Pathological and antiviral studies on *Broad bean mottle virus*affecting Faba bean plants in Ismailia Governorate

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Abstract:

Broad bean mottleBromovirus (BBMV) was obtained from naturally infected faba bean plants exhibiting blotchy mottle,vein-clearing and deformation. BBMV was able to infect limited host range ten out of thirty three tested plant species, and cultivarsbelonging to six different families. It was transmitted mechanically and not transmitted by seeds or aphids. The isolated virus was inactivated by 10 min exposure to 95°C, but not at 97°C, at a dilution of 10^{-3},and at3 weeks storage at room temperature. The BBMV was tested serologically against antibodies of *Broad bean stain virus* (BBSV) and (BBMV) using indirect ELISA. Positive reactions was obtained only with BBMV antiserum.The BBMV induced amorphous cytoplasmic inclusion bodies in infected cells .The Susceptibilityof some faba bean cultivars and genotype was also studied forvirus infection.The effectiveness of extracts from garlic cloves (GE) and onion (OE) as an antiviral against BBMV infection *in vivo* has been evaluated. The percentage of virus inhibition induced by GE and OE varied according to the time of treatment (1, 2 and 3 days).GE was more effective in reducing the percentage of infection produced by BBMV on faba bean plantsthan did OE.

Key words:faba bean (*Viciafaba)-broad bean mottle virus* (BBMV)-antiviral agents -garlic (*Allium sativum*), onion (*Allium cepa*)

Introduction

The virus was seen as being of minor importance until reports of its widespread occurrence began to appear in the 1970s and 1980s. BBMV was reported in faba bean crops in Portugal (Borges and Louro, 1974), Sudan (Murant et al., 1974), Morocco (Assou, 1978), and Algeria (Ouffroukh, 1985). Makkouk et al. (1988a) undertook a regional survey and found BBMV in faba bean crops in Egypt, Morocco, Sudan, Syria and Tunisia (Fortass and Bos,1992).BBMV was first described by Bawden et al. (1951) from a severely infected broad bean (Viciafaba) crop in Nottinghamshire, England. Faba bean yield losses have been reported to range from 37 to 84% (Makkouk et al., 1988b). The most prevalent of these viruses in Egypt are Faba bean necrotic yellow virus (FBNYV), Bean yellow mosaic virus (BYMV), Broad bean stain virus (BBSV), Broad bean true mosaic virus (BBTMV) and Pea seed borne mosaic virus(PSBMV)(Makkouk el al.,1994; El- Afifi and El- dougdoug,1997; Fegla el al., 2003; and El-Hammady et al., 2004). Little work had been reported on BBMV in Egypt (Makkouk et al.,1994; Fegla et al., 2003).Broad bean mottle virus (BBMV) (Bromovirus, Bromoviridae) is one of a number of viruses which has been found in Africa, Asia, Europe and the Middle East. Many investigators have been studied the effect of antiviral activity of garlic and onion on different plantviruses(Chowdhury and Saha ,1985;Othman et al.,1991; Melcher et al.,1992; Gangel, 2002; Chen et al., 2006 and Mohamed, 2010).

The aim of the present study is to isolate and identify the BBMV which affectingfaba bean plants and the effectiveness of extracts from garlic cloves and onion against the virus *in vivo* has been evaluated.

Materials and methods

Isolation and identification of BBMV:

Samples of faba bean plants exhibitingblotchy mottle, interveinalchlorosis andvein-clearing were collected from different fields of Ismailia Governorate, Ismailia Experimental Station , Agric.Res.Center ; killo11 and Agriculture College Farm. These samples were checked serologically against BBMV and BBSV antisera provided by

Serological Lab in Virus and Phytoplasma Research Department ,A.R.C. Plant samples which gave positive reaction in the indirect ELISA test with BBMVantiserum was used as a source of virus infection .Extracted sap of infected broad bean leaves was used to inoculate the following indicator hosts: *Viciafaba cv.* Local as systemic host , *Chenopodiumamaranticolor* , *Chenopodium album* and*Chenopodium quinoa* were used as a local lesion host.To obtain virus isolate in a pure form , the single local lesion technique was followed according toKuhn(1964) in biological purification of the virus isolate, these plants were inoculated with infected virus juice. Inoculated plants were kept in separate cages , as a source of virus infection. Chlorotic local lesion induced by BBMV on*Chenopodiumamaranticolor* was back inoculated to*Chenopodium album* and *Chenopodium quinoa* to obtain virus isolate in a pure form.

Reaction of plantsto BBMV:-

1-Faba Bean cultivars and genotypessusceptibility

A greenhouse-pot experiment was conducted to determine the response of some commercial faba bean cultivars to mechanical inoculation with the tested isolated virus. It was carried out under greenhouse conditions at Ismailia Experimental Station. Six faba bean cultivars (Nobaria,Misr 1, Giza 843, Giza 429, Giza 716 , and Local) obtained from the Agric.Res.Center, Ministry of Agriculture, Cairo, Egypt were used. Eighteen genotype of faba bean (R3-26, R2-16, R5-11, R5-13,R5-7, R5-26, 11,22,23,29,30,17-RED, 14-RED, 5-RED, 29-RED, 24-RED, 10-RED and 3-T) obtained from the Agronomy Dept .Fac. of Agric .S.C.U were used. Nine faba bean plants of each cultivar were sown (3 plants/pot, 3 pots/cultivar were served as replicates for virus inoculation. The same numbers of faba bean plants from each cultivar served as control to each treatment. Inoculated plants were observed daily for 6 weeks . Development and severity of symptoms were recorded.Symptom intensity values were using (-,+, ++, +++) represents no symptoms to relative mild, moderate to severe symptoms(Joseph and Hesham ,2002).

2-Host range and symptomatology

Plant species and cultivars belonging to 6 different families (Fabaceae, Asteraceae,

Chenopodiaceae, Solanaceae, Cucurbitaceae, Amaranthaceae) were mechanically inoculated with the virus isolate to study the host range.Ten seedlings from each were mechanically inoculated by the virus isolate .An equal numbers of test plants were left without inoculation to serve as controls. Inoculated plants were observed daily for 6 weeks . Development and severity of symptoms were recorded. Symptomless plants assessed into indicator plants or checked serologically.

Virus stability

To study the stability of BBMV, thermal inactivation point (TIP) , dilution end point (DEP) and longevity *in vitro* (LIV) were determined using the methods described by Noordam(1973).*Ch. amaranticolor* was used as an indicator host plant for BBMV infection.

Seed transmission

Five broad bean commercial cultivars, Local ,Misr1, Giza843, Nubaria and Giza 3 were used in this study. Healthy seedling of *Viciafaba*cultivarswere inoculated with BBMV isolate. Inoculated plants showing symptoms were left to produce seeds and were regularly sprayed with insecticide (Malathion, 1.5ml. 57% / L*)* to avoid transmission . Three hundred seeds obtained from infected plants for each cultivar were sown in big pots (3o cm) at the rate of 5 seeds/ pot and kept under greenhouse conditions .The resultant seedlings were examined for symptoms development. Percentage of seed transmission was determined and the experiment was repeated twice.

Insect transmission

Two aphid species namely *Myzuspersicae*(Sulz.), and *Aphisfaba*(Scop.)were reared on cabbage and faba bean seedlings, respectively , and grown inside glass cages covered with cheese-cloth. The glass cages were kept in the glasshouse,then the aphids were left for reproduction more than four weeks. Virus – free aphids starved for 1 to 3 hrs were allowed to feed for acquisition feeding period of 5 minutes on infected faba bean cvLocalleaves. The viruliferous aphids(five aphids / plant) were then allowed to feed on healthy seedlings for inoculation feeding period of 24 hrs. Insects were killed by the systemic insecticide used before. The same procedure was conducted for the control except that aphids were fed on virus free seedlings of Faba bean. The

inoculated seedling were kept in an insect proof cage in the greenhouse for symptoms appearance and the percentage of insect transmission were recorded.

Inclusion bodies

Epidermal strips from plants infected by BBMV and healthy broad bean plants were removed from the lower surface of the leaves, stained with phloxine and coomassie blue ,then mounted in distilled water and examined under a Light microscope at magnification of 3.5x40X, according to the method described by Muellur&Koening (1965). Cytochemical techniques were used for the determination of the chemical nature of inclusion bodies associated with BBMV infection.

The differential staining of protein and lipids were performed as follows:-

Lipid staining:

Epidermal strips of both infected and non- infected leaves were placed in 50% ethanol for 10 minutes and transferred to phloxine(Bos,1970) in 70 % ethanol for 3 minutes . The strips were then transferred to distilled water for 5 minutes, and examined with light microscope, using the method described by Robb (1964).

Protein staining:

Strips of infected and non infected broad bean leaves were immersed in the stain which containing 100 mg coomassie blue and 10 mg mercuric chloride in 100 ml distilled water for 15 minutes. The treated strips were then placed in 0-5% acetic acid for 15 minutes. The treated strips were washed in tap water for 15 minutes and mounted on glass slide and examined under light microscope at magnification of 3.5x40 X (Mazia*et al*., 1953) .

Effect of garlic cloves and onion extracts on BBMV infection *in vivo:-*

Extracts of garlic cloves (GE) and onion bulbs (OE)were prepared separately in distilled water (1:1w/v). All experiments were repeated twice. Four replicates were used for each treatment.

Preparation of garlic and onion extracts (GE and OE) on BBMV infection *in vivo* :

Cloves of garlic (*Allium sativum*cv. Balady)and bulbs of onion(*Allium cepa*cv. Balady) plants were ground in blender using sterilized distilled water (1:1w/v). The pulp was pressed through two layers of cheesecloth, and then the fluidextract was centrifuged at 1000 rpm for 30 min. The

supernatant was collected and stored at -20C(Mohamed, 2010).

Pre-inoculation treatment:

1 ml of each GE and OE extracts wasrubbed on leaves of faba beanseedlings ,then they were mechanically inoculated with BBMV infected sap (1ml/plant) at different intervals: 1, 2, and 3days, respectively. Distilled water was used as a control. The percentage of inhibition was calculated from the following formula according to Taha and Mousa (2000).

% Inhibition = (control -treatment) x100/ control

Results and discussion

Isolation and identification of BBMV

Naturally infected faba bean plants showing blotchymottle ,interveinalchlorosis andvein-clearing were collected from different fields of Ismailia Governorate . These samples were checked serologically against BBMV and BBSV antisera using indirect ELISA . Samples which reacted positively with BBMV was collected separately and used for virus inoculation. The virus isolate was biologically purified as mentioned before under Materials and Methods and reinoculated onto faba bean plants(cv. Local)which were then used as a propagative host for the virus isolate.

Reaction of plantsto BBMV:-

The tested plants reacted with different response and reaction of susceptibility to BBMV.

1-Faba Bean Cultivars and genotypes Susceptibility

All inoculated faba bean cultivars and genotypes were found to be susceptible to BBMV infection. Symptoms started to appear 12-15 days after inoculation by BBMV on local bean cv. which showed blotchy mottle and, interveinalchlorosis. Other tested cultivars of faba bean reacted with vein-clearing followed by interveinalchlorosis and blotchy mottle (Fig1). As indicated in Table (1)faba bean cultivars Local ,Nobaria, Giza 429 exhibited severe infection (3+) followed by Giza 843, Giza 716 (2+) , whereas Misr 1 showed mild symptoms.

Tested genotype of faba bean reacted with interveinalchlorosis, leaf roll and blotchy mottle(Fig 2).Generally all genotypes tested gave symptoms , except 24-RED , R3-26. The severity of these symptoms were varied according to the genotypes, some of them

exhibited sever (3+) symptoms (R2-16, R5-11, R5-13, R5-7, R5-26, 11 ,22,29,30) , where others showing moderate (2+) symptoms (17-RED, 5-RED, 29-RED, 3-T).While 23, 14-RED,10- RED genotypes developed mild symptoms (Tabl 2).

2-Host range and symptomatology:

The tested plants reacted with different response and symptoms appeared on the plants might be grouped into three categories:-

A-Plants reacted with systemic symptoms:

Symptoms started to appear 12-15 days after inoculation by BBMV.The following plants showed systemic symptoms : *Phaselous vulgaris*cv.Local showed mosaic;*Glycine max* cv. Giza 22 showed vein clearing, Giza 35 cv. showing mottle , Giza 111 cv. showing yellowing (Fig 3, and Table 3);*Lens culinaris*cv. Local showed mottle*; Pisumsativum* cv. Local showedlethal systemic wilt and*Sonchusoleraceus* showed vein clearing. These results are in agreement with those reported by many investigators(Makkouk*et al.*,1988a; Fortass and Bos ,1992; Fortass and Diallo ,1993; Brunt *et al*.,1997; Joseph and Hesham,2002) .This virus was isolated in previous studies from fababean , by other investigators in different countries (Bawden*et al.*, 1951; Allam and EL-Kady,1966; Gibbs, 1972;Walters and Surin 1973; Murant*et al.*,1974 ; Borges and Louro, 1974; Assou, 1978 ;Ouffroukh, 1985; Makkouk*et al.*, 1988a ; Fortass and Bos, 1992; Bos*et al.*,. 1992;Fortass and Diallo ,1993; and Brunt *et al.*,1997).

B-Plants reacted with local symptoms :

Upon inoculation with BBMV on *Chenopodiumamaranticolor* and *Chenopodium album*, local lesions were developed and only *Chenopodium quinoa*(chenopodiaceae) reacted with necrotic lesions (Fig4,andTable 4).This result was in agreement with those reported by Walters and Surin (1973); Gibbs(1981) ; Makkouk*et al.*(1988a) ; Bos*et al.*(1992) and Brunt *et al.*(1997).

No symptoms were observed on the following inoculated plants :*Vignaunguiculata*cvs. Local, Kafer El-Sheikh andBuff;*Lupinalbus* cv. Local;*Arachiahypogaea* cv. Local;*Trifoliumalexandri*cvs. Ismailia94, Ismailia1, Mosabtazot and Siwa(Fabaceae);*Lycoperisicon esculentum*cv.Local;*Capsicum annuum* cv. Local ;*Datura stramonium*;*Datura metal;Nicotiana rustica;*

*Nicotiana tabaccum*cvs. White Burley, Samsun and 45; *Solanum nigrum* cv. Local (Solanaceae); *Cucurpita peppo*cv. Local; *Cucumis sativus* cv. Local; *Citrullus vulgaris* cv. Local (Cucurbitaceae); *Gompherena globosa (Amaranthaceae).*

Table (1):- Reaction of faba bean cultivars to infection with BBMV.

Cultivar	Disease reaction	Symptom Intensity
Viciafaba cv. Local	VC - IC- BM- LR	+++
Nobaria	VC - BM	+++
Giza 429	VC - BM	+++
Giza 843	VC - MM	++
Giza 716	VC -MM	++
Misr 1	VC -MM	+

(VC) Vienclearing;(LR) Leaf roll;(IC) InterveinalChlorosis;(BM) Blotchy Mottle; (MM) Mild Mottle.

Fig (1):- Symptoms caused by BBMV on faba bean cultivars 1-Giza 429 cv., 2-Misr1cv.,3-Giza 716 cv.,4-Nobaria cv. showing vein clearing,5-

Local cv. showing blotchy mottle,6- Giza 843 cv. showing mild mottle.

Table(2):-Reaction of faba bean genotype to infection with BBMV.

Genotype	Reaction	Reaction class	Symptom Intensity
R3-26	-	Land Race 5	-
R2-16	M	Triple White	+++
R5-11	M	Misre 2	+++
R5-13	M	Giza 843	+++
R5-7	M	Remablanca	+++
R5-26	BM	Land race 4	+++
11	BM	H2	+++
22	BM-IC	Saffa 1	+++
23	IC -LR	H4	+
29	BM-VC	Giza 3	+++
30	IC- LR	Introduced variety	+++
17-RED	M	S12	++
14-RED	M	S15	+
5-RED	M	H8	++
29-RED	M	S2	++
24-RED	-	S5	-
10-RED	M	S9	+
3-T	M	Resistant	++

(M) Mosaic;(BM) Blotchy Mottle; (VC) Vien clearing;(LR) Leaf rolle;(IC) InterveinalChlorosis. H—(Across between two local lines)S—(Selection from local line under breeding program in the agronomy Dept .Fac. of Agric .S.C.U).

Virus stability

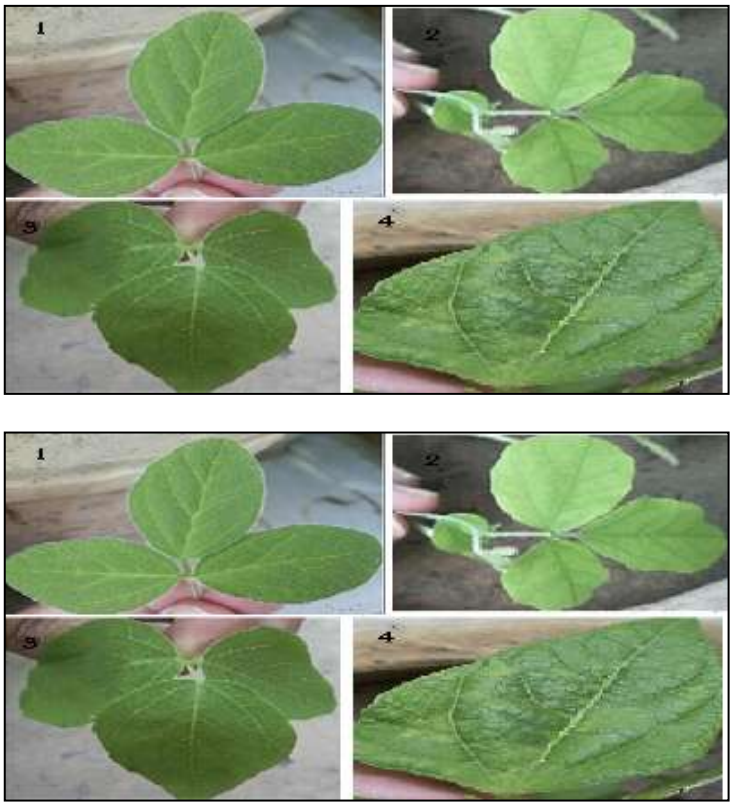
Thermal inactivation point (TIP) ,dilution end point (DEP) and longevity *in vitro* (LIV) ,were determined separately for BBMV. Results showed that (TIP) was between 95-97° C , DEP was 10^{-3} ,and LIV was 3 weeks at room temperature. Virus stability of BBMV was studied using Faba bean cultivar Local. This result are in harmony with Bawden*et al. (* 1951) , Gibbs(1972), and Brunt *et al.(*1997) (Table 5).

Fig (2):-Symptoms caused by BBMV on faba bean genotype 11, 29,R5-26 showing blotchy mottle, 23,30 showing interveinalchlorosis,and leaf roll ,22 showing interveinalchlorosis .

Table (3) Different hosts reacted with systemic symptoms to infection with BBMV.

Family	Species	Cultivar	BBMV
Fabaceae	*Phaselous vulgaris*	Local	M
	Glycine max	Giza 22	VC
		Giza 35	M
		Giza 111	Y
	Lens culinaris	Local	MO
	Pisumsativum	Local	lethal systemic wilt
Asteraceae	*Sonchusoleraceus*		VC

(MO) Mottle**. (M)** Mosaic **. (VC)** Vein clearing **.(Y)** Yellowing**.**



Fig(3):- Showing host range produced by BBMV . *1-Glycine max* cv. Giza35 cv. showing Mottle, 2- *Glycine max* cv. Giza 111 cv. showing Yellowing , 3- *Glycine max* cv. Giza 22 cv. showing vein clearing,4-*Phaselous vulgaris* cv. Local showing mosaic.

Table (4): Plants reacted with local symptoms to infection with the BBMV.

Family	Species	BBMV
Chenopodiace	*Chenopodiumamaranticolor*	CLL (dry pin- point)
	Chenopodium album	CLL
	Chenopodiumqunioa	CLL-NLL

(CLL) Chlorotic Local Lesion. **(NLL)** Necrotic local lesion

Fig (4):- Showing indicator hosts produced by BBMV .1,2- Chlorotic local lesions (Pin point) on *Chenopodiumamaranticolor* .3, 4- Chlorotic, and Necrotic local lesions on*Chenopodiumqunioa* , and5- Chlorotic local lesions on *Chenopodium album*.

Table (5) : Stability of Broad bean mottle virus.

Stability in vitro	Treatment result
Thermal inactivation point	95-97° C
Dilution end point	10^{-3}
Longevity in vitro	3 weeks at room temperature

Modes of transmission :-

a-Mechanical transmission

The results of mechanical transmission proved that the virus was easily transmitted mechanically to different hosts using infectious crude sap

b-Seed transmission

Seeds produced from inoculated plants of the five faba bean cultivars tested with the virus isolate were sown. The resultant seedlings were homogenized and used to inoculate the respective test plants to check the ability of these seeds to transmit the virus. Results showed that none of the inoculated test plant showed any symptomsSimilar results were obtained byBawden*et al.*(1951); Makkouk*et al.*(1988a) Brunt *et al.*(1997);and Fortass and Bos ,(1992).

c-Insect transmission

Results of insect transmission using *Myzuspersicae* (Sulz) and *Aphis faba*(Scop.)revealed that BBMV was not transmitted by aphids.Similar results were obtained byEfaisha(2005)and Simon and Glen (2008).

Inclusion bodies. Light microscopic examination of fresh stained epidermal cells of the underside leaves of healthy and BBMV infected fababean plants revealed amorphous cytoplasmic inclusion bodies in the infected cells. The cytoplasmic amorphous inclusion and nucleoli were stained blue with coomassie blue, whereas, nucleoplasm was almost colorless .These results are similar to the results obtained by Bos(1969);Makkouk*et al.* ,1988a and El-Afifi and El-Dougdoug(1997).While the amorphous inclusion and nucleoli were stained red with phloxine staining , whereas, nucleoplasm was almost colorless (Fig 5).

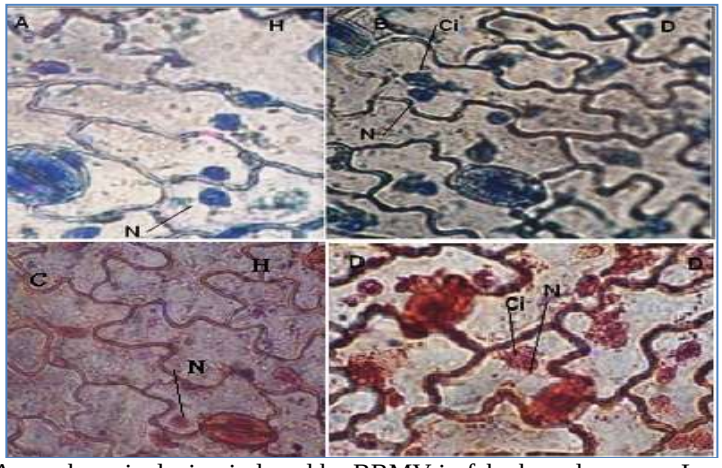


Fig (5):- Amorphous inclusion induced by BBMV in faba bean leaves cv.Local . N- Nucleous , CI- Cytoplasmic inclusion , A-healthy leaves, and B- infected leaves

stained by Coomassie blue , C- healthy leaves, and D- infected leaves stained by Phloxin (Magnification =3.5x40).

Effect of garlic and onion extracts (GE and OE) on BBMV infectivity *in vivo:*

The principal antimicrobial component ofgarlic oil is the sulfur compound diallylthiolsulfinate,which named allicin(Edward and Vincent, 1985). Onions also have medicinal and functional properties (Lanzotti, 2006).

Recorded results in Table(6) showed that the plant extracts (GE and OE) inhibited BBMV infection when used as a pre-inoculation treatment .Also, three days pre- inoculation treatment was most effective than did the pre- treatments using either GE or OE extracts .

Moreover, Garlik extracts had a higher inhibitory effect (77.8%) ,than did onion extracts (55.6%).Similar results were obtained by Chowdhury and Saha (1985);Othman et al.,(1991); Melcher et al.,(1992); Gangel (2002); Chen et al., (2006); Goncagul and Ayaz (2010) andMohamed (2010).

The most important chemical compounds of garlic are the organosulphur compound including allicin which was thought to be responsible for their potency against bacteria, Fungus, viruses and protozoa by the oxidation of aliphatic aldehyde into the corresponding carbonic acid, essential oil of garlic has the maximal efficiency of inhibiting hexenal oxidation(Ahmed ,2010).

Onions,in addition to organosulphur compounds, is rich in flavonoids (Augusti 1996; Griffiths et al., 2002)which are known to be synthesizedby plants in response to microbial infection (Dixon et al.,1983).

Hence, it is not surprising that they have been found to be efective antimicrobial substances . In addition plant flavonoids were shown to have antiamoebic and antigiardial activity (Calzada et al., 1999).

However, studies in vivo are needed to assess the true antioxidant and antiviral activities of these compounds to determine the metabolic pathways involved in their degradation.

Table (6): Effect of crude extract from garlic (*Allium sativum*cv. Balady) and onion (*Allium cepa*cv. Balady) plants on percentage of infection produced by BBMV on fababean plants *in vivo*treatment at different intervals.

Time intervals	% Percentage of infection				
	Control	Garlic	%Inhibition (%I*)	Onion	%Inhibition(%I*)
One day	80/100	60/100	25	70/100	12.5
Two day	100/100	40/100	60	60/100	40
Three day	90/100	20/100	77.8	40/100	55.6

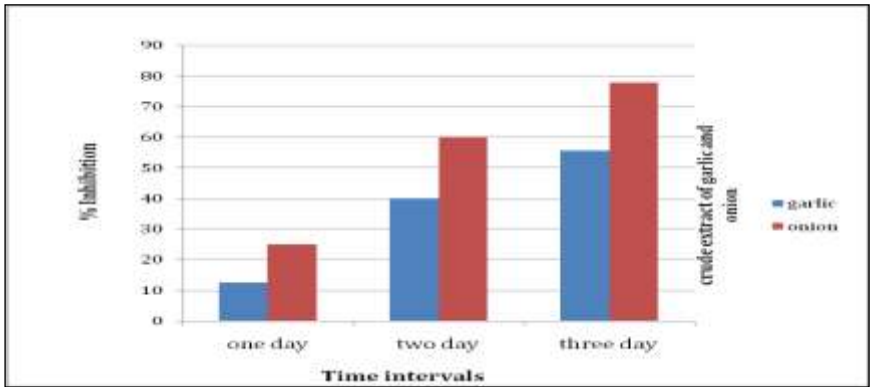


Fig. (6): Percentage of inhibition produced by infected sap of BBMV and extracts of garlic and onion *in vivo.*

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