Inhibition of *Broad bean mosaic virus* (BBMV) using extracts of Nigella (*Nigella sativa* L.) and Zizyphus (*Zizyphus spina-christi* Mill.) plants

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Abstract: The effect of Nigella and Zizyphus extracts (NE and ZE) as inhibitors against broad bean mottle virus (BBMV) infectivity *in vitro* and *in vivo* was studied. *Chenopodium amaranticolor* plant was used as a local lesion host for BBMV. Extracts of Nigella and Zizyphus plants were diluted by distilled water to10^{-1}, 10^{-2} and 10^{-3} before use. Crude extract and dilutions from 10^{-1} to 10^{-3} of Nigella plants gave percentages of inhibition 55.56, 47.22, 38.89 and 19.44 % respectively, when used after 7 days as a time intervals. All tested extracts of Nigella plants reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor*(infectivity of BBMV) and increased the percentages of inhibition against BBMV. Concerning Zizyphus plants, all tested extracts reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor* (infectivity of BBMV) and increased the percentages of inhibition against BBMV. These percentages of inhibition were increased to 44.44%. Crude extract and dilutions from 10^{-1} to 10^{-3} of Zizyphus plants gave percentages of inhibition 44.44, 36.11, 25.00 and 8.33 % respectively, when used after 7 days as a time intervals. It was found that, NE was more effective in reducing the local lesions produced by BBMV on *Chenopodium amaranticolor* than ZE. BBMV inhibition of pre-inoculation treatment was higher than that of post-inoculation treatment. In pre-inoculation treatment, the highest effect of NE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 25.71%). While, the highest percentage of inhibition of ZE against BBMV infectivity was 20.00 % in the crude extract and after 7 days. In post-inoculation treatment, the highest effect of NE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 22.85%). While, the highest percentage of inhibition of ZE against BBMV infectivity was 17.14 % in the crude extract and after 7 days. So, NE was more effective in reducing the local lesions produced by BBMV on *Chenopodium amaranticolor* than ZE.
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1. Introduction

Broad bean (*Vicia faba* L.) is one of the major legumes crops. Broad bean mottle disease is one of the world's main virus diseases in broad bean producing areas. **Broad bean mottle virus (BBMV**) is spread worldwide wherever broad bean plants are grown. Broad bean mottle virus (BBMV**)** was classified as a member of the bromovirus sgroup. BBMV was described by Bawden. BBMV has been reported in naturally infected broad bean in Egypt, England, Portugal, and Sudan, as well as in Syria and Tunisia **(Najar *et al*., 2000).** The percentage of RNA in BBMV particles has been estimated to be 23%. The molecular weights of the RNAs have been estimated to be 1.10 X 10^6 for RNA-1, 1.03 X10^6 for RNA-2, 0.90 X 10^6 for RNA-3, and 0.36 x 10^6 for RNA-4. Coat protein subunit molecular weights have been estimated to be 20.900 Da., 20.500 Da., and 16.400 Da. Virions isometric; not enveloped; 26 nm in diameter; Virions contain 21—23 % nucleic acid; 77—79 % protein; 0 % lipid. **The bromovirus** group was compiled by the ICTV in 1971. Viruses in this group have been transmitted experimentally by beetles and aphids. Bromoviruses possess four molecules of ssRNA: RNAI, with a molecular weight of 1.1x10^6; RNA-2, 1.0 x10^6; RNA-3, 0.7x10^6; and RNA-4, 0.3 x10^6**(Edwardson and Christie, 1991; Brunt, 1996 and Fujisaki *et al*., 2003).** Medicinal plants occupy a prominent economic position because of the continuous increasing demand for their medicinal products from the local and foreign markets. There are four species of Nigella indigenous to Mediterranean region and especially in Egypt. They are *Nigella arvensis,* L., *N. desert-boiss, N. assyriace-boiss* and *Nigella sativa,* L. The last one is very common spread while the other three species are very rare (Bailey, 1953). *Nigella sativa,* L. plant (Black cumin or Nigella) is an annual herb of the Ranunculaceae family. The seeds contain volatile and fixed oil which are used in pharmaceutical industry. The active ingredient is used as an antiasthmatic drug and to strengthen the immunity system. The seeds contain of about 20.6% proteins, about 30 % of a fixed oil and 0.40-0.45% of a volatile oil **(Hashim**

and El-Kiey, 1962; El-Alfy *et al.* 1975; Nafez *et al.*, 2009; and Mehdi *et al.*, 2010). *Zizyphus spina-christi,* Mill plant (Christ's thorn or Zizyphus) is a tree of the *Rhamnaceae* family. Zizyphus is a wild fruit tree which is widespread in the Africa. Zizyphus has a high content of sugar, protein, vitamin C**(ascorbic acid), magnesium calcium and iron(Kou *et al*., 2000; Danthu *et al.*, 2002; and Gan *et al*., 2002).** A few number of investigators used Negella or Zizyphus extracts to inhibit the plant virus infection **(Yamada and Imoto, 1987; Nikaido *et al*., 1990; Kano *et al*., 1991; Kimura *et al*., 1992; Yamashiki *et al*, 1994; Ikarashi *et al*, 2001; and Ietidal *et al.*, 2010).** Accordingly, the present study was designed to throw the light on the importance of application of Nigella and Zizyphus extracts (NE and ZE) as inhibitors against broad bean mottle virus (BBMV) infectivity *in vitro* and *in vivo*.

2. Material and Methods

Extracts of Nigella and Zizyphus plants were diluted by distilled water to10^{-1}, 10^{-2} and 10^{-3} before use. All experiments were repeated twice. Four replicates were used for each treatment.

2.1. Virus isolate

Virus inoculum was the crude sap obtained by trituration of frozen leaves of broad bean plants (*Vicia faba* L. cv.Giza 402) seedlings showing mosaic symptoms. These symptoms developed 14 days after inoculation with a single local lesion obtained from *Chenopodium amaranticolor* leaves that were inoculated with sap extracted from naturally infected broad bean plants (*Vicia faba* L. cv.Giza 402). Inoculation of leaves was carried out by rubbing with finger after their being dusted with carborandum as described by **Rawlins and Tompkins, (1936).**

2.2. Preparation of Nigella and Zizyphus extracts (NE and ZE)

Seeds of Nigella (*Nigella sativa* L.) and dried leaves of Zizyphus (*Zizyphus spina-christi* Mill.) plants were ground in blender. About 50 gm of each material was extracted by soaking in ethyl alcohol (75%) for 48 hours, then the flasks were shaken for 48 hours, and finally filtered. The solvent was evaporated under temperature not exceeding 50 C. The yielded extracts were kept in clean bottle in a refrigerator under cooling conditions till use **(Abd-Rabboh, 2000 and Abdel-Rahman, Saida 2001).**

2.3. Effect of Nigella and Zizyphus extracts (NE and ZE) on BBMV infectivity *in vitro*

For testing the effect of Nigella and Zizyphus extracts for different time intervals(1, 3, 5, and 7 days) on BBMV infectivity *in vitro*, 1 ml of the expressed sap containing virus was added to 1 ml of each of Nigella and Zizyphus extracts, mixed well and allowed to stand for 1, 3, 5, and 7 days. Distilled water was used as a control. Virus-NE and ZE mixtures and the control were inoculated into one month old *Chenopodium amaranticolor* at previously mentioned intervals. The developed local lesions were counted and the percentage of inhibition was calculated from the following formula according to **Taha and Mousa, (2000)**

% Inhibition = (control -treatment) x100/ control

2.4. Effect of Nigella and Zizyphus extracts (NE and ZE) on BBMV infectivity *in vivo*

2.4.1. Pre-inoculation treatment

1 ml of each NE and ZE concentrations was rubbed on leaves of *Chenopodium amaranticolor,* then they mechanically inoculated with BBMV infected sap (1ml/plant) at different intervals: 1, 3, 5, and 7 days respectively. Distilled water was used as a control.

2.4.2. Post-inoculation treatment

The former steps in pre-inoculation were applied except that, virus infected sap was applied first followed by NE and ZE treatments.

3. Results and Discussion

The current work was designed to evaluate the effects of some medicinal plants on BBMV infectivity *in vitro* and *in vivo*. The seeds of *Nigella sativa* L., commonly known as black seed, have been used in traditional medicine by many Asian, Middle Eastern and Far Eastern Countries to treat headache, coughs, abdominal pain, diarrhea, asthma, rheumatism and other diseases. The seeds of this plant are the most extensively studied, both phytochemically and pharmacologically. The extracts of the seeds have been shown to possess antioxidant, anticancer, and antimicrobial activities. Thymoquinone(TQ), the most abundant constituent of black seed, has been shown to be the active principle responsible for many of the seed's beneficial effects (**Hala *et al.*, 2006).**

3.1. Effect of Nigella and Zizyphus extracts (NE and ZE) on BBMV infectivity *in vitro*

The effect of crude and diluted extract from Nigella (*Nigella sativa* L.) plants on the production of local lesions symptoms produced by BBMV on Nigella plants is presented in **Table (1) and Fig. (2).** All tested extracts reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor*(infectivity of BBMV) and increased

the percentages of inhibition against BBMV. These percentages of inhibition were increased to 55.56%. Crude extract and dilutions from 10^{-1} to 10^{-3} of Nigella plants gave percentages of inhibition 55.56, 47.22, 38.89 and 19.44 % respectively, when used after 7 days as a time intervals.

Data obtained from **Table (2) and Fig. (3)** show the effect of crude and diluted extract from Zizyphus (*Zizyphus spina-christi* Mill.) plants on the production of local lesions symptoms produced by BBMV on *Chenopodium amaranticolor* plants.

All tested extracts reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor*(infectivity of BBMV) and increased the percentages of inhibition against BBMV. These percentages of inhibition were increased to 44.44%. Crude extract and dilutions from 10^{-1} to 10^{-3} of Zizyphus plants gave percentages of inhibition 44.44, 36.11, 25.00 and 8.33 % respectively, when used after 7 days as a time intervals.

NE was more effective in reducing the local lesions produced by BBMV on *Chenopodium amaranticolor* than ZE. Also, the obtained results were confirmed through inoculation on broad bean (*Vicia faba* L.) plants. The results were agree with that obtained on *Chenopodium amaranticolor* plants.

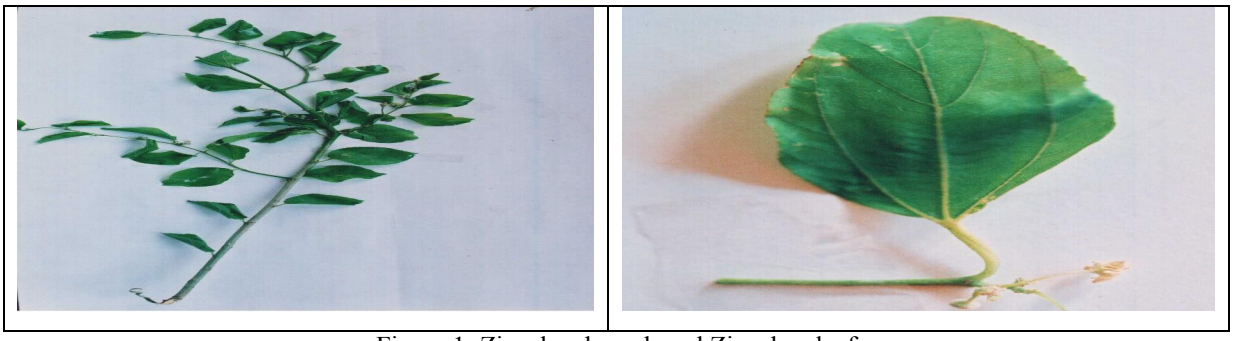

Figure 1. Zizyphus branch and Zizyphus leaf

Table 1. Effect of crude and diluted extract from Nigella (*Nigella sativa* L.) plants on local lesions number produced by BBMV on *Chenopodium amaranticolor in vitro* treatment at different intervals.

Time intervals	Mean number of local lesions								
	Control	Crude extract	% I*	Dilutions			% I*		
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
One day	35	26	25.71	28	31	33	20.00	11.42	5.71
Three days	30	17	43.33	19	22	27	36.67	26.67	10.00
Five days	32	15	53.12	18	22	28	43.75	31.25	12.50
Seven days	36	16	55.56	19	22	29	47.22	38.89	19.44

% I*: Percentage of inhibition

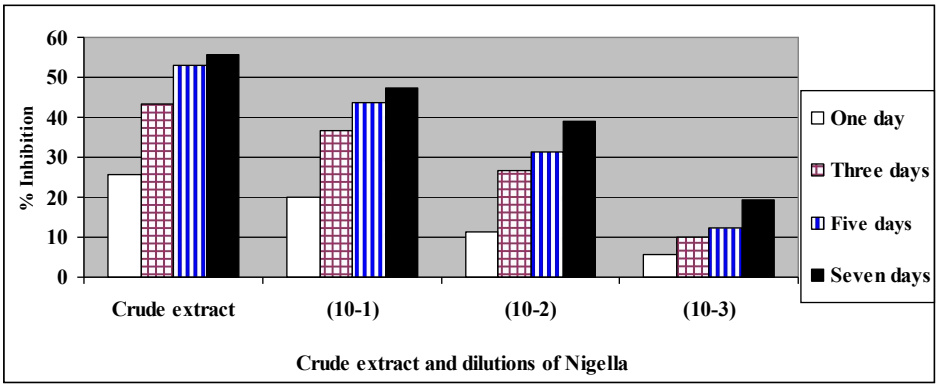

Figure 2. % of inhibition produced by crude and diluted extract of Nigella on BBMV infected sap *in vitro.*

Table 2. Effect of crude and diluted extract from Zizyphus (*Zizyphus spina-christi* Mill.) plants on local lesions number produced by BBMV on *Chenopodium amaranticolor in vitro* treatment at different intervals.

Time intervals	Mean number of local lesions								
	Control	Crude extract	% I*	Dilutions			% I*		
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
One day	35	28	20.00	30	32	34	14.28	8.57	2.85
Three days	30	20	33.33	22	25	29	26.67	16.67	3.33
Five days	32	19	40.62	22	26	30	31.25	18.75	6.25
Seven days	36	20	44.44	23	27	33	36.11	25.00	8.33

% I*: Percentage of inhibition

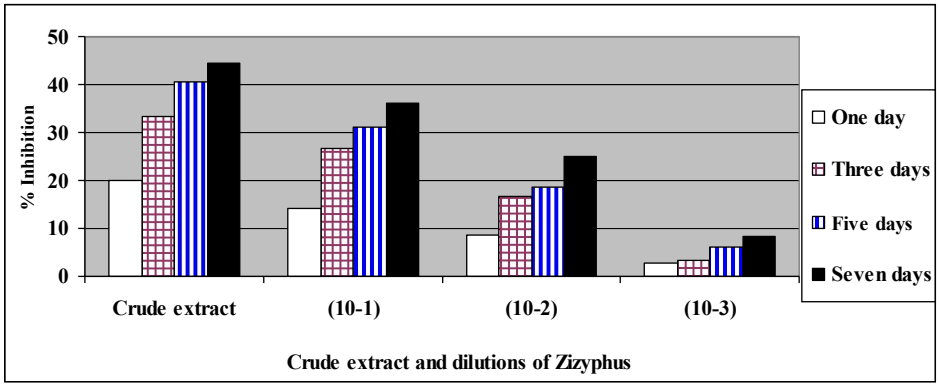

Figure 3. % of inhibition produced by crude and diluted extract of Zizyphus on BBMV infected sap *in vitro.*

3.2. Effect of Nigella and Zizyphus extracts (NE and ZE) on BBMV infectivity *in vivo*

The effect of crude and diluted extract from Nigella (*Nigella sativa* L.) plants on local lesions symptoms produced by BBMV on Nigella plants is presented in **Table (3) and Fig.(4).** All tested extracts reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor*(infectivity of BBMV) and increased the percentages of inhibition against BBMV. **In pre-inoculation** treatment, the highest effect of NE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 25.71 %). **Also in post-inoculation** treatment, the highest effect of NE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 22.85 %). **Thus**, pre-inoculation treatment was more effective in reducing virus infectivity than post-inoculation treatment.

Similar results were obtained in **Table (4) and Fig. (5)** using ZE. A higher inhibitory effect of ZE was obtained by **pre-inoculation** treatment than post-inoculation one. All tested extracts reduced the numbers of local lesions produced by BBMV on *Chenopodium amaranticolor*(infectivity of BBMV) and increased the percentages of inhibition against BBMV. In pre-inoculation treatment, the highest effect of ZE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 20.00 %). **Also in post-inoculation** treatment, the highest effect of ZE against BBMV infectivity was in the crude extract and after 7 days (percentage of inhibition was 17.14 %). **Thus**, pre-inoculation treatment was more effective in reducing virus infectivity than post-inoculation treatment. Also, the obtained results were confirmed through inoculation on broad bean (*Vicia faba* L.) plants. The results were agreed with that obtained on *Chenopodium amaranticolor* plants.

These results are in the same line with the earlier reports of many workers. **Ball *et al*., (1994) who** investigated the antiviral activity of Keishi-ni-eppi-ichi-to **(TJS-064)** on of influenza A2(H2N2). **TJS-064** contained *Ziziphus* sp., administered 1 day before, and 1 and 4 days after infection in mice infected with a lethal amount of influenza A2(H2N2). When mice were exposed to a 5 LD5O dose of the virus, 100% survived over a 25-day experimental period following TJS-O64 treatment; control mice had a mean survival time (MST) of 11.2 days, and all control mice died within 25 days. The MST of mice treated with TJS-064 following infection with a 10 LD50 dose of the virus was 17.4 days; treated mice had a survival rate of 50%, whereas control mice had a survival rate of 0%, and a MST of 8.7 days. No significant antiviral effect was observed when TJS-064 was administered to mice infected with larger doses of the virus. TJS-O64 did not exhibit disease

activities in vitro, and it is suggested that the antiviral effects of TJS-064 are expressed through the host's immune system.

Table 3. Effect of crude and diluted extract from Nigella (*Nigella sativa* L.) plants on local lesions number produced by BBMV on *Chenopodium amaranticolor in vivo* treatment at different intervals.

Time intervals	Mean number of local lesions																	
	Pre-inoculation									Post- inoculation								
	Control	Crude	% I	Dilutions			% I*			Crude	% I	Dilutions			% I*			
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}			10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	
One day	36	29	19.44	31	34	35	13.89	5.56	2.78	30	16.67	32	34	35	11.11	5.56	2.78	
Three days	32	25	21.87	27	30	31	15.62	6.25	3.12	26	18.75	28	31	31	12.50	3.12	3.12	
Five days	30	23	23.33	25	28	29	16.67	6.66	3.33	24	20	26	29	29	13.33	3.33	3.33	
Seven days	35	26	25.71	29	32	34	17.14	8.57	2.85	27	22.85	30	33	34	14.28	5.71	2.85	

% I*: Percentage of inhibition

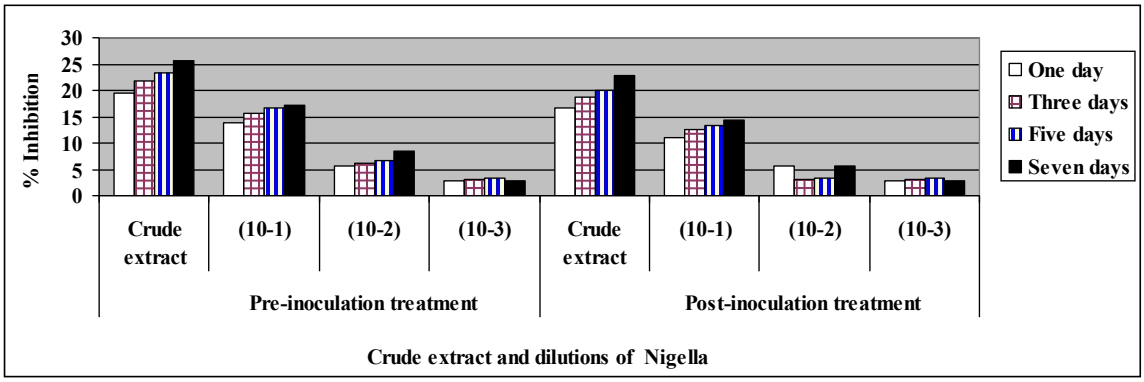

Figure 4. % of inhibition produced by crude and diluted extract of Nigella on BBMV infected sap *in vivo.*

Table 4. Effect of crude and diluted extract from Zizyphus (*Zizyphus spina-christi* Mill.) plants on local lesions number produced by BBMV on *Chenopodium amaranticolor in vivo* treatment at different intervals.

Time intervals	Mean number of local lesions																	
	Pre-inoculation									Post- inoculation								
	Control	Crude	% I	Dilutions			% I*			Crude	% I	Dilutions			% I*			
				10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}			10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	
One day	36	31	13.89	32	34	35	11.11	5.56	2.78	32	11.11	33	35	35	8.33	2.78	2.78	
Three days	32	27	15.62	28	30	31	12.50	6.25	3.12	28	12.50	29	31	31	9.37	3.12	3.12	
Five days	30	25	16.67	26	28	29	13.33	6.67	3.33	26	13.33	27	29	29	10.00	3.33	3.33	
Seven days	35	28	20.00	29	32	34	17.14	8.57	2.85	29	17.14	30	33	34	14.28	5.71	2.85	

% I*: Percentage of inhibition

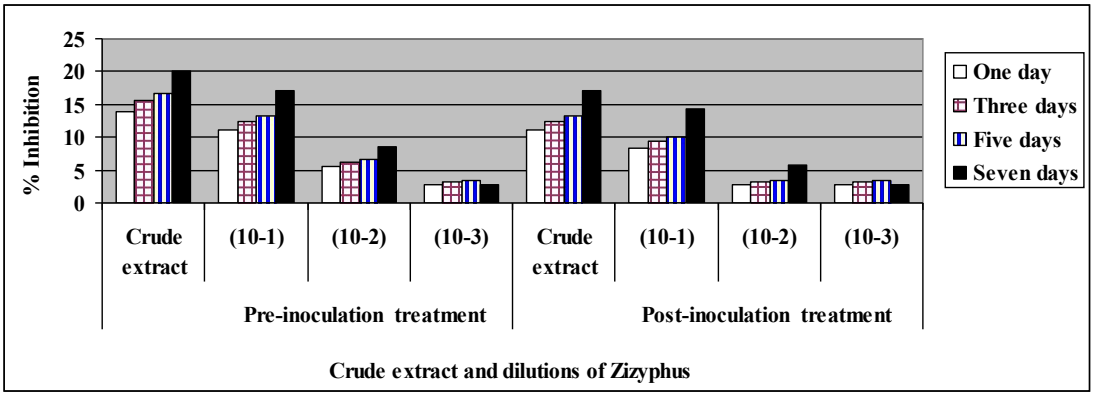

Figure 5. % of inhibition produced by crude and diluted extract of Zizyphus on BBMV infected sap *in vivo.*

Belford (1994) reported that**, c**hronic hepatitis can be caused by both hepatitis B and C viruses. Orthodox medical treatment for chronic hepatitis is mostly with the drug, interferon, however many patients relapse after initial improvement. Chinese herbs, either singly or in formulations, have potential as alternative methods for the treatment of chronic hepatitis. Medicinal plants which inhibit hepatitis B virus antigens, modulate the immune response, and/or exhibit hepatoprotective properties, are listed. One Chinese formulation, called Minor Bupleurum, which contains Zizyphus (*Ziziphus sp*), induces interferon, and exhibits hepatoprotective properties. **Salem and Hossain, (2000) and Salem, (2005)** illustrated that, murine cytomegalovirus (MCMV) is a herpes virus that causes disseminated and fatal disease in immunodeficient animals similar to that caused by human cytomegalovirus in immunodeficient humans. In our own experience, we have found that *in vivo* treatment with *N. sativa* induced a striking anti-viral effect against MCMV infection, indicating a promising therapeutic potential of *N. sativa* as an anti-viral remedy. It has been reported that viral infection induces apoptosis leading to lymphocyte depletion in the host, and that anti-oxidant agents can inhibit virus-induced apoptosis as well as the viral replication in target cells. Eventually, the anti-oxidant effect of the *N. sativa* may represent another mechanism that contributes to its anti-viral activity. Indeed, the anti-viral effects of *N. sativa* against MCMV infection open a new avenue for a novel anti-viral remedy. However, further studies are required to confirm this effect in other viral models, as well as to define which active ingredients exerting such anti-viral effects. It can be suggested that the anti-tumor effects of *N. sativa* oil might be mediated through anti-angiogenic effects through inhibition of local tumor invasion and metastasis *in vivo*. The anti-tumor effects of *N. sativa* oil might be attributed to the effect of TQ(seed constituent, thymoquinine), since administration of TQ in drinking water resulted in significant suppression of forestomach tumor induced by benzo(α)pyrene. Similarly, the same treatment regimens of TQ significantly inhibited the tumor incidence and tumor burden of 2-methyclonathrene induced soft tissue fibrosarcoma associated with reduction in hepatic lipid peroxides and increased enzyme contents and activities of GSH(Oxidant scavenger enzyme system including, glutathione). Using the same fibrosarcoma tumor model, administration of *N. sativa* extract 30 days after subcutaneous administration of methyclonathrene restricted fibrosarcoma tumor incidence, compared with control tumor-bearing mice, indicating to therapeutic potentials. **Shimizu (2000**) Mentioned that, the Chinese herbal medicine, sho-saiko-to, an officially approved prescription drug in Japan most commonly administered to outpatients with chronic hepatitis and liver cirrhosis(hepatic carcinogenesis). Its effects on hepatic fibrosis and hepatic carcinogenesis are reviewed. Sho-saiko-to is a combination of herbs containing Zizyphus: This combination contains the flavonoids, which have the ability to inhibit cell proliferation. A similarity of chemical structures was observed between these flavonoids and those of silybinin and quercetin. Silybinin and quercetin showed anti-fibrogenic properties *in vitro* and in animal models of hepatic fibrosis. It is noted that sho-saiko-to may have beneficial effects not only on hepatic fibrosis but also on hepatocellular carcinoma development in patients with liver disease. **Watanabe *et al.*, (2001)** showed that**, t**he Long Evans Cinnamon (LEC) rat is a well-characterized model of spontaneous hepatocarcinogenesis. It has been shown that dietary administration of lycopene or the herbal medicine sho-saiko-to (contains Zizyphus**)** has anticarcinogenic activity, although the mechanism by which these products protect against carcinogenesis is not well known.

The inhibitory effect of NE and ZE on the virus infectivity can be attributted to some constituents like; thymoquinone, an active constituent of *Nigella sativa* extract **(Kamal *et al.*, 1993; Merfort *et al.*, 1997; D'Antuono *et al.*, 2002; Iman *et al.*, 2006)**, betulinic acid **(Yasunari and Bharat, 2003)**, Saponins **(Alexandre *et al.*, 2004),** flavonoids quercetin, hyperoside, rutin and quercetin-3-*O*-[β-xylosyl-(1-2)-α-rhamnoside] 4′-O-α- rhamnoside **(Abdelaaty *et al.*, 2001; Taskin *et al.*, 2005 and Dadgar *et al.*, 2006)**, Nigellon and thymohydroquinone **(Kawther *et al.*, 2008)** or steroids **(Zhu *et al.*, 2010).**

The inhibitory effect of NE and ZE may flavonoids compounds. Therefore, the mechanism of inhibition may be related to virus-specific nucleic acid or protein synthesis **(Konig, 1986; Konig and Dustmann, 1986 and Serkedjiva, 1992).**

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