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Enhancing nematicidal activity of *Tagetes patula* extract using silica nanoparticles

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ABSTRACT

This study was carried out to investigate the biological effect of the methanol flower extract of *Tagetes Patula* (Asteracea) as a nematicide for root –Knot nematode of cucumber plant (AL-Rafidain cultivar) alone, and with the silica nanoparticles compared to the chemical pesticide. The experiment was applied in winter season during 2017 in glass houses in the Yusufiya area. A complete randomized design was used to analyze the data using five replicates. This study included the detection of secondary metabolites in alcoholic flowers crude extract using 80% methanol. Chemical tests showed positive results for alkaloids, phenols, flavonoids, terpenes and tannins. The concentrations (5%, 10%, 15%, 20%) of flowers extract were prepared and added to petri dishes containing nematode larvae. All concentrations showed a fatal, ascending, and compatible effect with increasing in time of treatment (24, 48 and 72) hr.The best concentration was 20% for 72 hours with a 98% mortality rate. The concentration and duration time of the subsequent experiments were adopted separately in addition to using nanoparticles (silica nanoparticles) in comparison with the chemical pesticide. Silica nanoparticles were prepared by tetra ethyl ortho silicate (TEOS) hydrolysis and ammonium hydroxide in ethanol. Particles examination appeared in a hollow spherical shape with a size of 33-38 nm. The treatment with flowers extract and silica nanoparticles showed the best results in fresh and dry weight, stem length and fruits yield(number of fruits) (118 g, 15 g, 120 cm, 95 fruit) respectively. On other hand this treatment reduced root-knot number which reached to 100% compared with control (untreated) and chemical nematicide.

Keywords: *Tagetes petula*, Nematicide, Plant extract, Nanoparticles.

1. INTRODUCTION

Nematode caused huge economic losses in agricultural crops through the past periods and to overcome these agricultural pests, many countries were adopted on synthetic pesticide since1940s till now [1]. These synthetic pesticides led to many problems in environment such as resistance of pest, contamination of the ecosystem, contamination of air, water and soil, and other many problems [2].

The use of plant crude extracts in crops protection from pests is an old practice [3].Substances from natural sources were used for controlling nematode (pyrethrum, rotenone and nicotine) and as biological control agent [4].Mean while many researches proved that pesticide of plant origin do not possesses any dangerous toxicity to humans and animals this fact led to the interest in natural substances of pest control, including intensified searches for new sources of botanical pesticide [5].

Tagetes flower component are widely used in commercial pesticide, this flower is rich in flavonoids, phenolic acid, and terpines (pyrethroids) [6]. Studies has been undertaken to produce nanoparticles incorporated with pesticides as a smart delivery system. Nanotechnology-based products and their applications in agriculture may include nano nutrients, nano pesticides, and nano scale carriers. Nano particles provides better penetration into the cell due to their high surface area and high reactivity these can activate plant and microbial activities resulting in more nutrient use efficiency [7,8].

The aim of the current study is to evaluate the efficiency of nematicidal activity of *Tagetes* flowers crud extract alone and promoted with silica nan-oparticles, in comparison with chemical nematicide.

2. MATERIALS AND METHODS

2.1 Detection of phytochemical compounds

Phytochemical test mainly included test for terpens, alkaloids, saponins, tannins, flavonoids, phytosterols, phenols, proteins and amino acids.

2.2 Preparation of experimental plant (cucumber)

This experiment was done to detect the effect of methanol extract of *Tagetes patula* flowers alone and with nanoparticle against root-knot nematode grown on cucumber roots. Plastic pots (5kgm) were filled with a sterile mixture of soil(loamy soil) and Peat-moss in a ratio (1:3). Soil mixture was serialized by autoclave then 1L of flowers extract was added at concentration 200 mg to the pots. Two weeks old of cucumber seedlings were planted in the pots under plastic house conditions (Fig 2-1). After one week from treatment 25 ml of larvae suspension (200 larvae/ml) was added to the pots. Five replicates were used for each treatment. Readings were recorded after 90 days of treatments. Results were analysed statistically according to complete randomized design (CRD).

2.3 Collection and drying of *Tagetes patula* flowers

The flowers of *Tagetes patula* were collected from the gardens of Baghdad University and from arboretum. The flowers were washed with clean tap water and then left at room temperature at (22-25) ˚C for (2-3) weeks for drying; dry flowers were grinded by electric miller.

2.4 Preparation of methanol flowers extract

Crude flowers extract were prepared by using methanol 80%, dried flowers powder (30 mg) was soaked in 300 mL of the solvent for 5 days at room temperature with an extraction ratio of (1:1). Extract was filtered through filter paper (whatman No.4) then concentrated in oven at (37-38C) until drying and kept in small container in cold and dry place for further uses.

2.5 Collection of nematode (*Meloidgyne incongnita*) from plant root

2.5.1 Preparation of sample

Ideally the sample should be processed as possible after collection. The root suspension should be passed through a coarse sieve (mesh openings of 10 mm or 1/4 inch) to remove any lumps, leaves and other materials. Nematode was collected from cucumber roots that infected with root-knot nematode planted in green house in private farm south of Baghdad. according to the way described by[9] the roots were cut into small pieces (2mm) in a blender and 1% hypocloric sodium solution(Nalco) were added and left stirring for 30sec at high speed. The root suspension then passed through coarse sieve(mesh opening of 10mm or 1/4 inch). Larvae collected by 75µm sieve and washed by distilled water to remove excess of hypochloric sodium solution then larvae kept in glass containers. Larvae numbers was estimated in one ml from suspension of nematode by using histology glass slide special for nematode. The hull of glass slide were filled with 1ml of larvae suspension then examined under light microscope at 10X and larvae accounted in 10 squares then multiplyed by 125 (constant number). The average number of larvae in 1ml was reached to 200 larvae/ml.

2.5.2 Effect of flowers extract on nematode larvae

The effect of flowers extract was tested on larvae of nematode by making series of concentrations (5%,10%,15%,20%), 5ml from each concentration was added to petri-dishes containing 1.5ml of larvae suspension(300 larvae). Petri-dishes then incubated at incubator in(2±25)c, three replicates were used for each concentration according to(CRD), the percentage for larvae mortality was accounted under microscope at(60x) after 24h, 48h and 72h [10].

2.6 Preparation of silica nanoparticles

Silica nanoparticles were prepared by hydrolysis and condensation of TEOS in ethanol, and presence of ammonia as catalyst[11]. First,solution containing appropriate quantities of absolute ethanol, ammonia and deionized water were stirred for 5 minutes to ensure complete mixing. Then a proper amount of TEOS in absolute ethanol was added to the above solution and the reaction proceeded at ambient temperature for 24 hours according to reactants concentrations. Thereafter the colloidal solution was separated by high-speed centrifuge, and the silica particles were washed by absolute ethanol for three times to remove undesirable particles, followed by drying in oven at 100 C for 2 hrs to prevent continuous reaction

2.7 Characterization of silica Nanoparticles

2.7.1 X-ray energy dispersive spectroscopy (EDS Analysis)

Energy dispersive X-ray analysis, also known as EDS, EDX or EDAX, is a technique used to identify the elemental composition of a sample or small area of interest on the sample. During EDS, a sample is exposed to an electron beam inside a scanning electron microscope (SEM). These electrons collide with the electrons within the sample, causing some of them to be knocked out of their orbits. The vacated positions are filled by higher energy electrons which emit x-rays in the process. By analyzing the emitted x-rays, the elemental composition of the sample can be determined. EDS is a powerful tool for microanalysis of elemental constituents.

2.7.2 Transmission electron microscope analysis (TEM)

TEM analyses of silica particles were carried out using JEOL JEM 2010 on silica particles to investigate the diameter of silica particles. To prepare samples for TEM analysis, silica powder is dispersed in absolute ethanol and a drop of silica colloid solution was placed

on a copper grid coated with carbon. The solvent was evaporated at room temperature, leaving the silica particles on the grid.

2.8 Scanning electron microscope (SEM)

SEM machine was used to characterize mean particle morphology, diameter of nanoparticles. The dried sample of silica NPs solution was sonicated with distilled water; small drop of this sample was placed on glass slide and allowed to dry. After that a thin layer of platinum was coated to make the samples conductive [12].

2.9 Atomic force microscopy (AFM) analysis

Atomic Force Microscopy was used to analyze silica NPs. A thin film of prepared silica NPs was deposited on a silica glass plate by dropping few drops of the silicaNPs on the plate and allowed to dry at room temperature in the dark. The deposited film glass plate was then scanned with the AFM.

2.10 Determination of silica nanoparticles concentration

2.10.1 Preparation of standard curve for silica nanoparticles

Serial concentrations of silica nanoparticles (3.5, 7, 10.5, and 14 µg /ml) were prepared from the stock solution by dilution with D.D.W. The absorbance was measured for each concentration by Atomic absorption spectrometer. The standard curve was plotted in Figure 1 between the concentration and absorbance. The absorbance of unknown sample was measured and applied on stander curve to determine the concentration of sample.

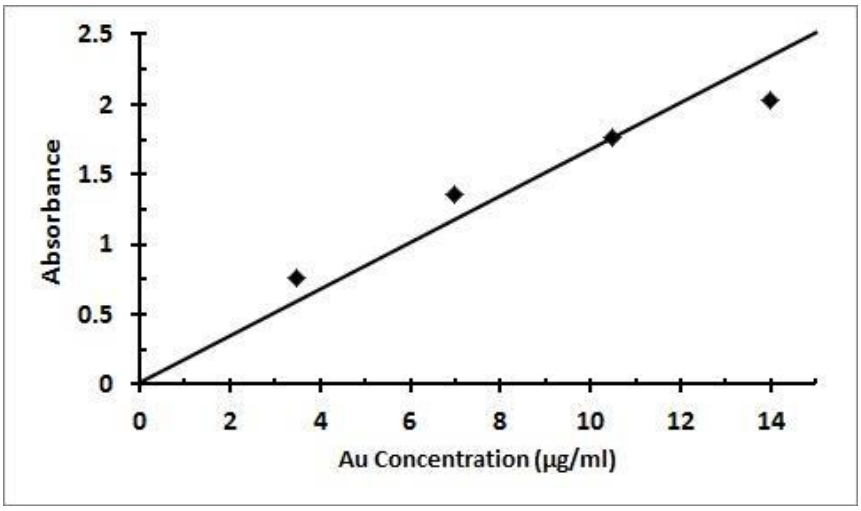

Figure 1: Standard curve of silica nanoparticles.

3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis

Methanol extracts were subjected to preliminary photochemical investigation for the detection of secondary metabolites [27]. Results obtained by chemical detection of methanole extract for *Tagetes patula* indicated the presence of terpenoid, tannins, phenols, flavonoids and alkaloid, while the results were negative for a phytosterol, saponins, protein and carbohydrate as shown in Table 1. The methanol used to prepare extracts of *Tagetes patula* this is due to the high polarity of methanol. The variation in the presence of these compounds may be due to the genetic nature of the plants beside the time of plant collection and the conditions of extraction.

Table 1: Active compounds detected in *Tagetes patula*

Active compounds	Methanol extract
Terpenoid	+
Alkaloids	+
Phenols	+
Protein	-
Tannins	+
Saponins	-
Flavonoids	+
Carbohydrate	-
Phytosterole	-

Table 2: Effect of different concentration from *Tagetes Patula* flowers extract on *Meloidgyne incongnita* larvae.

No of TRET.	Treatment	No. of mortality			Average
		R_1	R_2	R3	
A1	Control after 24h	0	0	0	0
A2	Cont. after 48h	0	0	0	0
A3	Cont. after 72h	0	0	0	0
A4	5% after 24h	52.82	49.81	52.52	51.71
A5	5% after 48h	52.32	52.81	51.83	52.32
A6	5% after 72h	52.40	53.31	54.32	53.43
A7	10% after 24h	73.31	73.54	76.81	74.55
A8	10% after 48h	76.30	78.32	80.22	78.28
A9	10% after 72h	77.21	78.21	82.25	79.22
A10	15% after 24h	84.45	88.31	92.91	88.55
A11	15% after 48h	92.31	96.71	97.82	95.61
A12	15% after 72h	94.81	96.81	98.93	96.85
A13	20% after 24h	93.64	93.97	97.91	95.17
A14	20% after 48h	97.32	97.76	98.31	97.79
A15	20% after 72h	97.82	98.83	99.73	98.80
		اقل فرق معنوي			3.227
		Least significant differences of means = L.S.D			

3.2 Nematicidal activity

From the results above we conclude that the increasing in concentration resulted in increasing of larvae mortality rate and it was time dependant. This scientific fact has been mentioned by[13] who indicated that the mortality percentage of nematode larvae *M. javanica* treated with leaf extract of *Moringa oleivera* increased with the increasing of concentration and the highest mortality rate for larvae was reached 30% in concentration 100% and the lowest mortality rate in 25% concentration was 16% he also stated that the rate of egg whites increased with concentration .

3.3 Estimation of cucumber root fresh weight treated with tagetes flowers extract and silica nanoparticles (SNPs).

Table 3 represents the results of cucumber roots fresh Weight for each treatment. Results showed that the treatment with chemical pesticide gave the lowest root fresh weight (100gm) in comparison with control (143gm) and the difference were significant($p \geq 0.05$) between them .On other hand root fresh weight of cucumber recorded 120gm, 118gm, and 119gm for the treatments (flowers extract alone, flowers extract with silica NPs, and silica NPs) respectively.

This results were in agreement with [14] who pointed that treating plant affected by root knot nematode with tagetes extract was significantly higher ($p \geq 0.05$) in fresh and dry weight compared to untreated plant.

Table 3: Fresh Weigh of cucumber roots treated with tagetes flowers extract and NPs

Treatment	Fresh Weigh (gm)					Average
	R_1	R_2	R_3	R_4	R_5	
Control	143	142	141	143.2	142	143.1
Pesticide	99	98	101	102	100	100
Flower extract	119	117	118	122	122	120
Flower extract+nanosilia	117	116	119	120	117	118
Nanosilica	116	118	119	120	120	119

3.4 Estimation of cucumber root dry weight treated with tagetes flowers extract and silica nanoparticles (SNPs).

Table 4 showed the results of root dry Weight for each treatment of cucumber plant under study. From the table below appears that readings for the treatments (flowers extract, flowers extract with silica NPs, and silica NPs alone) recorded 16.3gm, 15gm, and 16.1gm respectively and they were closer to chemical pesticide treatment (14.1gm) in comparison with control (untreated) (21.9gm) with significant differences ($p \geq 0.05$).

Table 4: Dry Weight of cucumber root treated with tagetes flowers extract and NPs

Treatment	Dry Weight (gm)					Average
	R_1	R_2	R_3	R_4	R_5	
Control	21.1	21.1	20.1	22.1	22.1	21.9
Pesticide	14.1	13.2	13.3	13.9	15.6	14.1
Flower extract	14.2	16.2	16.2	17.2	15.3	16.3
Flower extract+silica NPs	14	16	17	15	15	15
Silica NPs	16.2	16.1	16.9	17.1	15.9	16.1

3.5 Number of cucumber fruit yield treated with flower extract and silica Nano particles (SNPs).

Any disease affect plant can cause damage to it and may be decreases its product .One of these diseases are root-knot nematode that cause decrease in plant product as shown in Table 5 below which appeared that the yield recorded 43 with untreated plant (control) and 49 with plants treated with chemical pesticide in comparison with yields reached to 86.4, 95, 90 for treatments with flower extract, extract and silica NPs, and NPs alone respectively.

Table 5: Number of fruits yield according to each treatment

Treatment	Number of fruits					Average(yield)
	R_1	R_2	R_3	R_4	R_5	
Control	42	39	48	43	43	43
Pesticide	44	48	49	50	52	49
Flower extract	84	85	86	88	89	86.4
Flower extract+nanosilica	95	93	94	92	93	95
Nanosilica	89	88	87	91	93	90

3.6 Plant stem length treated with tagets flower extract and silica Nano particles(SNPs).

Stem length also affected by nematode and cause weakening and shortening in stem growth. Table 6 show that there are significant differences in stem length between treatments. best results in growth of stem length were obtained when plants treated with tagets flowers extract (128cm) and flowers extract with silica NPs (129cm) in comparison with control (102cm) and pesticide treatments (114cm).

The extract of tagetes plant was used as root knot nematicidal on tomato root and appeared that extract led to increase in plant growth parameters such as increase in stem length and fruit yield compared to untreated plants [15].

Table 6: Stem length for each treatment

Treatment	Stem length (cm)					Average
	R_1	R_2	R_3	R_4	R_5	
Control	100	102	103	103	99	102
Pesticide	110	111	115	116	118	114
Flower extract	124	125	129	130	129	128
Flower extract+nanosilica	129	130	131	128	127	129
Nanosilica	100	110	120	111	116	111

3.7 Characterization of silica Nanoparticles

3.7.1 X-ray energy dispersive spectroscopy (EDS)

Figures 2 showed one representative EDS analysis of SNP. It was observed from the EDS data that major constituent of the synthesized nano particulate material was Silicon (39.72%), Na (0.48%) and Oxygen (59.40%) as shown in Table 8. So it was confirmed that synthesized NPs were nothing but SNP.

Table 8: EDS data of Silica nanoparticles showing the major elements present.

Element	Wt%	Wt% Sigma
O	59.40	0.23
Na	0.48	0.08
Al	0.08	0.05
Si	39.72	0.22
S	0.32	0.06
Total:	100.00	

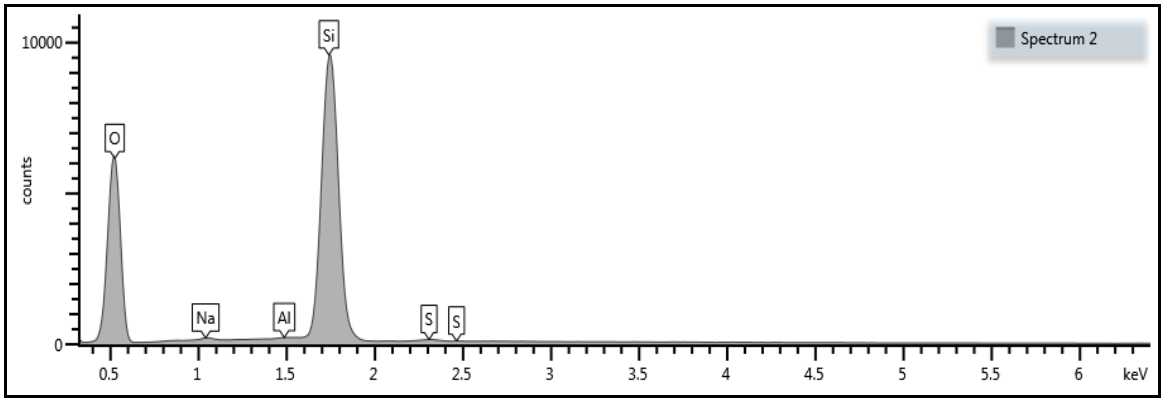

Figure 2: EDS of Silica nanoparticles showing the major elements

3.8 Transmission Electron Microscopy (TEM) analysis

The TEM image of prepared silica nanoparticles using TEOS extract is shown in figure 3, and the resulting image revealed formation mainly spherical nanoparticles with diameter ranged (33-40 nm) without any aggregates and dispersed, on one hand this is due to presence of the repulsive nature of the capping and stabilizing agent coated the surface of SNPs [16]. On the other hand biomolecule that was supporting formation of spherical shape [17].

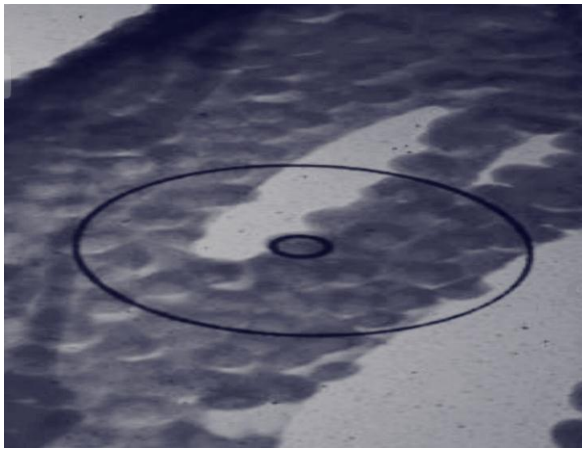

Figure 3: Transmission Electron Microscopic (TEM) image of silica nanoparticles.

3.9 Scanning electron microscope (SEM)

SEM was tested to complete the nanoparticles characterization protocol and for confirmation of the results obtained by TEM, the SEM images was showed in figure 4, and when analysis the SEM images that the same shape and diameter of silica nanoparticles had been appears in TEM images.

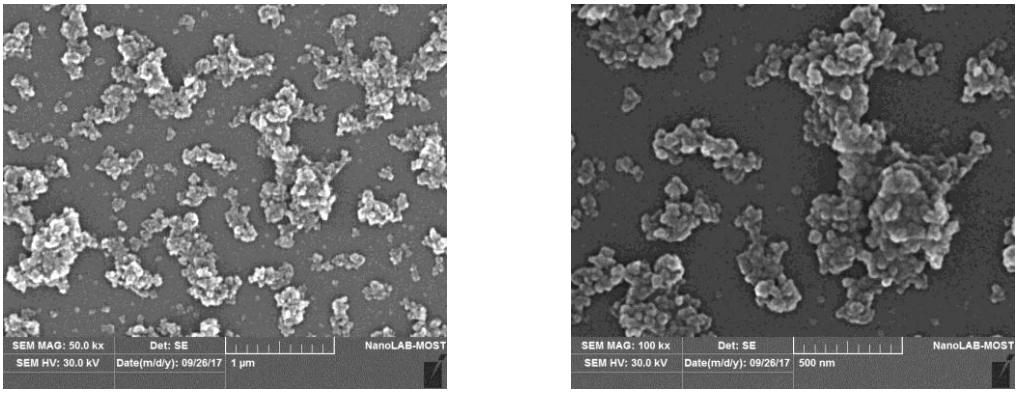

Figure 4: SEM image of silica Nanoparticles synthesis by hydrolyses and condensation of TEOS in ethanol

3.10 Atomic force microscopy (AFM) analysis

The AFM scanning was used to detect the topographic distribution of silica nanoparticles prepared by hydrolysis and condensation of TEOS in ethanol. The images were obtained by AFM that show in figure 5 and revealed the particles shape (spherical) and homogeneous distribution Sio2NPs with no agglomeration was observed.

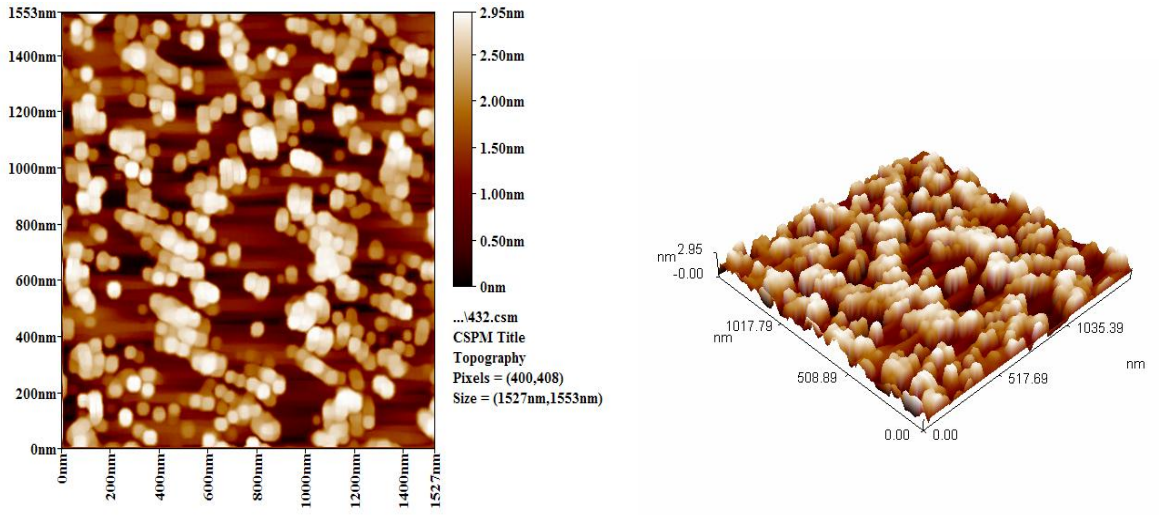

Figure 5: AFM image of silica Nanoparticles synthesis by hydrolyses and condensation of TEOS in ethanol.

4. CONCLUSION

The results of the present study showed that methanol extract of *Tagetes patula* flowers have nematicidal activity and using of silica nanoparticles as a carrier enhance this property. The study also reveals the fact that the use of this natural insecticide in the optimum dosage can replace the use of chemical one.

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