.07	2.87	1.90	1.57
$LSD_{0.05}$	0.04	0.03	0.02	0.05	0.03	0.05	0.04	0.03	0.04	0.05	0.02	0.05	0.04	0.05	0.03

3- Antibacterial Effects of fractions of Crude Ethyl Acetate-Ethyl Alcohol Solvent System Extracts of *A. precatorius*

The methanolic extractives of *Abrus precatorius* was subjected to silica gel column chromatography, containing a suspension of 250 gm of silica gel in 5% ethyl acetate-absolute alcohol and using ethyl acetate-absolute alcohol solvent system. Results of the antibacterial effects of different fractions of seed extract were presented in Table 3. Data revealed that the 20% ethyl acetate-absolute alcohol fraction was more effective against the tested bacteria followed by 40% ethyl acetate-absolute alcohol fraction, while the 95% ethyl acetate-absolute alcohol fraction was less effective against tested bacteria. Also, the *P.c.c.* was the least sensitive to the extract fractions compared to the other tested bacteria.

4- Antibacterial Effects of Di-(2-ethylhexyl) phthalate (DEHP) of *A. precatorius*

Results of the bactericidal activity of di-(2-ethylhexyl) phthalate (DEHP), which was isolated from *A. precatorius,* against the three tested bacteria were shown in Table 4.

Table 4. The bactericidal activity of di-(2-ethylhexyl) phthalate (DEHP) against tested bacteria strains (mean ± SD)

Treatment	Conc (mg/L)	A verage of inhibition zone		
		P.c.c	*R.s.*	*S.s.*
Control	0	0.00	0.00	0.00
Oxolinic Acid	100	3.59±0.10	3.62±0.09	3.87±0.12
Streptrol	100	3.54±0.09	3.81±0.12	3.84±0.11
A. precatorius	500	1.97±0.08	2.13±0.07	2.33±0.09
	1000	2.10±0.07	2.30±0.07	2.60±0.09
	1500	2.30±0.08	2.70±0.09	2.93±0.10
	2000	2.43±0.07	2.97±0.09	3.27±0.10
	2500	2.77±0.08	3.30±0.10	3.67±0.11
	3000	3.20±0.09	3.57±0.10	4.00±0.11
$LSD_{0.05}$		0.035	0.029	0.034

P.c.c.: *Pectobacterium carotovora* subsp *carotovora*, *R.s.*: *Ranostonia solanacearum*, *S.s.*: *Streptomyces scabies,* $LSD_{0.05}$; least significant difference was calculated at $P < 0.05$.

Di-(2-ethylhexyl)phthalate caused significant inhibitory effect on the growth of the bacterial strain; *P.c.* subsp *carotovorum* compared with the non-treated control. Also, the results indicated that *P.c.* subsp *carotovorum* was the more sensitive to DEHP than *R.*

solanacearum and *S. scabies*. Additionally, the data indicated that increasing the concentrations of Di-(2-ethylhexyl)phthalate from 500 to 3000 mg/L, significantly increased the inhibition the bacterial growth. The DEHP at concentration more than 2500 mg/L gave similar inhibition results to the tested standard bactericides.

DISCUSSIONS

Results of antibacterial activity of the methanolic extract of *Abrus precatorius* were consistent with previous reports (Mistry et al., 2010; Amuta *et al.*, 2011; Jaina and Gautam, 2011). Results showed that the tested bacterial strains were sensitive to extracts of different solvents at certain levels. The antibacterial activity of the extracts of *A. precatorius* might be due to the presence of phyto-constituents namely; sterols, triterpenes, flavonoids, alkaloids, resins, phenols, and glycosides and that was supported by many authors Agina *et al.* (2000), Parekh and Chanda (2007), Adeleye *et al.* (2008), Prashith *et al.* (2010), Choudhari *et al.* (2011), and Taur and Patil (2011).

The main compound of *A. precatorius* extract was confirmed and identified by GC-MS analysis (Figure 1) as di-(2-ethylhexyl) phthalate (DEHP) from seeds of *A. precatorius*. The DEHP is used as synthetic plasticizer and was also reported in *Aloe vera* (Lee *et al*., 2000), *Alchornea cordifolia* (Mavar-Manga *et al*., 2008), Iris plants (Asghar *et al.*, 2011), and *Mallotus tetracoccus* (Ramalakshmi and Muthuchelian, 2011). The ethyl acetate extract and identified the compound (DEHP) showed an *in vitro* broad spectrum antibacterial activity against the tested bacteria. These results were in agreement with Habib and Karim (2009) who found that di-(2-ethylhexyl)phthalate has antibacterial activity against the gram positive *Staphylococcus aureus*, *Bacillus subtilis*, and *Sarcina lutea* and the gram negative *Escherchia coli*, *Shigella sonnei*, *Shigella shiga* and *Shigella dysenteriae* bacteria.

CONCLUSIONS

The methanolic extract of *Abrus precatorius* showed antibacterial properties. This antibacterial activity might be due to the presence of sterols, triterpenes, flavonoids, alkaloids, resins, phenols, and glycosides. The fractionation of the methanolic extractives revealed that the 20 % ethyl acetate : absolute alcohol fraction showed an *in vitro* broad spectrum antibacterial activity against the tested bacteria. Moreover, the main compound of the seed extract of *A.*

precatorius was identified and confirmed by GC-MS analysis as (di-(2-ethylhexyl) phthalate (DEHP)). The DEHP had antibacterial activity against the tested bacteria in a concentration-dependent manner.

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الملخص العربي

كفاءة مستخلصات نبات *Abrus precatorius* كمبيدات بكتيرية والتحليل الكيميائي لتلك المستخلصات بإستخدام جهاز الـ GC-MS

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المستخلصات الطبيعية مصدر خصب وامن كبدائل أو منشطات للمبيدات. تم إجراء الدراسة الحالية لاختبار كفاءة المستخلصات الميثونيلية (methanolic extract) وخلات الايثيل مع الايثانول (ethyl acetate-ethanol) والمركب الرئيسي لنبات *Abrus precatorius* ضد بكتريا الـ (Pcc) *Pectobacterium carotovorum* subsp *carotovorum* ، (Rs) *Ralstonia solanacearum* ،والـ *Streptomyces scabies*. أوضحت النتائج أن المستخلص الميثانولي ثبط نمو بكتريا الـ Pcc أكثر من الاثنين الاخريين وظهر ذلك بقطر تثبيط أكبر. كما ان تحليل المكونات الطبيعية الموجودة في المستخلص وجود مركبات flavonoids ، alkaloids ، resins ، phenols ، glycosides ، sterols ،وtriterpenes والتي قد يكون لها دور كبير في التاثير ضد البكتريا الذي تم تسجيله. كما أوضحت النتائج أن مستخلص خليط خلات الايثيل-الايثانول (80 : 20) لبذور نبات الـ *A. precatorius* تاثيرا مثبط عالي ضد Sc بالمقارنة بـ Pcc أو Rs. بالاضافة الي ذلك فقد أظهر تحليل GC-MS analysis أن المركب الرئيسي فى هذا النبات هو مركب الـ di-(2-ethylhexyl)phthalate (DEHP) وتاثير هذا المركب ضد البكتيريا المختبرة كان جيدا ومعتمد علي التركيز المستخدم من هذا المركب. ان تنقية وعزل المركبات (المستخلصات) الطبيعية النباتية تعتبر خطوة مهمة في اتجاه تعريف وعزل مركبات جديدة قد يكون لها دور كمبيدات للافات.