Isolation and Structural Identification of Compounds with Antioxidant, Nematicidal and fungicidal Activities from *Punica granatum L. var. nana.*

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ABSTRACT-Pure compounds have been isolated from methanolic extract of *Punica granatum* L. var. *nana*. The crude extract and pure isolated compounds have been tested for their antioxidant, nematicidal and fungicidal activities against three nematode species, *i.e. Meloidagyne incognita*, *Rotylenchulu reniformis* and *Pratylenchus penetrans* as well as three phytopathogenic fungi, *i.e Fusarium oxysorum* f.sp. *lycopersici* ,*Rhizoctonia solani* and *Sclerotium rolfsii*. The methanolic extract exhibited free radical scavenging activity against DPPH radicals (IC_{50}=25.97µg/ml^{-1}). Activity guided separation resulted in isolation of three active compounds namely; Cyanidin 3,5-diglucoside (IC_{50}=5.2 µg/ml^{-1}), Galloyl glucoside (IC_{50}=4.1 µg/ml^{-1}) and 2-methyl-pyran-4-one-3-*O*-*β*-D-glucopyranoside (IC_{50}=5.5 µg/ml^{-1}).

The structures of the isolated compounds were elucidated by UV, ^{1}H and ^{13}C – NMR spectroscopic techniques. The fungicidal and nematicidal activities of the isolated compounds were performed *in vitro* and *in vivo.*

The bioactive components of *Punica granatum* L. var. *nana* (A, B and C) resulted in significant reduction to the linear growth and sclerotial viability of the three tested fungi. Also, they caused exhibited larvacidal and ovacidal to the tested nematode. Moreover, these compounds resulted in increasing plant weight and length of tomato plants and reducing number of nematodes / 250 cm^3, galls , egg masses and developmental stage as well as the infection by any of the three tested fungi when tomato plants grown in soil infested with *M. incognita* and any of the tested fungi. This effect was increased by increasing the concentration of the tested bioactive components.

Key words: *Punica granatum* L. var. *nana*, Lythraceae, secondary metabolites, phenolic compounds, Anthocyanins, Fungicidal and nematicidal activity.

1 INTRODUCTION

People have used plants for food and medicinal purposes for thousand years and have acquired extensive knowledge of their properties (Brouwer *et al*., 2005). Many higher plants accumulate extractable organic substances in quantities sufficient to be economically used in management of diseases in Agricultural and Medicinal fields. Plants have been a rich source of pesticides and medicine drugs because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection.

The demand for nature –based biopesticides, predominantly those derived from plants is rising steady all over the world. This is because of increased public awareness of environment and the pollution potential and health hazards related to many conventional (synthetic) pesticides. Biopesticides are cost effective, safer, readily available, biodegradable and therefore more environment-friendly and will offer alternative to those conventional pesticides (Ranasing, 2007). *Punica granatum* L. var. *nana* is a dwarf variety of *P. granatum* popularly planted as an ornamental plant in gardens and larger containers and used as a bonsai specimen tree. It could well be a wild form with distinct origin. The variety has a very compact pomegranate from with showy double flowers. It does not usually produce edible fruit (Hellyer, 1978 and Gupta and Diskshit, 2010).

Pomegranate is a symbol of life, longevity, health, feminity, fecundity, knowledge, morality, immortality and spirituality, if not Divinity (Mahdihassan, 1984). In Aurvedic medicine; The pomegranate is considered "a pharmacy unto itself".

The pharmacological properties of various extracts of different parts of this plant have been reported by many investigators including antifungal activity against plant pathogenic fungi (Tehranifar *et al*., 2011 and Al-Askar, 2012).

The pharmacological properties of various different parts of this plant have been attributed to its high content of bioactive secondary metabolites, such as polyphenols glycosides, triterpenes, sterols, flavonoids, anthocyanins, triglycerides, tannins and alkaloids (Tantray *et al*., 2009). The tree could be divided into several anatomical compartments (1) bark (2) root (3) peel (4) flowers (5) juice (6) seed (7) leaves.

Despite several previous studies on the phytochemistry and pharmacological actions of all *Punica granatum* L. components, to date there is no any research about the variety *nana*.

The objective of this study was to evaluate *in vitro* both the nematicidal activity of this plant extract against three nematode species, *Meloidegyne incognita Rotylenculus reniformes* and *Pratylencus penetrans*, causing root knot, and canker in many economically important crops such as vegetables , cotton, ….etc ; and the fungicidal activity against three phytopathogenic fungi, *Fusarium oxysporum*, f. sp. *Lycopersici*, *Sclerotium rolfsii* and *Rhizoctonia solani*, causing root rot and wilt in many economic important crops such as tomato, beans ……etc; along with the isolation and structural elucidation of the active constituents responsible for these activities.

2 MATERIAL AND METHODS

1. Plant material:

Leaves of *Punica granatum* L. var. *nana* (Family; Lythraceae) were collected at the flowering stage during May of 2009 from El-Tahreir garden, on the front of Bani-Suef general hospital, Bani-Suef

governorate. The botanical identification of the collected plant (Fig.1) was authenticated by Dr. T. Labeb, Herbarium of Orman garden, Hort. Res. Instit. ARC, Giza, Egypt. A voucher specimen (p.g.n.1) is deposited at the Herbarium of the Biochemis. Dept., Fac. Agric., Fayoum Univ.

The leaves of the plant were thoroughly washed with tap water, air dried in the shade and then powdered by laboratory mill to 24 mesh. Powdered material was maintained in an air tight container at room temperature (28± 2º C) and protected from light until used.

2. Preparation of plant extract:

Dry powdered leaves of the plant (350 g) were exhaustively macerated with Me OH / H_2O (4:1) at room temperature (28± 2º C) for several times until the solvent became colourless. The obtained extract was filtered by Whatman No.1 filter paper and the filtrate was evaporated in vacuum. The residue (75g) was defatted using chloroform and the remaining solid (71.3g) was evaluated for their biological activities.

Fig (1): *Punica granatum* L. var. *nana.* (local name : flower pomegranate).

3. Assessment of biological activities:

3.1.The tested nematodes:

The three nematode species, root-knot nematode *Meloidagyne incognita*, reniform nematode *Rotylenchulu reniformis* and lesion nematode *Pratylenchus penetrans* were isolated and propagated locally in Nematol. Dept., Plant Pathol. Res. Instit., ARC, Giza , Egypt.

3.1. a. Determination of nematicidal activity:

Nematode eggs of *M. incogentia* and *R. renformis* were recovered from roots by agitation in 0.5 % sodium hypochlorite solution. The total number of eggs was counted under stereoscopic micro scope and expressed as number of eggs per one ml water (Jenkins, 1964).

3.1.b. Bioassay procedure:

The nematicide effects of the defatted methanolic extract on the hatchability of eggs and mortality of juveniles of *M. incognita*, *P. penetrans* and *R. reniformis* were assayed according to the method described by Al-Sayed *et al.*,(1996) as follows:

One ml of each nematode; *M. incognita*, *P. penetrans* and *R. reniformis* containing approximately 500 fresh newly hatched juveniles or 500 individual eggs of each. The eggs of each nematode was separately transferred into Petri dishes (5 cm diameter) containing 1ml of aqueous solution of each concentration tested (400, 800 and 1600 $\mu g.ml^{-1}$) and then incubated at 25±ºC. Dead and survived juveniles of *P. penetrans* were counted after 48h and for both *M. incogentia* and *R. renformis* were counted after 72h under a stereoscopic microscope and percentage of mortality was determined . Three replicates of each concentration were prepared and three Petri dishes containing one ml distilled water were used as control.

3.2. Fungicidal activity:

3.2.a. The tested fungi :

Pure cultures of the three phytopathogenic fungi, *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* were previously isolated from tomato plants and identified in the Integ. Cont. Manag. Res. Dept., and the identification was kindly confirmed by the Mycol. Classifi. and Identifi. Res. Dept., Plant Pathol. Res. Instit., ARC, Giza , Egypt.

3.2.b. Determination of fungicidal activity

The fungicidal activity of the extracts and isolated compounds was determined *In vitro* and *in vivo* by two different techniques as follows:

3.2.c.Effect on the linear growth:

Droplet (1µl) of the tested leaf extract concentration ranged from 25-1600 $\mu g.ml^{-1}$MeOH was mounted on the surface of the nutrient glucose agar medium and spread using sterilized glass road in Petri-dishes just after solidification. Disks, (4 mm Ø) of each of the three different tested fungal growth (7 days old) were mounted on the medium in the Petri-dishes. The plates were incubated at 25°±1 C for 7 days. Linear growth around the disks was measured and efficacy percentages were calculated referred to the control treatment.

3.2.d. Inhibition of spore & sclerotial germination:

The effect on the spore germination of tested fungi was determined .The spores of *F. o.* f. sp. *lycopersici* was collected from 10 days old cultures by gently brushing the surface of the growth in the presence of 10 ml sterile distilled water . Also, the sclerotia of both *R. solani and S. rolfsii* were collected from 10 days old cultures . The slide – germination fungicide bioassay technique described by Sharvrelle (1979) and adapted by Ashmawy (1997) was followed. A droplet (1µl) of the tested substance concentration (25,50,100 and 200 $mg.l^{-1}$/ Me OH), was mounted on the surface of the slide, using an adjustable micropipette then the droplets were allowed to dry. After drying, one 1µl of spore suspension of *F. o.* f. sp. *lycopersici* was placed on the slide on the substance residues and then the slides were incubated in a humidity chamber at 25±1ºC. Spore germination was microscopically examined tell we get a constant germination number. Spores put on sterilized water were counted as control treatment. Four replicates were used for each treatment. The percentages of spore germination were calculated for every replicate and treatment and the average was recorded .

Sclerotial germination of both *R. solani and S. rolfsii* was carried out by but 25 sclerotia in each Petri-dish containing nutrient glucose agar medium amended with the different concentrations of the tested extracts. The dishes were incubated at 25±1ºC. Sclerotia put on nutrient glucose agar medium without any extract were used as control treatment. Four replicates were used for each treatment. The percentages of sclerotial germination were calculated for every replicate and treatment and the average was recorded .

3.2.2. *In vivo* test of the tested fungi:

Tomato transplants of six weeks old (*Lycopersicon esculentum*) cv. super marmend were immersed in the different tested concentrations of pomegranate leaves extract separately for 10 min. before transplanting in infested soil with the tested fungi . Tomato transplants immersed in water only (without treatments) were also transplanted in infested soil and served as control treatment. Pots were consists of the following treatments:

1-*M. incognita*+ compound A , *P. penetrans*+ compound A and *R. reniformis* + compound A.
2-*F. o.* f.sp. *lycopersici* + compound A , *R. solani* + compound A and *S. rolfsii* + compound A .
3- *M . incognita*+ *F.o.* f.sp. *lycopersici*+ compound A .
4- *M. incognita*+ *R. solani*+ compound A.
5- *M. incognita*+ *S. rolfsii* + compound A .
6- Tomato with all infestation individuals without compound A.

The aforementioned treatments have been repeated with the other two isolated compounds, *i.e.* B and C. Each treatment was replicated three times. Pots were watered twice per week .Tomato seedlings were infested with nematodes by using 2000 freshly extracted juveniles that were poured around the roots of tomato transplants (7 days after transplanted).

Forty five days after transplanting the percentages of disease incident were recorded. Also, tomato roots were carefully pulled off and then washed to get rid of the adhering soil particles to determine height and weight of plants also the number of nematode larvae in 250 cm^3 of soil, number of galls, egg-masses, RF (Reproductive factor) and DS (Developmental stages) were determined according to Norton (1978).

Reproductive factor (RF) = $\frac{\text{No. eggs + developmental stages + Free nematode in soil}}{\text{Initial nematode population}}$

Developmental stages (DS) = number of developed juveniles (second, third and fourth stages) embedded in the roots.

All treatments of the experiments were statistically arranged in a complete randomize design according to Snedecor and Cochran (1989), where mean values were compared using L.S.D. at 5% level.

4. Preliminary phytochemical screening:

The preliminary screening of the defatted methanol extract for the following classes of phytoconstituents was preformed according to the methods described by (Farnsworth, 1966) using the indicated detection tests:

1- Saponins were identified by Froth test.
2- Sterols and/or triterpenoids were identified by Liebermann-Burchard test.
3- Tannins were identified by Ferric Chloride 5%.
4- Flavonoids were identified by Aluminium Chloride 5%.
5- Alkaloids were identified by modified Dragendorff's reagent.
6- Glycosides and / or Carbohydrates were identified by Molisch test.
7- Anthocyanins were identified by NH_3/ HCl.

5. Isolation and structure identification of the active compounds:

5.1. Analytical and preparative Thin Layer Chromatography (TLC):

Analytical and preparative TLC was carried out on Merck pre-coated silica gel plates (F254 thickness = 0.25 mm and 2.0 mm respectively) using the following Solvent systems.

1- n-Butanol - Acetic acid – Water (4:1:5) upper layer.
2-Ethyl acetate – Acetic acid – Formic acid – Water (100:11:11:27).
3-Chloroform – Methanol – Water (75:25:2, 70:30:5 and 65:35:2).
4-Chloroform – Methanol (80:20).
5-Dichloromethane – Methanol – Water (50:25:5).
6-Chloroform – Acetone (50:6).

Spots on TLC were detected under UV lights (254 and 365 nm) and / or by spraying with concentrated H2SO4 followed by heating at 105℃ for 5 min. and / or by Spraying with; $FeCl_3$ to detect tannins; $AlCl_3$ 5% to detect flavonoids and NH_3/HCl to detect anthocyanins. Sugars were detected by spraying with nphthoresorcinol phosphoric acid followed heating at 105°C for 10 min.

5.2. Isolation of the bioactive compounds:

The bioactive defatted methanol extract was subjected to the isolation of the antioxidant components as follows:

Twenty grams of the defatted methanol extract residue were loaded onto a chromatographic column (5 cm × 100 cm) packed with silica gel (800g, 230 – 400 mesh, Merck) and eluted with a gradient of chloroform : methanol : water (80:20:0, 75:25:2 and 70:30:5 V/V/V; 3L for each eluent). Thirty 100 ml fractions of each eluent were collected and analyzed by TLC. According to differences in composition monitored by TLC system ($CHCl_3$: MeOH: H_2O, 70:30:5), 12 fractions were obtained and then tested for antioxidant activity. The three most bioactive fractions No. 4, 5 and 8 were further purified as shown in (Fig.2) to give three compound designated as A, B and C. The purity of these compounds was established by the resolution of each one as a single spot in four different TLC solvent systems.

5.2.1. Structure identification of the purified compounds:

The purified compounds were characterized by detection tests (as mentioned previously in 3. Preliminary Phytochemical Screening), acid hydrolysis and spectroscopic methods.

5.2.1.1. Acid hydrolysis:

The purified compound to be hydrolyzed (2mg) was heated with aqueous 10% HCl (2ml) in a sealed tube at 100 °C water bath for 4 hours. The aglycone was extracted with diethyl ether and analyzed by TLC with chloroform – acetone (50:6). The aqueous layer was neutralized with N, Ndioctylamine (10% in $CHCl_3$). After evaporation to dryness, the sugars were identified by TLC with dichloromethane – methanol – water (50:25:5) by comparison with Rf of authentic samples.

5.2.1.2. Spectroscopic Methods:

5.2.1.3. Nuclear Magnetic Resonance (NMR) Spectroscopy:

^1H and ^{13}C NMR spectra were recorded in deuteron methanol (CD_3OD) on a varion Mercury VXR, 300 and 500 spectrometers (500 MHz for ^1H and 75 MHz ^{13}C) the chemical shifts (ppm) were related to the solvent. The spectroscopic NMR experiments were performed at the Nuclear Magnetic Resonance Laboratory, National Research Center, El-Dokki, Cairo and the Central Laboratory, Faculty of Science, Cairo University.

5.2.1.4. Mass Spectroscopy (MS):

Mass spectra were recorded on a GCMS. QP 1000 EX Shimadzu Mass spectrometer at 70e.v. The MS experiments were carried out at Biochemical Genetics Laboratory, National Research Center, El-Dokki, Cairo.

5.2.1.5. Ultraviolet Spectroscopy (UV):

The UV spectra were recorded on Cecil 3000 series spectrophotometer according to (Mabry *et al.*, 1970).

6. Statistical analysis:

Data were statistically analyzed using the standard procedures for complete randomize block and split designs as mentioned by Snedecor and Cochran (1989). The averages were compared at 5% level using least significant differences (L.S.D) according to Fisher (1948).

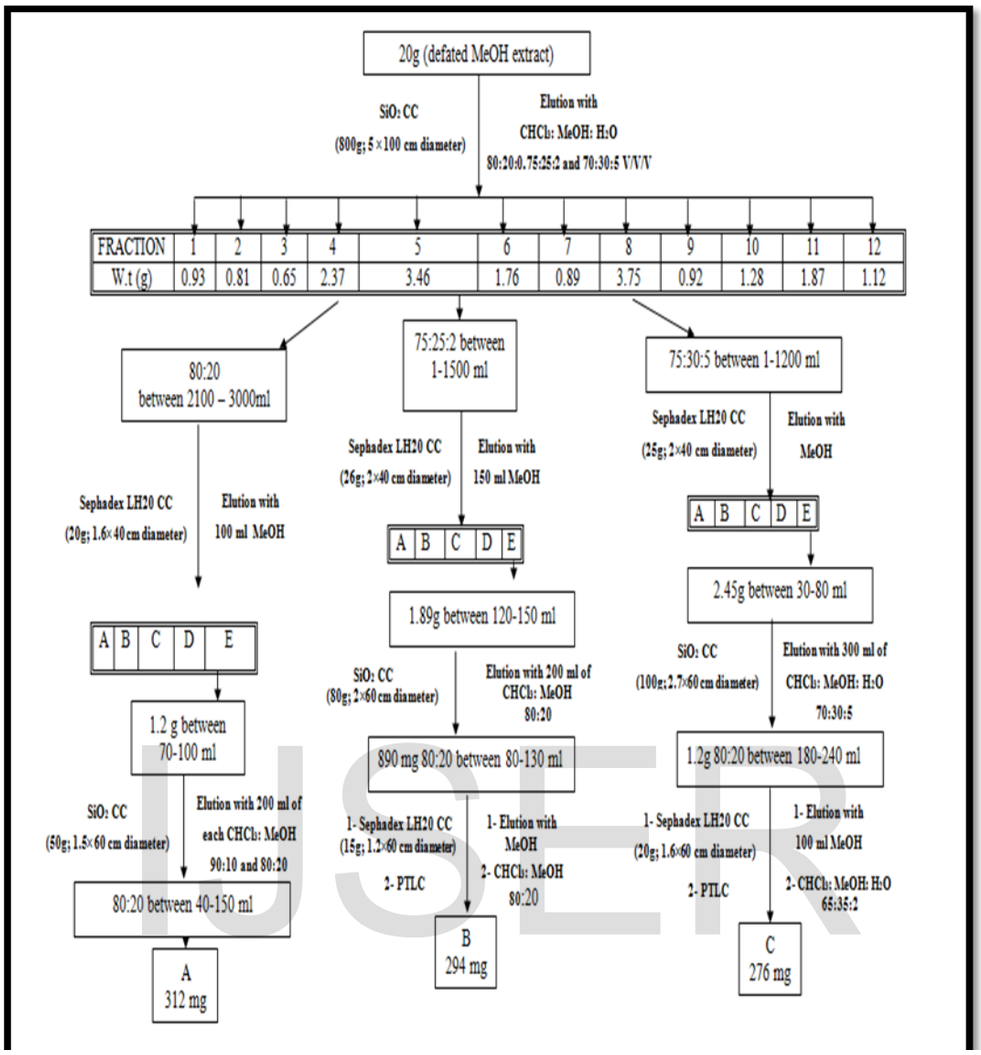

Fig. (2) Follow diagram for the isolation of the active compounds A-C.

3 RESULTS AND DISCUSSION

1- Fungicidal activity :

The antifungal efficacy of the defatted methanolic extract on the linear growth of the three well- known phytopathogenic fungi, *i.e. Fusarium oxysporum* f.sp. *lycopersici* , *Rhizoctonia solani* and *Sclerotium rolfsii* was studied *in vitro* and the results are shown in Table (1).

The obtained results show that the defatted methanol extract exhibited fungicidal activity against the three tested fungi at concentration ranging from 25 to 1600 ppm. It is , also, clear that the efficacy of the defatted methanol extract on the linear growth of the three tested fungi ,*i.e. F. o.* f.sp. *lycopersici*, *R. solani* and *S. rolfsii* was increased with increasing the concentration from 25 to 1600 $\mu g.ml^{-1}$. The percentage of inhibition of the tested fungi increased from 23.8, 28.8 and 16.3 to 83.8, 85.3 and 73.8%, respectively. The data obtained revealed that the defatted methanolic extract was more effective against the fungus *R. solani* than the other tested fungi. Recently several plant extracts have been reported as botanical fungicides against the three phytopathogenic fungi *F. oxysporum.* f.sp. *lycopersici*, *R. solani* and *S. rolfsii* (Mamdouh and Mohamed, 2007; Belal *et al.*, 2007; Jasso de Rodriguez *et al.*, 2007; Sharma and Kumar, 2009; Satish *et al.*, 2009; Emam *et al.*, 2010; Castillo *et al.*, 2010; Osorio *et al.*, 2010; Mahlo *et al.*, 2010; Bhardwaj, 2012; Hadian, 2012 and Plodpai *et al.*, 2013).

Table (1): *In vitro* fungicidal activity of the defatted methanol extract on the linear growth (mm.) of the tested fungi

Concentration ($\mu g.ml^{-1}$)	*F. oxysporum* f.sp. *lycopersici*		*R. solani*		*S. rolfsii*		Mean
	Linear growth	% Efficacy	Linear growth	% Efficacy	Linear growth	% Efficacy	
25	6.1	23.8	5.7	28.8	6.7	16.3	6.2
50	5.8	27.5	5.2	35.0	5.4	32.5	5.4
100	5.1	36.3	4.9	38.8	5.0	37.8	5.0

200	4.8	40.0	4.1	45.8	4.7	41.3	4.6
400	4.3	47.8	3.6	55.8	4.1	48.8	4.0
800	3.2	60.0	2.8	65.0	3.2	60.0	3.1
1600	1.3	83.8	1.6	85.3	2.1	73.8	1.7
Control	8.0	0.0	8.0	0.0	8.0	0.0	8.0
Mean	4.8	------	4.5	------	4.9	-----	

L.S.D. at 5 % for:
Concentration (C)=1.3 , Fungi (F)= n.s. and CxF= 3.4

2- Nematicidal activity :

The nematicide effect of the defatted methanol extract on the hatchability of eggs and mortality of juveniles of the three nematods *M. incognita*, *R. reniformis* and *P. penetrans* were studied *in vitro* and the results are shown in Table (2).

As shown in (Table 2) the methanolic extract exhibited larvacidal and ovacidal activities at concentration ranging from 400 to 1600 $\mu g.ml^{-1}$. In general, the extract with higher concentration i.e. 1600 $\mu g.ml^{-1}$ showed more activity when compared with lower concentration i.e. 400 $\mu g.ml^{-1}$. This observation showed that, an increase in concentration represent a supplementary input of different active compounds (Wabo *et al*., 2011). The data obtained also revealed that methanolic extract was more efficient against the juveniles than eggs as the % of inhibition at the same concentrations were the highest and more effective against the juveniles of *P. penetrans* than the other nematode species i.e. *M. incognita*, *R. reniformis* as the percentage of juvenile mortality was the highest at all tested concentration. During the last two decades various plant extracts have been reported as botanical nematicides against the three nematodes tested (Tiyagi and Alam, 1995; Begum *et al.*, 2000; Oka *et al.*, 2001; Tsay *et al.*, 2004; Ahmad *et al.*, 2004; Ibrahim *et al.*, 2006; Belal *et al.*, 2007; El-Badri *et al.*, 2008; Abo- Elyousr *et al.*, 2010; Hussain *et al.*, 2011; Kayani *et al.*, 2012).

Table (2): *In vitro* nematicidal activity of the defatted methanolic extract on the hatchability of eggs and juveniles mortality of nematodes.

Tested nematodes	Concentration ($\mu g.ml^{-1}$)	%Inhibition of egg hatching	% Mortality of juveniles
M. incognita	400	21	33
	800	40	51
	1600	56	83
R. reniformis	400	25	34
	800	41	58
	1600	55	74
P. penetrans	1600	N.t.	92
	800	N.t.	66
	400	N.t.	44
L.S.D. at 5 %		3.0	4.0

N.T. = Not tested.

The methanolic extract of *P. granatum* L. var. *nana* shown marvelous inhibitory effect against egg hatching and juvenile of test nematodes, which might be due to the presence of various secondary metabolites such as Flavonoids, Terpenoids, phenols…..etc in the plant sample. Some phytochemical have also been reported in Literature and possess nematicidal activity (Begum *et al.*, 2000; Oka *et al.*, 2000; Calvet *et al.*, 2001; Belal *et al.*, 2007 and Shakil *et al.*, 2008). Hence , this extract could be used for protection plant against the three nematodes tested which causes rot not and die back in various economically important crops such as vegetables, cotton, citrus..……etc. (Kayani *et al.*, 2012).

4. 2. Preliminary phytochemical screening:

Screening test for Saponins, Sterols, Triterpenoids, Tannins, Flavonoids, Anthocyanins and Alkaloids in the defatted methanolic extract of the dry powdered leaves of *Punica granatum* L. var. *nana* were made and results are given in Table (3), which reveals the presence of all the aforementioned phytoconstituents.

3 .Preliminary phytochemical screening:

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It is noteworthy that many plant derived compounds belong to the classes of secondary metabolites such as Saponins, Flavonoids, Tanins, Anthocyanins and Alkaloids have been used as botanical pesticides such as fungicides, insecticides and nematicides as well as natural antioxidant and hapatoprotective agents in the agricultural and medical fields. (Marston *et al.*, 1988, Begum *et al.*, 2000, Chatterjee, 2000; Mnaa *et al.*, 2008; Sudjaroen *et al.*, 2005; Feng *et al.*, 2010; Bhardwaj, 2012 and Abdel-Salam *et al.*, 2012).

Therefore, the biological activities of this plant may be attributed to the presence of a compound or more belongs to one or more of these classes of plant secondary metabolites.

Table (3): Phytochemical screening tests for constituents of *P. granatum* L. var.*nana* methanoliv extract.

Constituent	Detection test	Result
Saponins	Froth test	(+)
Sterols or Triterpenoids	Liebermann –Burchard test.	(+)
Tannins	Ferric Chloride 5%.	(+)
Flavonoids	Aluminium Chloride5%.	(+)
Alkaloids	Modified Dragendorff's reagent.	(+)
Glycosids	Molisch test	(+)
Anthocyanins	NH_3 / HCl	(+)

4. Structure identification of the antioxidant compounds:

Bioactivity guided separation of the antioxidant defatted methanol extract of the dried leaves of *P. granatum* L. var. *nana* by using column chromatography and preparative TLC as described in the Materials and Methods resulted in the isolation of three pure compounds designated as A, B, C. These compounds were identified as follows:-

4.1. Compound A :

Compound showed absorption maxima (λ_{max}) at 282 and 518 nm, which were in agreement with the absorbance pattern of cyanidin glycosides (Strack and Wray, 1989). The appearance of bathochromic shift upon the addition of 5% $AlCl_3$ or NaOAc reagents to the methanolic solution of this compound demonstrated the presence of Free ortho-dihydroxyl groups on the B-ring and free hydroxyl group on the A-ring of cyaniding skeleton (Harborne, 1958). The ^{13}C- NMR spectrum (Fig.5 and Table4) displayed 27 carbon signals, 15 of which were assigned to cyaniding aglycone, while the remaining 12 were due to two sugar units.

It was obtained as light red amorphous powder (312 mg; 1.56%) and gave a positive color with NH_3 / HCl on TLC suggesting it is an anthocyanin compound.

The ESI-MS spectrum (Fig. 3) showed molecular ion peak $[M-1]^+$ at m/z 610 which together with ^1H and ^{13}C- NMR spectroscopic data (Table 4 and Figs. 4 and 5) suggested that the molecular formula as $C_{27}H_{31}O_{16}$.

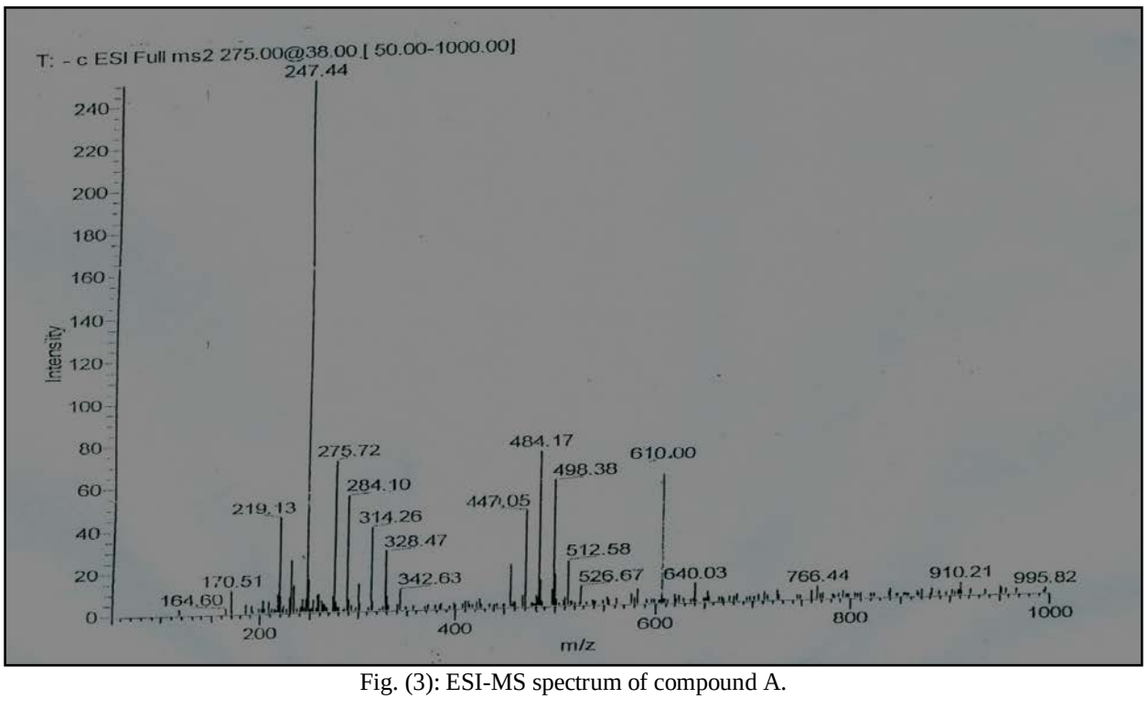

Fig. (3): ESI-MS spectrum of compound A.

Table (4) ^{1}H and ^{13}C – NMR Spectral data of compound A in CD_3OD.

Position	δ_C	δ_H
Aglycone moiety	-	-
1	-	-
2	162.9	-
3	144.0	-
4	136.0	7.7s
5	159.5	-
6	99.9	6.4d
7	166	-
8	95.5	6.9d
9	158.3	-
10	111.2	-
1'	120	-
2'	117.9	7.6d
3'	145.9	-
4'	150.1	-
5'	116.2	7.2d
6'	126.9	7.1dd
Sugar moiety		
1''	102.9	4.3d
2''	72.5	3.32m
3''	78.1	3.54m
4''	69.4	3.59m
5''	76.4	3.28m
6''	62.1	3.8m , 3.6m
1'''	103.8	4.5d
2'''	72.8	3.32m
3'''	77.9	3.54m
4'''	69.8	3.59m
5'''	76.1	3.28m
6'''	61.9	3.8m , 3.6m

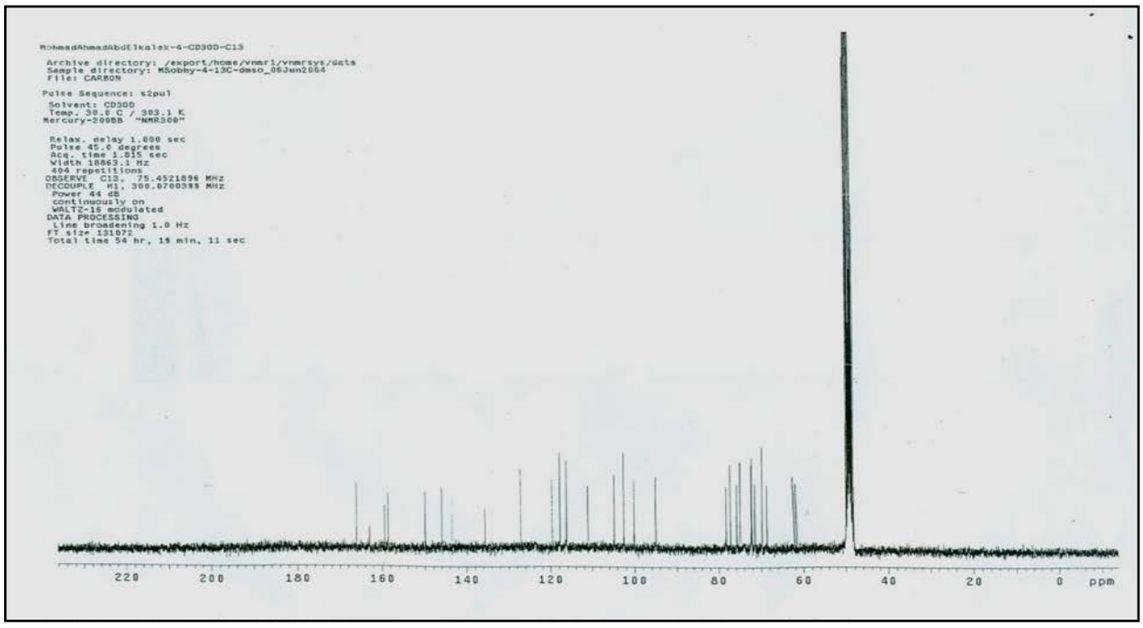

Fig. (4): H ^{1} –NMR spectrum of compound A in CD_3OD.

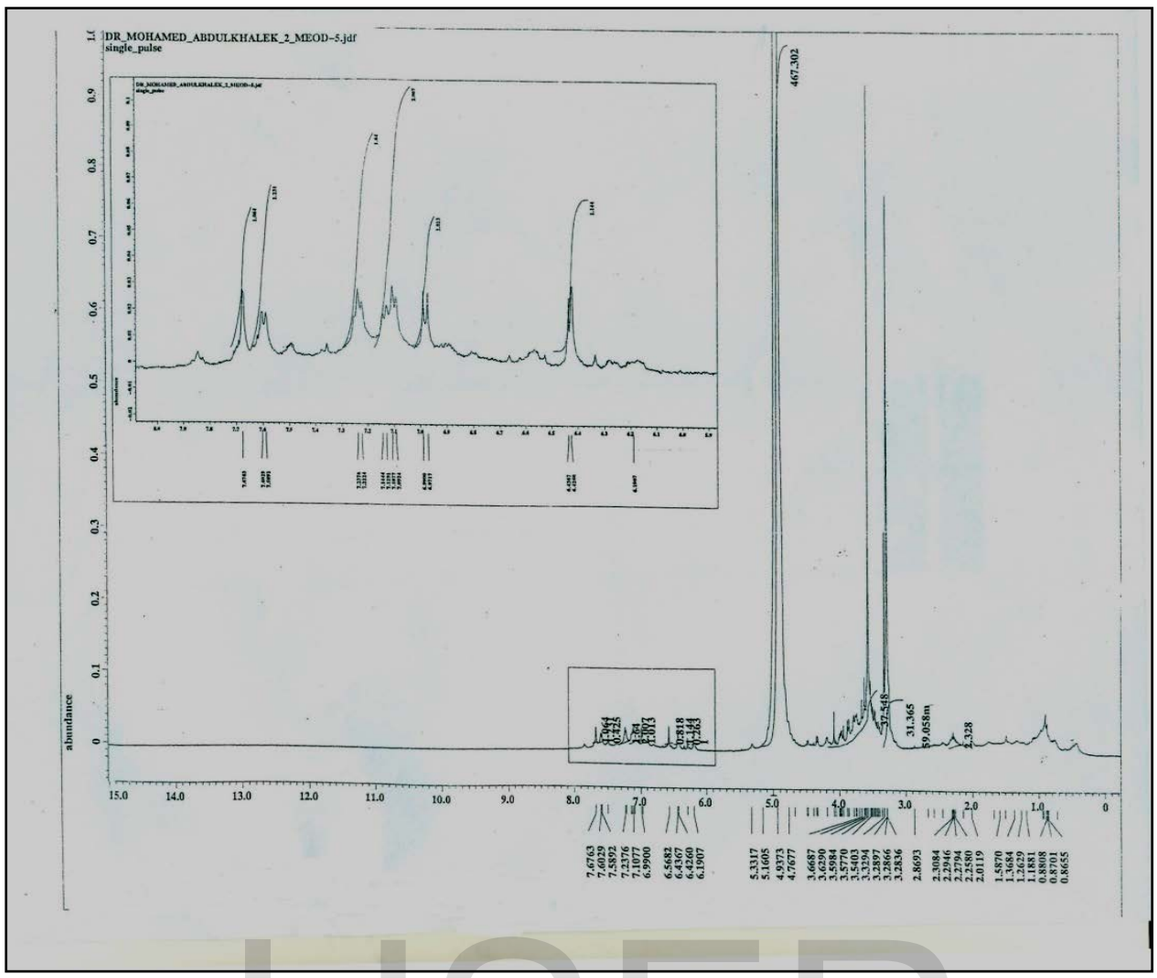

Fig. (5): 13C- NMR spectrum of compound A in CD3OD.

On acidic hydrolysis it gave D-glucose on TLC by direct comparison with authentic samples. The presence of two terminal *β*-D-glucopyranose in the molecule were established by the appearance of two anomeric proton signals in the ^{1}H-NMR spectrum (Fig.4) at δ 4.3 ppm (d,J=7.7Hz) and 4.5 ppm (d,J=7.7Hz) and proton signals between 3.28-3.8 ppm (m) and carbon signals in the ^{13}C- NMR spectrum (Fig.5 and Table 4) at 102.9, 72.5, 78.1, 69.4, 76.4 and 62.1 ppm (Glu1) and at 103.8, 72.8, 77.9, 69.8, 76.1 and 61.9 ppm (Glu2), in addition to the fragment ion peaks at 447.0 m/z (M-1-Glu) and 284 (M-1-2Glu) in the ESI-MS spectrum. The presence of cyaniding aglycone in the molecule was deduced from the ^{1}H and ^{13}C- NMR data of compound A, which included a one H singlet at δ 7.7 ppm, two 1H doublet at δ 7.6 and 7.2 ppm, a one H doublets of doublet at δ 7.1 ppm and two 1 H doublets at δ 6.9 and 6.4 ppm ascribed respectively to H-4, H-2^{-}, H-5^{-}, H-6^{-}, H-8 and H-6 Of cyanidin with their corresponding carbons resonating at δ 136.0, 117.9, 116.2, 126.9, 99.9 and 95.5 ppm (Table 4).

The carbon chemical shifts of the two sugars (*β*-D-glucopyranose) in the molecule (Table 4) indicated that there is no linkage between the two sugars and these sugars must occupy two different positions.

The positions of the two *β*-D-glucopyranose at C-3 and C-5 were established from the ^{13}C- NMR chemical shifts of the C-3 and C-5 positions at δ 144.0 and 159.5 ppm (Fig. 5 and Table 4) and the UV-visible spectral E_{440} / E_{vis} = 24.8% analysis (Harborne, 1958, Santagati *et al*,. 1984 and Hernandez *et al.*, 1999) as well as from mass spectrum (the fragment ion peak of 447(M-1-Glu) and 284(M-1-2Glu).

Thus the structure of compound A (Fig. 6) was characterized as cyanidin 3,5 di glucoside. This compound which isolated for the first time from *Punica granatum L. var. nana* is a known compound which was previously reported (Harborne 1958 ; Santagati *et al*., 1984 and Hernandez *et al*., 1999).

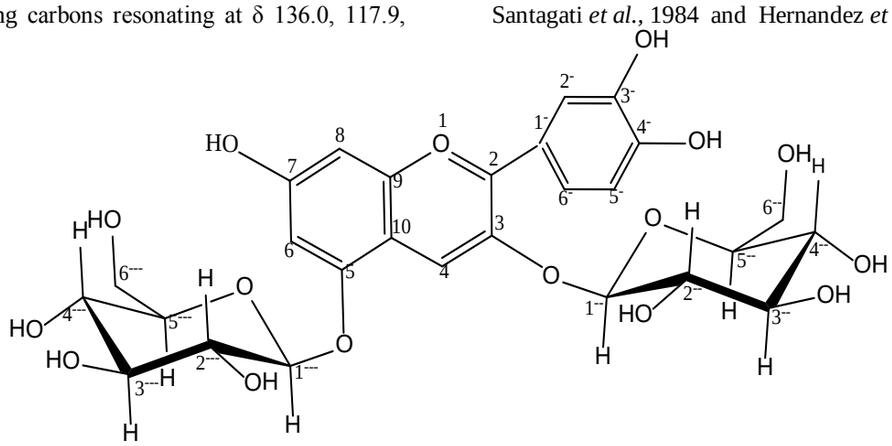

Fig. (6): Structure formula of compound A. Cyanidin 3,5-diglucoside ($C_{27}H_{31}O_{16}$).

4.2. Compound B:

It was obtained as a white amorphous powder (294 mg; 1.47 %) that gave positive color reaction with 5% $FeCl_3$ test on TLC suggesting it is a phenolic compound.

The electron spray ionization mass spectrometric (ESI-MS) spectrum of this compound (Fig. 7) showed a molecular ion peak $(M+1)^+$ at m/z of 333, which together with ^1H and ^{13}C- NMR spectroscopic data (Table 5 and Figs. 8 and 9) suggested that the molecular formula as $C_{13}H_{16}O_{10}$.

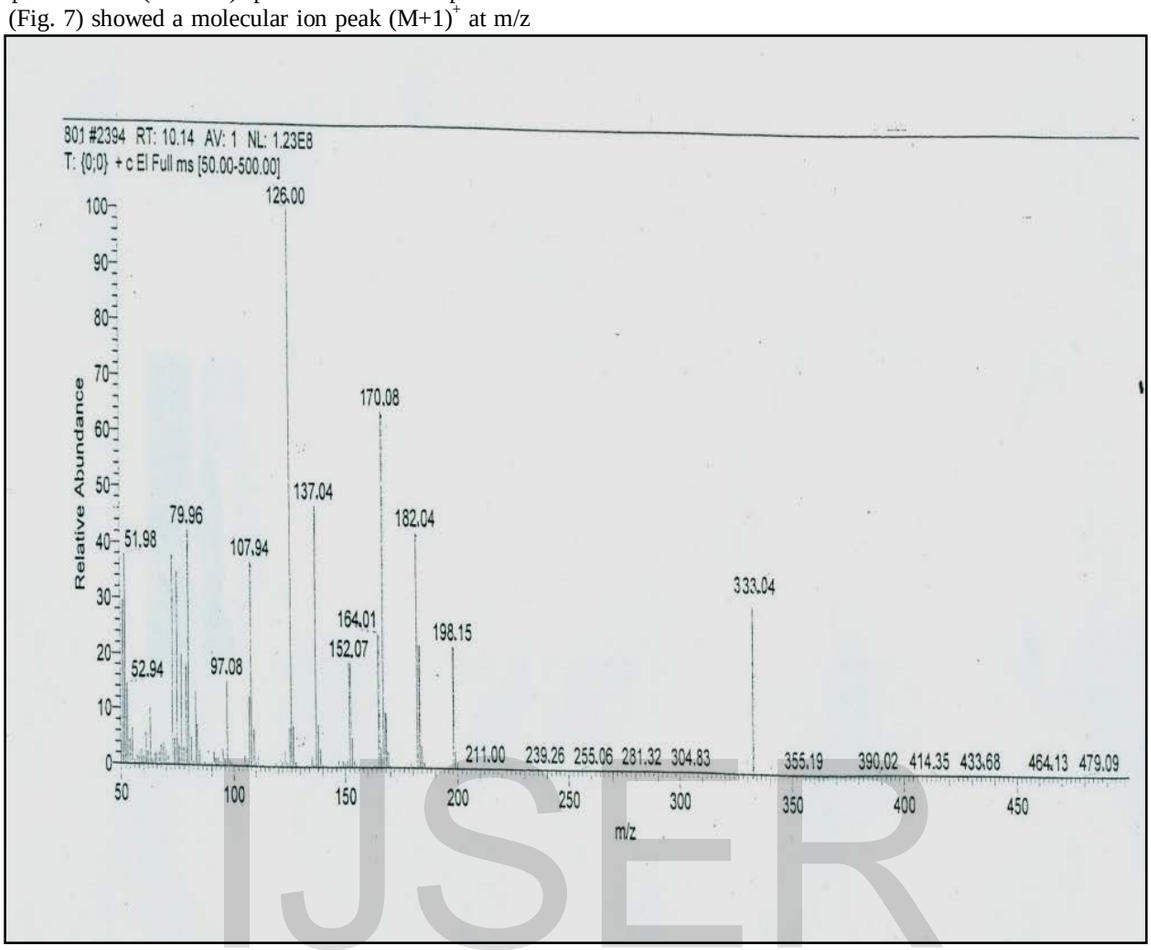

Fig. (7): ESI-MS spectrum of compound B.

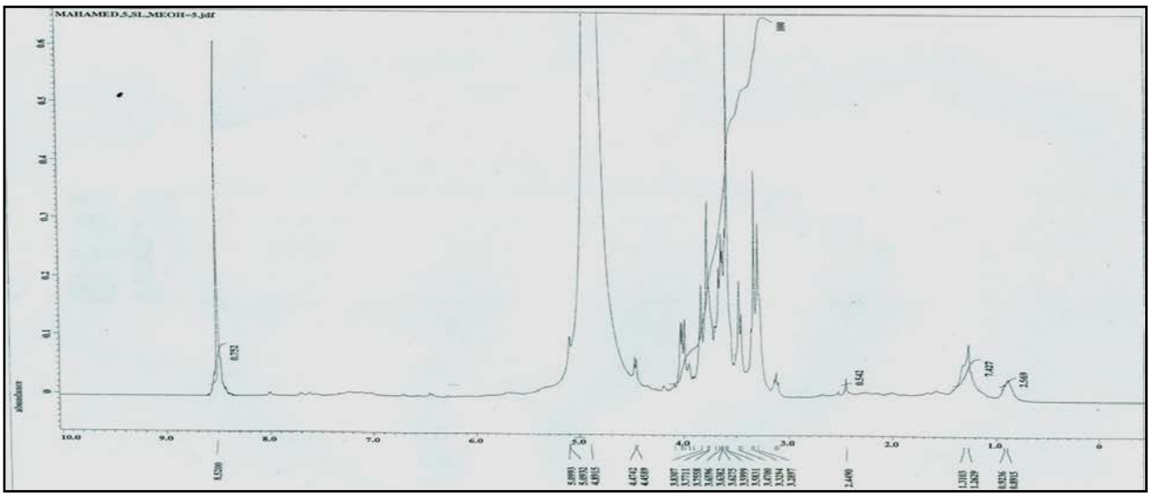

Fig. (8): ^1H- NMR spectrum of compound B in CD_3OD.

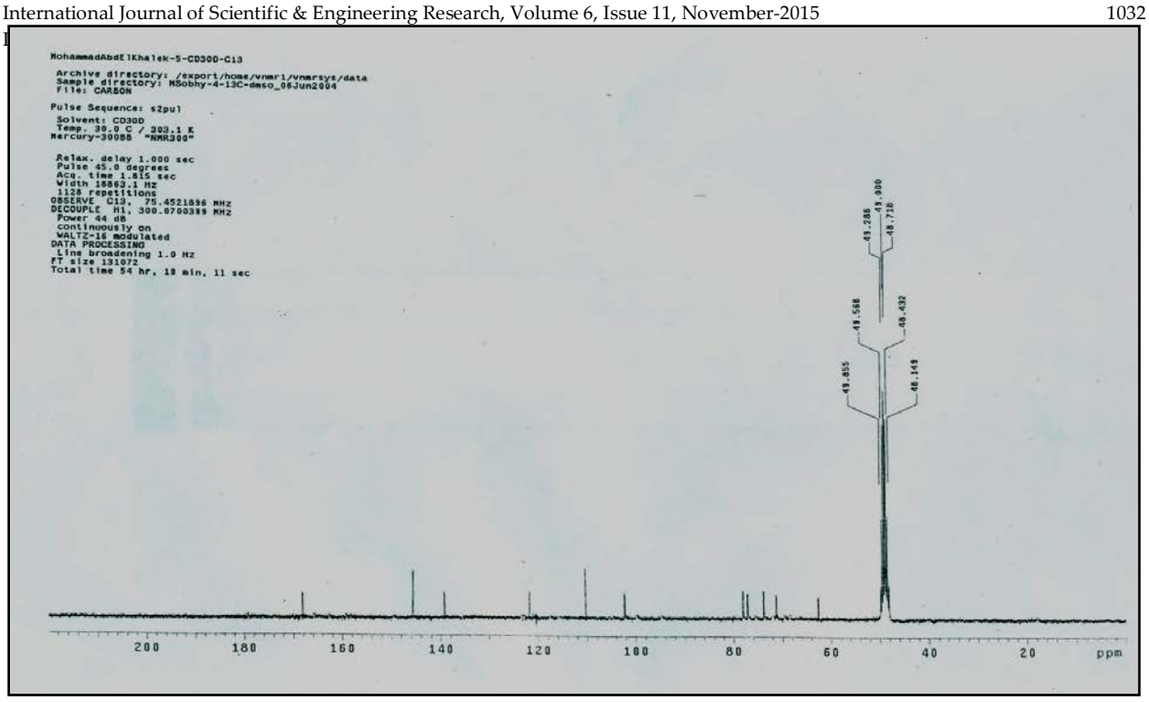

Fig. (9): ^{13}C- NMR spectrum of compound B in CD_3OD.

Table (5) ^{1}H and ^{13}C – NMR Spectral data of compound B and compound C in CD_3OD.

Compounds	Compound B		Compound C	
Position	δ_C	δ_H	δ_C	δ_H
Aglycone moiety	-	-	-	-
1	122.1	-	-	-
2	110.7	8.52s	157.3	-
3	145.9	-	117.4	-
4	138.9	-	177.2	-
5	146.9	-	143.6	6.5d
6	110.7	8.52s	164.6	8.0d
7	168.4	-	15.9	2.44s
Sugar moiety				
1'	102.3	4.74(d_J= 8.0Hz)	105.5	4.8d
2'	75.4	3.32- 3.65m	75.4	3.31m
3'	78.5	3.32- 3.65m	78.5	3.37m
4'	71.2	3.32- 3.65m	71.2	3.43m
5'	78.0	3.32m	78.0	3.25m
6'	62.6	3.80m , 3.64m	62.6	3.81m , 3.65m

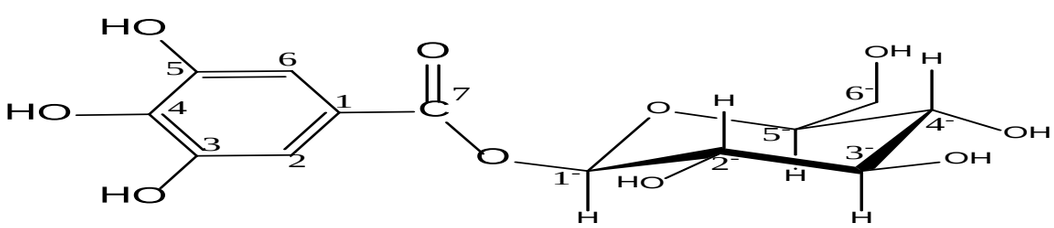

Fig. (10): Structure formula of compound B ($C_{13}H_{16}O_{10}$) Galloyl glucoside.

4.3. Compound C:

It was obtained as white fine powder (276 mg; 1.38%). The ESI-MS spectrum Fig. (11) showed molecular ion peak (M+1) at m/z 289 which together with ^{1}H and ^{13}C – NMR; **(Table 5 and Figs. 12 and 13)** suggested that molecular formula as $C_{12}H_{16}O_8$.

Acid hydrolysis of this compound afforded D-glucose as a sole sugar on TLC by direct comparison with an authentic samples. The presence of *β*-D-glucopyranose as the sole sugar moiety in the molecule was deduced from NMR and mass spectra **(Figs. 12 and 13)** by the appearance of only one doublet signal at δ 4.8 ppm with J= 7.4 Hz ascribed to anomeric proton and multiple signals at δ 3.25-3.81 ppm ascribed to five protons glucosyl in the ^{1}H-NMR spectrum with their corresponding carbons resonating at δ 105.5, 75.4, 78.5, 71.2, 78.0 and 62.6 ppm in the ^{13}C-NMR spectrum, in addition to the diagnostic fragment ion peaks of mass spectrum at m/z 164, 126 for sugar and aglycone moieties.

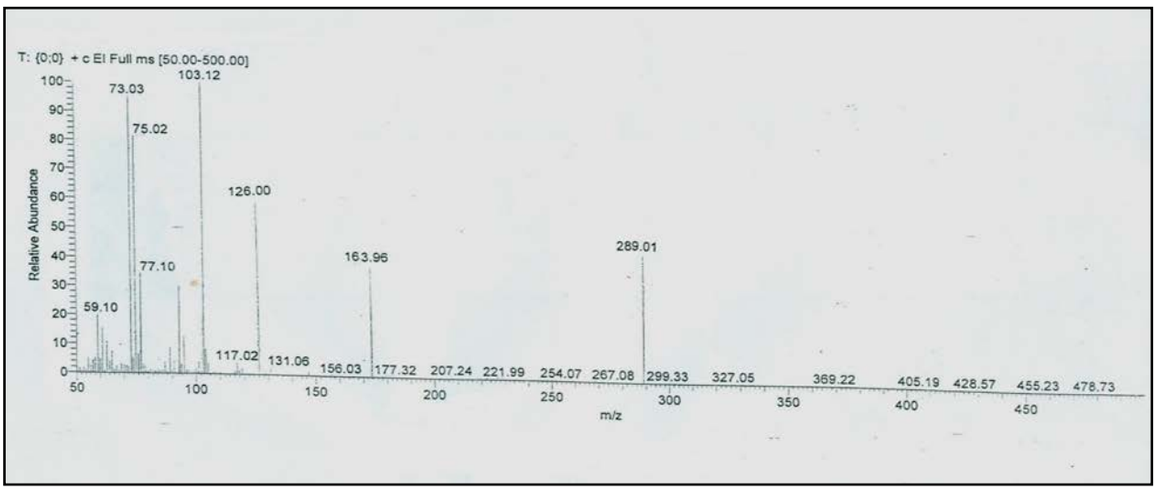

Fig. (11): Mass spectrum of compound C.

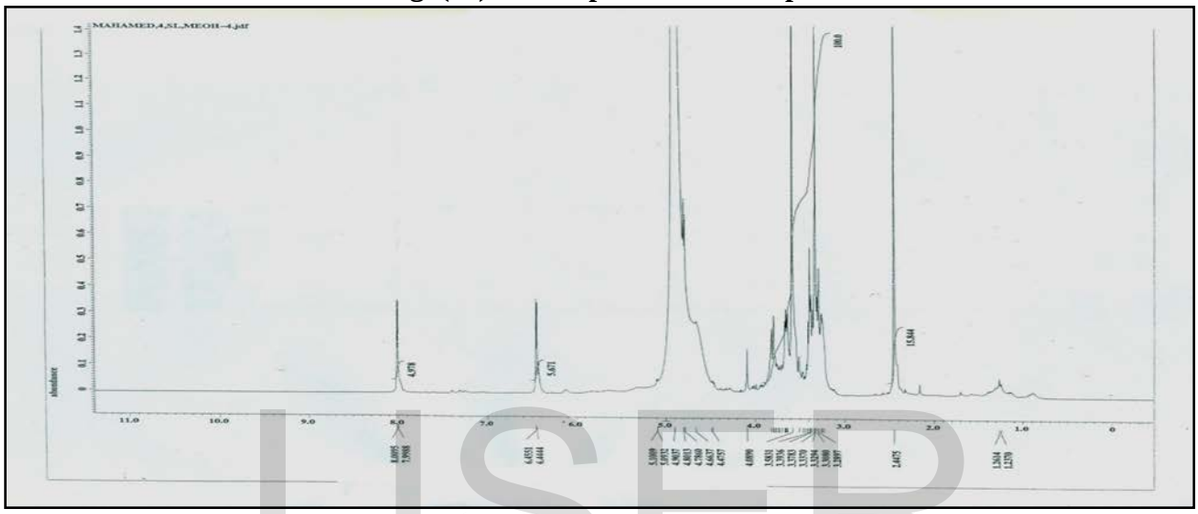

Fig.(12): ^{1}H- NMR spectrum of compound C in CD_3OD .

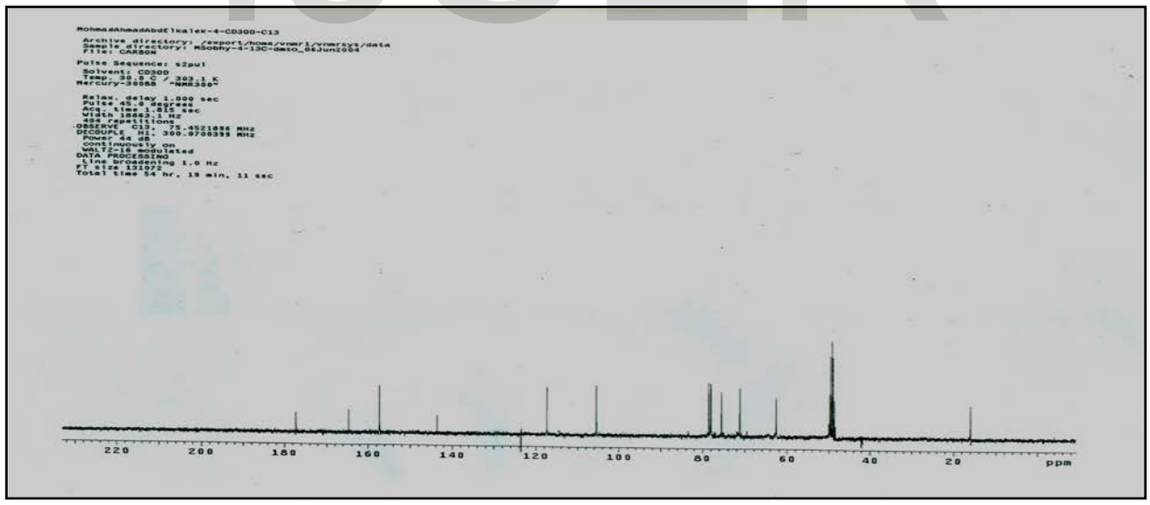

Fig. (13): ^{13}C- NMR spectrum of compound C in CD_3OD.

The ^{13}C-NMR spectrum **(Fig. 13)** displayed 12 carbon signals. Out of which 6 carbons accounted for the sugar moiety (Glu). The remaining 6 carbons were due to the aglycone moiety. In the aglycone part of the molecule, the ^{13}C-NMR spectrum revealed the presence of methyl group (C7; δ 15.9 ppm), three quaternary carbon including carbonyl group (C4; δ 177.2 ppm; CO) and two methine group including oxymethine (C6; δ 164.6 ppm) characteristic for 2,3 disubstitud pyran-4-one ring. This observation was further supported by the ^{1}H-NMR spectrum which exhibited signals for two doublets (each for 1H) centered at δ8.0 ppm (H6; J= 5.3Hz) and δ6.5 ppm (H5; J= 5.3Hz) and one singlet at δ2.44 ppm (H7; 3Hs) for methyl group. The glycosylation site at C-3 and the methyl group at C-2 were deduced from the chemical shifts of the C-2 and C-3 positions (δ117.4 and 157.3 ppm). On the basis of the above finding compound C (Fig.14) was identified as 2-methyl-pyran-4-one-3-O-β-D-glucopyranoside.

This compound which isolated here for the first time from *P. granatum* L. var. *nana* is a known compound recently isolated only from the leaves of *Punica granatum* (Balwani *et al.*, 2011). It is interesting to note that this is the first report about the antioxidant activity of this compound (IC50 = 5.8 µg.ml-1).

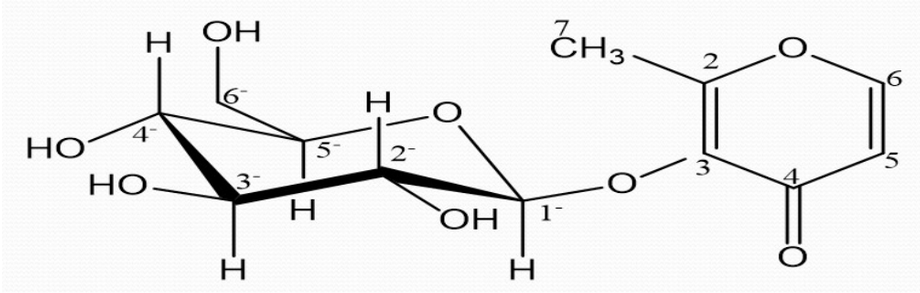

Fig. (14): Structure formula of compound C ($C_{12}H_{16}O_8$).

5. Effect of the tested extracted bionematicides on M. incognita*:*

The effect of the tested extracted bionematicides on *M. incognita* is showed in Table (7). Results illustrate that the tested extracted
bionematicides resulted in considerable decrease in the number of galls, egg-masses and juveniles in 250 cm^3 soil comparing with untreated control.

The highest concentration (200 ppm) gave the highest decrease in root- knot nematode galls, egg-masses and also juveniles , which recorded 20.0, 13.0 and 280 for compound (A) , 29.0,18.0 and 200 for compound (B) and 50.0, 19.0 and 480 for compound (C) comparing with 935.0, 625.0 and 1480 in untreated control, respectively

Table (7) Effect of the tested extracted bionematicides on *M. incognita* infecting tomato (cv. super marmend).

Treatments (ppm)	No. of galls /2 g. roots	No. of egg masses /2 gm roots	No. of juveniles in 250 cm^3 soil
A (25)	166	158	840
A (50)	83	73	610
A (100)	31	22	400
A (200)	20	13	280
B (25)	53	92	540
B (100)	40	34	380
B (50)	48	55	500
B (200)	29	18	200
C (25)	107	129	870
C (50)	88	114	720
C (100)	66	48	650
C (200)	50	19	480
Control	935	625	1480
LSD 5%	8.8	8.5	14.7

6. Effect of the tested extracted *bionematicides on R.reniformes :*

Effect of extracted compounds on tomato infected with *R. reniformes* show that increasing the concentration of the tested compounds led to decrease in egg-masses and individuals in soil, also the highest concentration of the three extracts was more effective than the low concentration (Table,8).

7. Effect of the tested extracted bionematicide on P. penetrans :

Effect of the tested extracted compounds on tomato infected with *P. penetrans* (Table, 9) show the same trend of above that increasing the concentration led to decrease in egg-masses and individuals in soil , also the highest concentrations of the three extractions were more effective than the others . The effect of extracted compounds was more effective on *P. penetrans* than the others two nematodes genera .

Table (8): Effect of the tested extracted bionematicides on *R.reniformes* infecting tomato (cv. Super marmend).

Treatments (ppm)	No. of egg masses /2 g roots	No. of juveniles in 250 cm^3 soil
A(25)	98	602
A(50)	72	309
A(100)	33	230
A(200)	10	170
B(25)	93	380
B(50)	70	243
B(100)	42	198
B(200)	15	92
C(25)	49	348
C(50)	38	215
C(100)	25	97
C(200)	9	42
Control	117	1260
LSD 5%	3.1	8.2

Table (9): Effect of the tested extracted bionematicide on *P. penetrans* infecting tomato (cv. Super marmend).

Treatments (ppm)	No. of eggs/ 5 g. roots	No. of juveniles in 250 cm^3 soil
A(25)	82	518
A(50)	39	342
A(100)	17	214
A(200)	9	173
B(25)	93	609
B(50)	45	431
B(100)	21	350
B(200)	11	216
C(25)	88	535
C(50)	52	406
C(100)	28	226
C(200)	7	138
Control	241	611
LSD at 0.5 %	4.7	6.8

8. Effect of the tested extracted biofungicides on the percentage of spore and sclerotial germination of the tested fungi:

Table,10) shows the antifungal activity of the three tested extracted compounds *in vitro* against the three tested fungi at concentrations of 0.0, 25, 50,100 and 200 $\mu g.ml^{-1}$. All the tested extracts had different degrees of antifungal activity against the tested fungi compared with the control. The highest antifungal activity was recorded for extract (A) at the concentration of 200 $\mu g.ml^{-1}$. Meanwhile, extract (B) at 200 $\mu g.ml^{-1}$, came in the second and the extract(C) came in last even when used at a high concentration. The extracts of *Punica granatum* L var. *.nana* could be recommended as a potent bio-fungicide against fungal plant diseases (Tehranifar *et al.*, 2011 and Al-Askar, 2012).

Table (10): Effect of the tested extracted biofungicides on the percentage of spore and sclerotial germination of the tested fungi .

Treatments (ppm)	% Inhibition of germination of					
	F. oxysporum		*R. solani*		*S. rolfsii*	
	Spores	**Efficacy%**	Sclerotia	**Efficacy%**	Sclerotia	**Efficacy%**
A(25)	61.2	22.72	31.2	67.29	68.4	23.83
A(50)	56.2	29.04	25	73.79	57.3	36.19
A(100)	37.5	52.56	12.5	86.89	18.5	79.39
A(200)	12.5	84.21	6.2	93.50	7.2	91.98
B(25)	68.7	13.26	50	47.58	69.4	22.71
B(50)	56.2	29.04	37.5	60.69	58.2	35.19
B(100)	43.7	44.82	12.5	86.89	38.7	56.90
B(200)	18.7	76.38	6.2	93.50	12.5	86.08
C(25)	73.4	7.32	78.2	18.02	62.5	30.40
C(50)	62.5	21.08	12.5	86.89	43.7	51.34
C(100)	37.5	52.56	6.2	93.50	18.7	79.17
C(200)	25	68.43	0.0	100	12.5	86.08
Control	79.2	---------	95.4	---------	89.8	---------
LSD at 5%	3.1	---------	3.9	---------	3.5	---------

9. Effect of the tested bioactive components on the interaction between *M. incognita* and *F. o.* f.sp. *lycopersici* :

Data presented in Table (11) reveal that the tested bioactive components (A, B and C) of *Punica granatum* L. var. *nana* resulted in increasing plant weight and length and reducing number of nematodes / 250 cm^3, galls , egg mases and developmental stage as well as the infection by *F. o.* f.sp. *lycopersici* when tomato plants grown in soil infested with both *M. incognita* and *F. o.* f.sp. *lycopersici* . This effect was increased by increasing the concentration of the bioactive components of pomegrate from 100 to 200 ppm. On the other hand, tomato plants grown in soil unamended with the bioactive components of pomegrate and infested with any of M. incognita and *F. o.* f.sp. *lycopersici* and their combination showed low value of plant weight and length and great increase in of the estimated critiria of number of nematodes / 250 cm3, galls , egg mases and developmental stage as well as the infection by *F. o.* f.sp. *lycopersici.*

The highest effect refer to the treatment with compounds A and C, which gave no disease incidence with Fusarium interaction with *M. incognita* . Whereas, the percentage of disease incidence increased to 60% without treatments and 45% in case of the infection with Fusarium alone . Moreover, the effect on nematode infection was decreased clearly in galls , egg-masses and developmental stages at the highest concentration .Meanwhile, the effect was differed among the three extracted compound .

10. Effect of the tested bioactive components on the interaction between *M. incognita* and *R.solani:*

Data presented in Table (12) show that the tested bioactive components (A,B and C) resulted in increasing plant weight and length and reducing number of nematodes / 250 cm^3, galls , egg mases and developmental stage as well as the infection by *R.solani* when tomato plants grown in soil infested with both *M. incognita* and *R.solani* . This effect was increased by increasing the concentration of the bioactive components of pomegrate from 100 to 200 ppm. On the other hand, tomato plants grown in soil unamended with the bioactive components of pomegrate and infested with any of *M. incognita* and *R.solani* and their combination showed low value of plant weight and length and great increase in the estimated critiria of number of nematodes / 250 cm^3, galls , egg mases and developmental stage as well as the infection by *R.solani*.

The highest effect refer to the treatment with compounds C, A and B, which gave 2.0 , 3.0 and 5.0% disease incidence with Rhizoctonia interaction with *M.incognita* , respectively . The infection with the two pathogens (nematode + fungus) gave 78% disease incidence and 45% in case of the infection with Fusarium alone. The effect on nematode infection was decreased clearly in galls , egg-masses and developmental stages at highest concentration(200 ppm), which recorded 20.0 ,8.0 and 6.0 , respectively in treatment of compound (A). Whereas, it recorded 91.0,68.0 and 41.0 in case of the infection with the nematode alone.

Table (11): Effect of the tested bioactive components on the interaction between *M. incognita* and *F. o.* f.sp. *lycopersici* on weight and height of tomato plants, No. of nematodes per 250 cm^3 , egg-masses , developmental stages of the nematode and disease incidence by the fungus .

Treatments (ppm)	P.w.(g)	P.h.(cm)	No./250cm^3	Galls	E.m.	D.s	D.I.F %
A(100)	24.3	23.0	490	43.0	27.0	15.0	10.0
A(200)	25.7	23.4	300	22.0	15.0	9.0	0.0
B(100)	28.2	22.7	518	55.0	40.0	24.0	15.0
B(200)	27.8	24.5	409	31.0	23.0	18	1.0
C(100)	25.2	23.8	341	28.0	30.0	20.0	11.0
C(200)	29.7	26.4	292	51.0	18.0	12.0	0.0
Nematode alone	15.0	17.0	1300	91.0	68.0	41.0	-
Fungus alone	16.2	17.5	-	-	-	-	45.0
Nema.+fungus	12.4	15.7	2090	310	212.0	98.0	60.0

P.w = Plant weight . P.l = Plant height, No. = No. of nematodes per 250 cm^3, E.m =Egg-masses , D.s =Developmental stages, D.I.F =Disease incidence by *F. o.*f.sp. *lycopersici.*

Table (12): Effect of the tested bioactive components on the interaction between *M. incognita* and *R.solani* on weight and height of tomato plants,No. of nematodes per 250 cm^3 , egg-masses , developmental stages of the nematode and disease incidence by the fungus .

Treatments at (ppm)	P.w.(g)	P.h.(cm)	No./250cm^3	Galls	E.m.	D.s	D.I.R %
A(100)	21.6	20.0	340	33.0	19.0	13.0	15.0
A(200)	23.5	20.3	190	20.0	8.0	6.0	3.0
B(100)	20.2	17.8	226	35.0	32.0	18.0	13.0
B(200)	23.6	19.7	201	21.0	13.0	16.0	5.0
C(100)	23.2	22.3	307	33	25.0	17.0	18.0
C(200)	24.7	26.0	200	23	13.0	9.0	2.0
Nematode alone	15.0	17.0	1300	91	68.0	41.0	-
Fungus alone	12.6	13.0	-	-	-	-	65.0
Nema.+fungus	12.4	15.2	1580	211	34.0	54.0	78.0

P.w =Plant weight , P.l = Plant height , No. = No. of nematodes per 250 cm3 , E.m = egg-masses ,D.s =Developmental stages , % D.I.R= Disease incidence by *R.solani* .

11. Effect of the tested bioactive components on the interaction between *M. incognita* and *S. rolfsii*:

Data presented in Table (13) reveal that the bioactive components of *Punica granatum* L. var. *nana* resulted in increasing plant weight and length and reducing number of nematodes / 250 cm^3, galls , egg mases and developmental stage as well as the infection by *S.rolfsii* when tomato plants grown in soil infested with both *M. incognita* and *S. rolfsii* . This effect was increased by increasing the concentration of bioactive components of pomegrate from 100 to 200 ppm. On the other hand, tomato plants grown in soil unamended with the bioactive components of pomegrate and infested with each of *M. incognita* and *S. Rolfsii* and their combination showed low value of plant weight and length and great increase in the estimated critiria of number of nematodes / 250 cm^3, galls , egg mases and developmental stage as well as the infection by *S.rolfsii*.

The highest effect refer to the treatment with compounds C, A and B at 200 ppm, which reduced disease incidence in the interaction between Sclerotium and *M. incognita* to 7.0 , 8.0 and 9.0%, respectively . The percentage of disease incidence increased to 78% without any treatments and 65% in case of infection with *S.rolfsii* alone . Moreover, the effect on nematode infection was decreased clearly in galls , egg-masses and developmental stages at the highest concentration.

Table (13): Effect of the tested bioactive components on the interaction between *M. incognita* and *S. rolfsii* on weight and height of tomato plants, No. of nematodes per 250 cm^3, egg-masses , developmental stages of the nematode and disease incidence by the fungus.

Treatments at (ppm)	P.w.(g)	P.h.(cm)	No. /250 cm^3	Galls	E.m.	Ds.	D.I.S%
A (100)	25.5	24.1	460	51.0	31.0	22.0	13.0
A (200)	28.2	21.4	240	30.0	18.0	15.0	9.0
B (100)	24.2	29.4	392	65.0	35.0	41.0	13.0
B (200)	26.3	27.3	321	31.0	23.0	23 .0	8.0
C (100)	22.6	21.4	407	43.0	29.0	24.0	18.0
C (200)	25.1	28.5	337	28.0	18.0	12.0	7.0
Nematode alone	15.0	17.0	1300	91.0	68.0	41.0	-
Fungus alone	12.6	13.0	-	-	-	-	65.0
Nema.+fungus	12.4	15.2	1580	211.0	34.0	54.0	78.0

P.w= Plant weight , P.h = Plant height , No. = No. of nematodes per 250 cm3, E.m =egg-masses , D.s =Developmental stages , D.I.S.= Disease incidence by *S. rolfsii*

It has been found the bioactive components of *Punica granatum* L. var. *nana* resulted in increasing plant weight and length and reducing number of nematodes / 250 cm3, galls , egg masses and developmental stage as well as the infection any of the three tested fungi when tomato plants grown in soil infested with *M. incognita* and any of the tested fungi. This effect was increased by increasing the concentration of the tested bioactive components from 100 to 200 ppm. On the other hand, tomato plants grown in soil unamended with the bioactive components of pomegrate and infested with each of *M. incognita* and the three tested fungi and their combination showed low value of plant weight and height and great increase in the estimated criteria of number of nematodes / 250 cm3, galls , egg masses and developmental stage as well as the infection by any of these fungi.

The mode of action of the tested bioactive components of *Punica granatum* L. var. *nana* may be due to their direct toxic effect , activation of enzymes responsible for disease resistance and / or induced systemic acquired resistance to both nematode and fungi (Sudheesh *et al.* ,2005; Ibrahim *et al.*,2006; Tehranifar *et al.*,2011 and Abd El- Salam *et al.*(2012). Some phytochemical have also been reported in Literature and possess nematicidal activity (Begum *et al.*, 2000; Oka *et al.*, 2000; Calvet *et al.*, 2001; Belal *et al.*, 2007 and Shakil *et al.*, 2008).

The obtained data are of great interest , where the bioactive components of *Punica granatum* L. var. *nana* could be used as an alternative safe trial for managing the

infection by nematode and soil borne fungi . The obtained results are in accordance with the obtained data by different plant extracts by Hernandez *et al*. (1999); Osorio *et al*. (2010) and Abd El- Salam *et al*.(2012).

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