Research Article

# Nematicidal Activity of Withanolides from *Withania coagulans* (Stocks) Dunal against *Meloidogyne incognita* (Tylenchida: Heteroderidae)

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**Abstract** | Plants are the one of the magnificent and captivating source of natural bio-pesticides. The currentstudy was performed for phytochemical analysis and to assess the nematicidal and nematostatic potential of crude extract of *Withania coagulans*. It is known for its withanolide class of chemical compounds. The biological assessment of two primary fractions of *W. coagulans* crude extract, chloroform and butanol, is the focus of the current study. Crude extracts were tested against *Meloidogyne incognitain vitro* conditions. The second stage juveniles ($J_2$) were exposed at 24, 48 and 72 hours in different concentrations of plant extracts. During *in-vitro* condition inhibition of egg hatchability and $J_2$ juveniles' mortality varied according to the concentration of plant extract. WC=1 showed 100% mortality after 48 hrs of exposure at 1% with the lethal concentration ($LC_{50}$) values 0.168 mg/land all four concentration are 100 % toxic at 72 h of exposure. W-56D proved to be 100 % lethal at 0.1 and 0.5 % concentration after 48 h of exposure with ($LC_{50}$) 0.065 mg/l. The lowest mortality was found from WC-3 and the mortality rate was found to be 30-50 %with ($LC_{50}$) values 0.135 mg/l, 0.187 mg/land 0.145 mg/lafter 24, 48 and 72 hrs of exposure. Compound C1 also proved to havehigh level of virulent effect rangingbetween 75 – 100 %. The isolated compounds were analysed through spectroscopy methods such as Fourier-transform infrared spectroscopy (FTIR), Ultraviolet–visible spectroscopy (UV), Mass spectrometry (MS), and Nuclear Magnetic Resonance (NMR). The structure of isolated chemical components employing bioassay-directed fractionation of both extracts. Concentration of the extract was directly proportional to the mortality of second stage juveniles, the resulted, six withanolides comprising coagulin E, G and J, withaperuvin C and triterpenoid, betulinic acid and the pure isolated bio active constituents also showed the 100% significance mortality with the constant concentration of 1 and 0.5 mg/lagainst the *M. incognita*. The present study revealed that withanolides compounds efficiently controlled and reduced population of root- knot nematodes.

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## Introduction

Plants are a major source of naturally occurring pesticides that are proven to use as harmless alternatives to chemical pesticides (Ujvary, 2002). Plants and their extracts are being used by around three-quarters of the world's population for health management (Premanathan *et al*., 2000; Gabhe *et al*., 2006). Plant extracts comprising volatile chemicals constituents, specially essential oils (Brown and Morra, 1997), have been reported to show in secticidal, antibacterial, and nematicidal activity (Digrak *et al*., 1999; Okoko *et al*., 1999). Root-knot nematode *Meloidogyne incognita* Kofoid and White (Chitwood) (Tylenchida: Heteroderidae) is most important plant parasitic nematode distributed worldwide that affectsthe quality and quantity of the crops produce in terms of financial break, especially in developing countries (Ismail *et al*., 2021). Promising chemical control strategies including nematicidestemik, aldicarb and furadonare harmful andhave toxic side effects;therefore, they areeither banned and restrictedor at least discouraged to be appliedin agricultural fields. Owingto security and ecological concern, alternative substitutes have a potential to suppress agricultural pests and are not toxic to human cells. Such as use of botanical pesticides into integrated pest control systems will decrease the use of synthetic pesticides that would otherwise be unneeded. This approach has a considerable influence in natural products based nematicides by screening naturally occurring chemical bio-compounds identified as plant secondary metabolites (Huang *et al*., 2019; Montiel-Rozas *et al*., 2018). Keeping in view the need of our country, bio-pesticides from the natural sources are safe and ecofriendlybiocontrol alternative. This study was focused on the isolation of chemical constituents of bioactive compounds of *Withania coagulans* Dun. (Stock.). The major chemical constituent of *W. coagulans* is withanolides with extensive kind of pharmacological properties (Saima *et al*., 2020; Maryam *et al*., 2012). *W. coagulans* has been selected on the basis of their nematicidal activities in preliminary bioassay (Nadra *et al*., 2019).

## Materials and Methods

*Plant collection*

*W. coagulanse* (Whole plant) was collected from Hub (25.0265° N, 66.8860° E), Balochistan, Pakistan, identified by Dr. Sher Wali Khan, plant taxonomist, voucher specimen No. 46257, deposited in herbarium of Department of Botany, University of Karachi, Karachi Pakistan.

Plant extracts: *W. coagulans* (4 kg) plant was soaked in methanol solvent for 48 h, then filtered and evaporated on a high vacuum rotary evaporator. The plant's crude gummy material (about 140 g) was obtained and dissolved in distilled water to make a suspension; then, a fractionation method was carried out on the basis of solvent polarity. The organic solvents utilized in this procedure were n-hexane, chloroform (CHCl3), ethyl acetate (EtOAc), and n-butanol (BuOH). The polarity order of organic solvent that were used in this procedure are n-hexane, chloroform (CHCl3), ethyl acetate (EtOAc), n-butanol (BuOH).

*Experimental procedure*

Ultraviolet visible spectroscopy was calculated as lambda max $\lambda_{max}$ nm on a UV 240 Shimadzu in methanol solutions. Infrared spectroscopy was recorded with the help of JASCO A-302-spectrometer and a spectrum was obtained in cm-1 as KBr discs. 1H-NMR (400 MHz) and 13C-NMR (100 MHz), spectrum was performed on a Bruker AV-500 machine with Deuterated methanol solvent, and the data is given in (ppm). Two dimensional nuclear magnetic resonance spectroscopes were trace on a NMR (Bruker AMX 500) spectrometer. EI-MS mass spectrometry data were recorded on a MAT-312 instruments, (Finnigan MAT-112), at 70 eV and main fragment ions are presented as m/z (%). (TLC). Thin layer chromatography was achieved on prepared silica gel cards in 254 nm UV of E. Merck. The all pure compounds were purified on RPHPLC, JAI, LC908W, Japan Analytical Industry Co. Ltd. was used with a ODS H-80 or L-80 column (YMC, Japan). Chromatography was accomplished on silica gel different column silica column M mesh), MPLC column chromatographychromatography(E. Merck, type 70-230 and 230-400 (Silica Wallogel C-300 HG) reversed phase column chromatography were performed on Diaion HP-20 resin Polyamide, and final purification and separation was achieved on HPLC (RPHLC) recycling preparative (JAILC-908 W, Japan Analytical Industry).

*Extract preparation*

The CHCl3 fractions werepreprared on the basis of three different pH (pH = 3, 7 and 10). The chloroform crude fraction was obtained on (pH-3)

(19.0 g) was loaded on flash silica gel with solvent system (chloroform/hexane). Three major fractions (WC =1, 2, and 3), the mobile system of column chromatography was 100%, 50% CHCl3/ hexane and 75% CHCl3 / n-hexane. Fraction-2 (MC= 2) 50% chloroform/ hexane (7.3g) repeated silica gel column chromatography to elute different fractions (Fr. 1-7). Fraction 1 (3.3 g) was subjected to column chromatography and fractionated on normal phase recycling high performance liquid chromatography (HPLC) and yielded this 5 fraction (HPLC-A-E) and then purified on thin layer chromatography; this led to the isolation of compounds 1-4. The CHCl3 fraction (WC=4) was further subjected to polyamide column chromatography, and eluted with Aceton/CHCl3. Four sub fractions were obtained by polyamide column chromatography. The eluted fraction WC=4A (20% Aceton/CHCl3) (4.8 g) was loaded to a silica gel column, and five main fraction (Fr = WCpH-A-E) were obtained. Fraction WC-pH-B (25mg) was further fractionated on normal phase recycling HPLC (20% isopropyl alcohol/ hexan) and obtained five fractions. Then repeated separately pencil column chromatography on silica gel, with 8% CHCl3/Hexan and resulted compound 5 was obtained.

*Nematicidal toxicity*

The seven withanolides compounds were used to evaluate the nematicidal toxicity against *Meloidogyne incognita* second-stage juvenile ($J_2$) which was obtained from pure culture on tomato plants (*Solanumlycopersicum*) grown in a green house. The egg-masseswereextracted and kept in glass cavity block with fresh water incubated for egg hatching at ambient temperature for 72 h. For treatments 100 juveniles ($J_2$) freshly hatched were kept in 3 × 3 glass cavity block separately for each concentration, compounds and replicates. Entire experiment was repeated with four replicates. The stock solution of plant extract was prepared by using 10 mg/ml with 5% Dimethyl sulfoxide (DMSO). The experiment was performed at 1%, 0.5%, 0.25 % and 0.125%concentrations at a rate of 1 mlper each cavity block. DMSO (5%) alone and spring water was used as a control. The percentage death rate was measured using a stereoscopic microscope after every interval of 24, 48, and 72 hours. Nematodes were examined after completing the experimental period that were found dead once no movement was observed after mechanical nudge by using sterilized needle.

*Data analysis*

Nematicidal special effects were assessedby ANOVA multifactor analysis of variance; if the ANOVA was significant, differences in treatments were calculated through Duncan's multiple range test ($P< 0.05$) using statistical software SPSS. The lethal concentration and confidential limit values were evaluated with probit analysis PROC PROBIT routine of (SAS, 2000).

## Results and Discussion

The root-knot nematodes are one of the most significant obstacles to the production of adequate food resources (Hemlata and Jyoti, 2018). Natural isolated compounds from the plants, play a vital role intended for the management of damage caused by RKN nematodes. Expensive synthetic chemical nematicides have been used for the protection of plants from the nematodes; on the other hand, theyhave very high risk and toxic effects for the environmental and human health, for controlling agricultural pest these synthetic chemical have expected alarming range (Adegbite and Adesyan, 2005).The alternative route is bio chemical compounds can act as a potential, safe and inexpensive (Javed *et al.*, 2007). The medicinal biochemical constituents of plants act as effective replacements for nematode caused losses. Many plants have been evaluated and recognizedas the sources of nematicidal constituents (Dawar *et al.*, 2008). This study was intended to evaluate the nematicidal prospective of medicinal plant *W. coagulans* against *M. incognita*. Seven pure compounds were evaluated inthis research project. Withanolide and butalinic acid appeared to be the most active against *M. incognita*. Compound-1, 3 (WC=1), (W-56D) showed potential effect as it caused 90-100% mortality of the nematode after exposure of48-72hours. Compound -2 (WEA=11) was found to be 77-97% mortality against the nematicidal assay, as the other withanolides also showed the great efficiency 92-100% and 72-90% mortality after the exposure time.

*Isolation of pure bio active chemical constituents from Withania coagulans*

The phytochemical study was carried out on the methanolic extract of the whole plants of *Withania coagulans* leading to six withanolides (1-6) and one Betulinic acid (7) (Chandramu *et al.*, 2003) that have been isolated and structurally characterized. This procedure was followed with the reported data of previously isolated withanolides compounds (Saima

*et al*., 2020). Preliminary solvent–solvent extraction of the methanolic extract *W. coagulans* (ca. 120 g) resulted in the isolation of four major fractions (hexane, ethyl acetate, n-butanol and water). Five withanolides 1-5 were obtained from the chloroform fraction (pH = 3). While the other two withanolide (6-7) were obtained from n-butanolic fraction of the plants. Spectroscopic data indicated that six compounds 1-6 were known withanolides, and one was identified a butanilic acid belonging to the triterpenoid class (Figure 1).

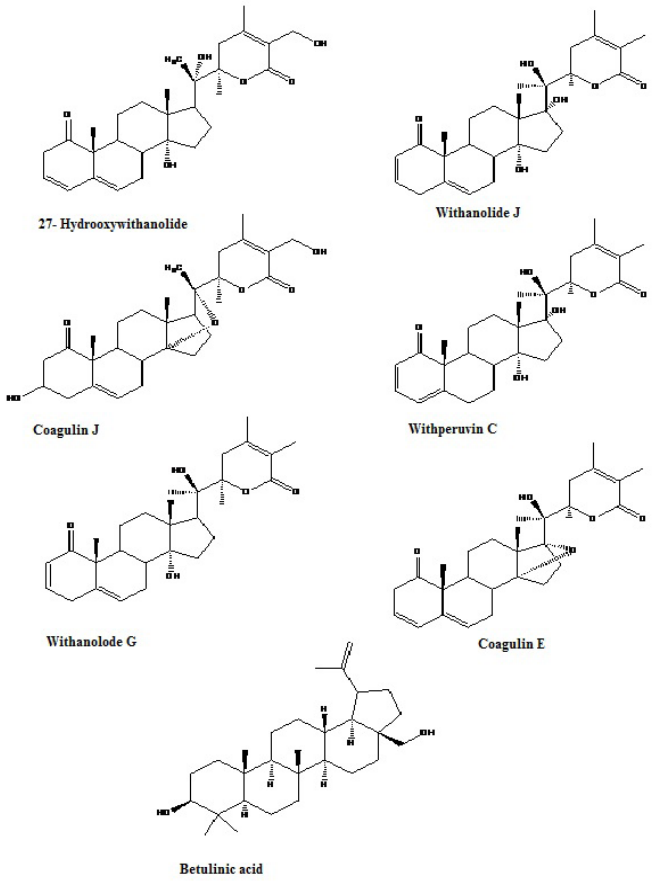


**Figure 1:** *Structure of pure compounds of Withania coagulans against Meloidogyne incognita*

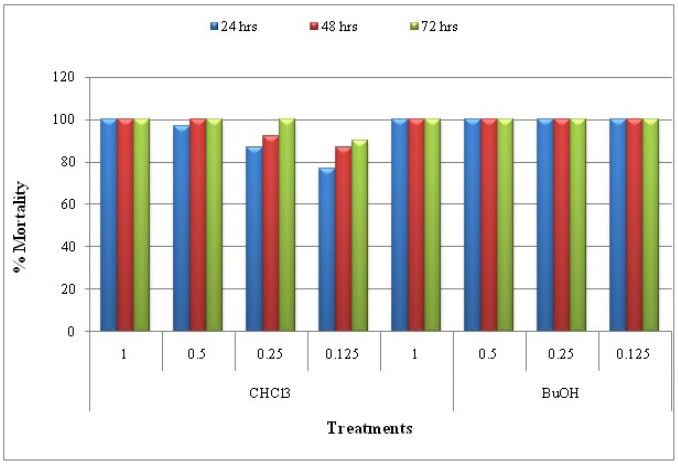


**Figure 2:** *Mortality (%) of Meloidogyne incognita against crude extract.*

*Nematicidal toxicity*

At all four concentrations on BuOH extracts no significant difference found and strong nematicidal activeness 100% mortality was achieved. Butanolic extracted compound WC=1 showed 100% mortality after 48 hrs of exposure at 1% with the lethal concentration ($LC_{50}$) values 0.168 mg/land at all four concentration are 100 % toxic at 72 h of exposure (Figure 2). W-56D proved to be 100 % lethal at 0.1 and 0.5 % concentration after 48 hrs of exposure with $LC_{50}$value 0.065 mg/l. The lowest mortality was found from WC-3 and the mortality rate was found to be 30-50 % (Figure 3) with $LC_{50}$ values 0.135 mg/l, 0.187 mg/land 0.145 mg/lafter 24, 48 and 72 hours of exposure (Table 1). Compound C1 also proved to be high level of virulent effect range between 75–100 %. The mortality was increased with increased time of exposure indicating the time dependent response of the compounds.

**Table 1:** *LC50 values of W. coagulanse compounds against M. incognita.*

| S. No | Compounds codes | Chemical name of compounds | Duration (hrs) | LC50 mg/L |
|---|---|---|---|---|
| 1. | W56D=1 | 27-hydroxy withanolide | 24<br>48<br>72 | 0.092(0.421-0.652)<br>0.065(0.4655-0.320)<br>0.051(0.3627-0.210) |
| 2. | WEA=11 | Withanolide J | 24<br>48<br>72 | 0.276(0.178-1.612)<br>0.153(0.838-0.234)<br>0.133(0.458-0.233 |
| 3. | WC=1 | Coagulin J | 24<br>48<br>72 | 0.175(1.234-1.236)<br>0.168(0.0602-1.285)<br>- |
| 4. | C=1 | Withperuavin C | 24<br>48<br>72 | 0.143(0.699-1.812)<br>0.131(0.507-1.02)<br>0.134(0.599-1.98) |
| 5. | C=2 | Withanolide G | 24<br>48<br>72 | 0.052(0.014-0.280)<br>0.033(0.012-0.205)<br>0.036(0.015-0.252) |
| 6 | WC=2 | Coagulin E | 24<br>48<br>72 | 0.143(0.820-1.321)<br>0.186(1.062-1.551)<br>0.223(0.452-0.82) |
| 7. | WC=3 | Betulinic acide | 24<br>48<br>72 | 0.1335(1.091-1.627)<br>0.187(1.085-1.524)<br>0.145(0.784-1.359) |

*$LC_{50}$ expressed as mg/L; CL, Confidence limit; are given in parenthesis.*

The study has proventhat with the class of withanolides and triterprnoid from *W. coagulans*, plant parasitic root knot-nematodes couldbe controlled by consuming this bio chemical, isolated from *W. coagulans*. This

route of management may help minimize the harmful synthetic chemical nematicides. These isolated chemical constituents from *W. coagulans* which was confirmed significance activity would be exposed to advance search for promising application in nematode controlling.

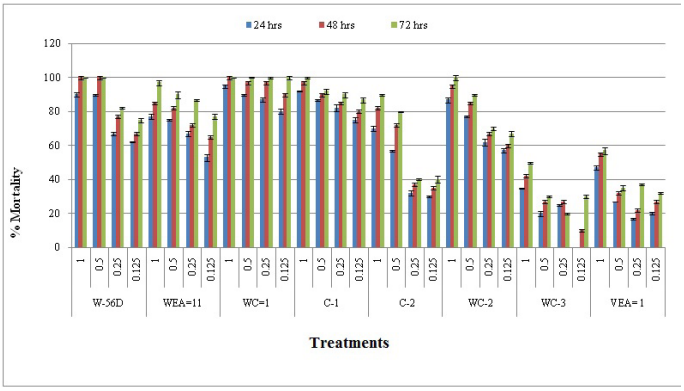


**Figure 3:** *Mortality reponse of M.incognita against compounds of Withania coagulanse.*

## Conclusions and Recommendations

The class of triterpenoides and withanolides were identified as major secondary metabolites of *Withania coagulans*. Isolated withanolides were evaluated for their bioactive potential and showed nematicidal activity. These discoveries bode well for the future discovery of novel nematicides derived from natural sources.

## Novelty statement

The investigation on plant origin pesticides is essentially vital for the progress of new botanical pesticides, especially in view of the vast worldwide flora.

## Author's Contribution

**Saima mehar:** Done plant collection and extraction of copounds and wrote experimental procedure about it.
**Salma Javed:** Done nematicidal activity and statistical analysis.

*Conflict of interest*
The authors have declared no conflict of interest.

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