Toxicity of derivatized citrulline and extracts of water melon (*Citrullus lanatus*) rind on root knot nematode (*Meloidogyne incognita*)

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Constituents of watermelon (*Citrullus lanatus*) rind were extracted in ethanol and derivatized to produce white water soluble crystals designated as derivatized citrulline. The derivatized citrulline, chromatographic fractions and ethanolic crude extracts from *Citrullus lanatus* (Thunb.) were assessed in the laboratory and screenhouse on possible toxicity to *Meloidogyne incognita* (Kofoid & Chitwood). The structure elucidation of the citrulline derivative (CTRL/DRVT) and chromatographic fractions (CMTG/FRCT) were established with spectral data from infrared (FTIR), $^{1}H/^{13}C$-NMR spectroscopy as well as gas chromatography mass spectrometry (GCMS). The infrared spectral data of the derivatized citrulline revealed a prominent absorption bands at 3431 cm^{-1} which is characteristic of N-H stretching vibration of primary amides. The bands at 1506 and 1456 cm^{-1} further support the presence of an amide group. The major compounds identified from GC-MS analysis of fractions include hydroxycinnamic acid (17.16%) p-anisic acid (16.10%) and chlorogenic acid (16.22%). ^{1}H-NMR data showed chemical shifts at δ 0.85, 0.88, 1.26, 1.33, 1.37, 1.60, 4.6, 4.6 ppm. The resonance at 0.8 - 1.37ppm is attributed to methyl and methylene protons. The down field signals at 4.6 ppm are ascribed to olefinic protons. The derivatized citrulline, had a significant action on percentage mortality of *M. incognita* juveniles. 19.10% mortality was achieved after 24 hours of juvenile exposure as opposed to 5.57% and 6.13% noted in the fractions and carbofuran (CBFN) respectively. At day six, percentage juvenile mortality in derivatized citrulline was remarkably similar to the observed value in carbofuran. Egg hatch in derivatized citrulline, fractions and carbofuran was inhibited maximally, while hatches were recorded in crude extract and control. In the screenhouse, vegetative growth of *Corchorus olitorius* (L) plants treated with derivatized citrulline, was appreciable and nematode population was significantly ($P \leq 0.05$) reduced in *C. olitorius* plants treated with chromatographic fractions and derivatized citrulline. The derivatized citrulline prepared in this study by semi-synthetic method can serve as an alternative to environmentally hazardous synthetic nematicides.

Keywords: *Citrullus lanatus*, citrulline, *Meloidogyne incognita*, hydroxycinnamic acid, bio-pesticide

Significant economic losses are caused to a wide variety of crops by plant parasitic nematodes (Dong and Zhang 2006; Fabiyi et al. 2018; Fabiyi 2020; Fabiyi et al. 2020a). Root-knot nematodes (*Meloidogyne* spp) are serious pest of vegetables in Nigeria, where they cause yield loss in most vegetable production areas (Fabiyi and Atolani 2011; Fabiyi et al. 2012; Fabiyi 2021a; Fabiyi 2021b). The most destructive nematode responsible for enormous yield reduction in *Corchorus olitorius* is the root-knot nematode, *Meloidogyne incognita* (Sasser 1989; Fabiyi 2016). Root-knot nematode has been reported as one of the world's most damaging agricultural pests (Curtis 2007). It causes about 5% of global crop loss and over 2000 plants have been indicated to be susceptible to *M. incognita* (Abad et al. 2003; Agrios 2005). The effect is characterized by numerous and pronounced galls on the roots of infected plants (Jain 1992). A highly toxic nematicide used in nematode control is carbofuran (2,3-dihydro-2, 2-dimethyl-benzofuran-7-yl methylcarbamate). Because of its high water solubility (351 ppm) and low adsorption coefficient, carbofuran is relatively mobile in soil and surface runoff (Lau et al. 2007). Consequently, carbofuran has the potential to contaminate lakes, streams, and groundwater (Goad et al. 2004). Environmental issues have increasingly limited the use of carbofuran (Rich et al. 2004). This study was initiated as a result of urgent concern for the enormous damage caused by nematode infection of crops, thereby reducing crop quality and yield. Also the environmental

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hazards consequent upon extensive and indiscriminate use of synthetic nematicides justify the search for alternative nematicidal compounds (Atolani et al. 2014a). The effect of derivatized citrulline extract from watermelon (*Citrullus lanatus*) rind was investigated on *M. incognita in vitro* and on *C. olitorius* infected with *M. incognita* in the screenhouse, to establish its potential as substitute for carbofuran. Watermelon a tropical fruit of the family cucurbitaceae grown in Asia and Africa is a popular fruit among Nigerians (Koocheki 2007). It is delicious, and a great source of much-needed water and electrolytes (Umesh Rudrappa 2009). It is an excellent source of vitamin A, which is a powerful natural anti-oxidant. 100 g fresh fruit provides 569 mg or 19% of daily required levels of this vitamin. It is also rich in antioxidants like lycopene, beta-carotene, lutein, zeaxanthin and cryptoxanthin (Melo et al. 2006; Umesh Rudrappa 2009). The lycopene content is superior to raw red tomatoes (Bauer 2002; Umesh Rudrappa 2009). The rind is eaten as a vegetable in some South American countries and also utilized in the production of pectin (Leong and Shui 2002; Umesh Rudrappa 2009), but the rind is usually a solid underutilized waste in Nigeria (Ahmed 1996; Lewinsohn et al. 2005).

Materials and methods

Extraction and synthesis

Watermelon, bought from Ilorin metropolis (Nigeria), was peeled and the rind (2 kg) was macerated with a mortar and pestle and extracted with 95% ethanol for 3 days. The extract was decanted and allowed to stand for 2 hours. The supernatant was decanted and 2 g of methylamine hydrochloride was added to one half of the extract solution. The reaction mixture was refluxed (using a reflux condenser over water bath) for 1 hour; and allowed to cool to room temperature (25 ± 2 ℃). Sodium nitrite (1 g) was added to the solution with continuous stirring. The reaction mixture was cooled in an ice/salt bath. This cold reaction mixture was added slowly with stirring into an ice-cold solution of 10 mL concentrated H_2SO_4 and 10 g ice. A whitish network of crystals was obtained and filtered by suction. The crystalline product was collected and dried inside a Petri dish. The product was insoluble in organic solvents, but readily soluble in water and stable to aerial oxidation. A second part of the ethanol extract was concentrated on rotary evaporator under vacuum and a portion of the crude concentrated extract was fractionated over silica gel (70 – 230 mesh grade). The elution was carried out with n-hexane; polarity of eluting solvent was later increased to hexane/dichloromethane 2:1 and finally dichloromethane alone. Eleven fractions were collected and were later pooled together on the basis of thin layer chromatography results (TLC) (pre-coated silica gel plates-DC-Alufolien 60 F_{254} Merck Darmstadt Germany). Coloured spots were detected by spraying with vanillin reagent. This afforded three major fractions, and the fractions which were soluble in water was selected for nematicidal test.

In vitro nematicidal assay

Pure culture of *M. incognita* eggs was extracted from roots of 55 day old *Celosia argentea*, using the sodium hypochlorite (NaOCl) method of Hussey and Barker (1973). The number of eggs per volume was estimated by taking 1 mL aliquots of egg solution three times and counting under a stereo microscope using Doncaster's (1962) counting dishes. Aliquots of 250/mL eggs were transferred separately into Petri dishes at 5 ml each. The experiment consisted of four treatments, derivatized citrulline (CTRL/DRVT), carbofuran (CBFN), chromatographic fractions from ethanol extract (CMTG/FRCT) and the pure crude ethanol extract of water melon rind (EtOH/CRD) at four levels with three replicates. The experimental design was a complete randomised design with a total of 48 Petri dishes. Each treatment was dissolved in 200 mL water at 30, 50 and 80 mg. The Petri dishes with ordinary water and juvenile or eggs served as control (0 concentrations). Counting was done at 24 hours' interval under the stereo microscope for juvenile mortality up to a period of six days, while egg hatch was observed for 5 days. The juveniles which did not respond to the touch of the picking brush were considered dead. The toxicity of the substances used was assessed as a percentage of the hatched eggs and dead juveniles (Abbasi et al. 2008; Fabiyi et al. 2020b).

Screenhouse trial

Sandy loam topsoil was collected and heat sterilised at 60 ℃ for 1 hour and was allowed to rest for 1 month to stabilise; 48 perforated 15 L plastic pots were each filled with 8 kg of the soil. *Corchorus olitorius* seedlings, 2 weeks old, were transplanted from the nursery into the experimental pots. Two days after transplanting, approximately 1,000 eggs of *M. incognita* in 25 mL solution extracted from *C. argentea* were inoculated in a small hole close to the base of each seedling (Fabiyi 2019). Treatments were dissolved in 200 mL water at 30, 50 and 80 mg and applied in banded form at the base of each plant. Data were collected on plant height, number of leaves and branches, nematode population in 250 g soil sample and nematode population in 20 g root sample.

Statistical analysis

Data collected were subjected to analysis of variance and where necessary, means were separated using Duncan's multiple range test at 5 % level of probability.

Spectroscopic analysis

Infra-red spectra were recorded on 8400 Fourier Transform Infrared (FTIR). The GC-MS analysis was done on Agilent 7890A GC-MS equipped with a quadrupole mass spectra detector and an auto-sampler with the following settings; injector, 200 ℃; interfaced temperature, 250 ℃; solvent cut time, 2.50 min; relative detector mode, ACQ mode; scan; start time and end time; 3 minutes and-56 minutes; event time, 0.50 seconds; scan speed, 1428 units. The characteristic mass fragmentation patterns of the fractions were compared with the patterns recorded in NIST library search for MS fragments. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) were determined using JEOL 400MHz. The chemical shifts were recorded in ppm relative to TMS, while the coupling constants are in Hz.

Results

Identification of the constituents of derivatized citrulline and chromatographic fractions were based on combination of data obtained from ultraviolet-visible, infrared, $^{1}H/^{13}C$-NMR spectroscopy as well as gas chromatography mass spectrometry. Infrared data ν_{max} 3674 (O-H), 3431 (N-H), 3417 (N-H), 2941, 2869 (CH), 1657 (C=C), 1680 (C=O), 1506 (C-N), 1456 (CH_2), 1441 (CH_2), 1388 (CH_3), 1054, 991 cm^{-1}. From Table 1, the GC-MS analysis of fractions reveals the presence of 4-hydroxybenzoic acid (13.12%), vanilic acid (6.18%), chlorogenic acid (16.22%), coumaric acid (8.14%), p-anisic acid (16.10%), hydroxycinnamic acid (17.16%), erucic acid (6.00%), vaccenic acid (9.01%) and cinnamic acid (8.07%). From the ^{1}H-NMR (400MHz $CDCl_3$), resonance at 0.8-1.37ppm is attributed to methyl, and methylene protons. Down field signals at 4.6ppm can be ascribed to olefinic protons. A doublet observed for the proton of an amide group was seen at 4.8ppm. ^{13}C-NMR reveals a carboxyl carbon at 178.2ppm and a quaternary carbon atom was indicated at 45.5ppm. This result supports the fact that constituent of the water melon rind had been derivatized to account for the new functional groups.

The toxicity of citrulline derivative and the chromatographic fractions from *C. lanatus* extract on juveniles of *M. incognita* is depicted in Table 2. Derivatized citrulline exhibited higher toxicity on *M. incognita* juveniles than the fractions and the water melon ethanol extract. After 24h of juvenile exposure to treatment, 19.10 % mortality was observed as opposed to 5.57 % and 6.13 % observed in the fractions and carbofuran respectively. Percentage juvenile mortality was significantly (P ≤ 0.05) higher in citrulline derivative than in other treatments. Mortality was however almost not observed in the control. The highest (80 mg) concentration of treatments was significantly more toxic than all the other concentrations. From Table 3, egg hatching capacity of *M. incognita* was significantly (P ≤ 0.05) affected by the treatments. Hatching was inhibited in all treatments but EtOH/CRD and control experiment recorded some hatchings.

The comparative heights of treated and untreated plants are shown in Table 4. Significant (P ≤ 0.05) variation was observed in plant height. Plants treated with watermelon citrulline derivative at the highest concentration produced the tallest plant from the 4th week after planting to the 14th week after planting, this was however not significantly (P > 0.05) different from carbofuran treated plants. Number of leaves and number of branches were more in plants administered with citrulline derivative and fractions, while fewer leaves and branches were observed in untreated control plants and plants treated with the lowest concentration (Table 5 and 6). Nematode populations in 250 g soil sample and 20 g root were very low in citrulline derivative and carbofuran treated plants, this was however followed by plants treated with fractions. The number of galls induced by *M. incognita* on *C. olitorius* plants was more in control plants giving an index of 5.00 while the index was as low as 0.45 in plants treated with fractions and 0.00 in citrulline derivative treated plants (Table 7).

Table 1: GC/MS analysis of fractions

GC peak number	Compound	Rt (min)	Peak area (%)
1	Cinnamic acid	2.36	8.07
2	Vanilic acid	3.28	6.18
3	Coumaric acid	3.55	8.14
4	4-hydroxybenzoic acid	4.10	13.12
5	Vacenic acid	4.48	9.01
6	Chlorogenic acid	5.21	16.22
7	Erucic acid	5.51	6.00
8	Hydroxycinnamic acid	6.37	17.16
9	p-anisic acid	6.56	16.10

Table 2: The effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on percentage mortality of *Meloidogyne incognita* juveniles

Treatments	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**
CTRL/DRVT	19.10[a]	27.22[a]	40.12[a]	60.15[a]	71.00[a]	89.00[a]
CMTG/FRCT	5.57[b]	10.78[b]	21.02[b]	30.09[b]	45.28[b]	70.00[b]
EtOH/CRD	0.00[c]	0.00[c]	0.00[d]	0.00[d]	6.20[d]	14.47[b]
CBFN	6.13[b]	11.24[b]	20.46[b]	30.31[b]	40.09[c]	88.65[a]
Level/Mg						
0	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.43[d]
30	4.18[c]	7.17[c]	11.39[c]	19.13[c]	28.16[c]	54.09[c]
50	10.25[b]	14.72[b]	22.04[b]	30.78[b]	39.04[b]	65.27[b]
80	15.67[a]	22.56[a]	30.21[a]	41.19[a]	50.10[a]	76.12[a]

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test. Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Table 3: Effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on percentage egg hatch of *Meloidogyne incognita* juveniles

Treatments	**Day1**	**Day2**	**Day3**	**Day4**	**Day5**
CTRL/DRVT	0.00[c]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
CMTG/FRCT	1.04[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
EtOH/CRD	5.12[a]	7.03[a]	11.01[a]	16.22[a]	19.12[a]
CBFN	0.00[c]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Level/Mg					
0	8.12[a]	13.01[b]	28.11[b]	32.19[b]	39.75[b]
30	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
50	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
80	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Table 4: Effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on plant height of *Meloidogyne incognita* infested *Corchorus olitorius* in the screenhouse

Treatments	4th WAP	6th WAP	8th WAP	10th WAP	12th WAP	14th WAP
CTRL/DRVT	21.02[a]	59.74[a]	85.69[a]	104.23[a]	141.09[a]	150.67[a]
CMTG/FRCT	17.08[b]	51.27[b]	74.43[b]	92.66[b]	118.65[b]	131.05[b]
EtOH/CRD	11.37[c]	20.87[c]	31.15[c]	52.06[c]	69.03[c]	77.18[c]
CBFN	20.59[a]	60.12[a]	86.11[a]	103.70[a]	140.89[a]	149.01[a]
Level/mg						
0	2.17[d]	8.13[d]	13.29[d]	20.54[d]	27.65[d]	36.92[d]
30	5.21[c]	18.45[c]	24.87[c]	34.02[c]	50.49[c]	60.19[c]
50	9.34[b]	25.12[b]	36.03[b]	46.18[b]	61.23[b]	74.05[b]
80	13.19[a]	32.60[a]	45.29[a]	58.26[a]	72.67[a]	89.62[a]

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test. Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Table 5: Effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on number of leaves of *Meloidogyne incognita* infested *Corchorus olitorius* in the screenhouse

Treatments	4th WAP	6th WAP	8th WAP	10th WAP	12th WAP	14th WAP
CTRL/DRVT	38.56[a]	63.18[b]	92.23[b]	126.07[a]	138.10[a]	171.13[a]
CMTG/FRCT	30.07[b]	48.45[c]	69.04[c]	90.30[b]	111.06[b]	140.42[b]
EtOH/CRD	17.33[c]	31.06[d]	40.71[d]	60.09[c]	70.24[c]	95.22[c]
CBFN	39.00[a]	62.67[a]	92.12[a]	125.84[a]	137.64[a]	171.19[a]
Level/mg						
0	9.35[d]	14.26[d]	26.44[d]	32.61[d]	40.33[d]	51.67[d]
30	15.54[c]	30.28[c]	40.39[c]	57.50[c]	71.05[c]	85.87[c]
50	21.17[b]	39.19[b]	52.14[b]	68.63[b]	82.76[b]	96.67[b]
80	29.45[a]	48.56[a]	61.17[a]	79.41[a]	93.45[a]	107.74[a]

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test. Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Table 6: Effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on number of branches of *Meloidogyne incognita* infested *Corchorus olitorius* in the screenhouse

Treatments	4th WAP	6th WAP	8th WAP	10th WAP	12th WAP	14th WAP
CTRL/DRVT	3.32a	7.01a	16.68a	24.35a	31.00a	31.00a
CMTG/FRCT	1.23b	5.45b	12.34b	17.42b	22.23b	22.23b
EtOH/CRD	0.00c	2.32c	5.00c	10.17c	15.07c	15.07c
CBFN	3.07a	6.78a	17.03a	24.16a	30.55a	30.55a
Level/mg						
0	0.00b	0.46d	0.85d	1.31d	4.28d	4.28d
30	0.00b	1.04c	3.21c	6.29c	10.19c	10.19c
50	0.00b	3.00b	7.01b	10.28b	14.05b	14.05b
80	1.05a	6.13a	10.12a	15.56a	21.33a	21.33a

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test. Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Table 7: Effect of different concentrations of chromatographic isolates, citrulline and crude extracts of watermelon rind and carbofuran on population of *Meloidogyne incognita* after harvest

Treatments	Nematode population in 250g soil	Nematode Population in 20g root sample	Root gall index
CTRL/DRVT	2.03b	1.10b	0.45b
CMTG/FRCT	7.53c	1.12 b	0.45b
EtOH/CRD	17.15d	5.27c	1.00c
CBFN	0.99a	0.16a	0.00a
Level/mg			
0	451.38d	32.64d	5.00d
30	76.23c	13.05c	2.00c
50	12.19b	7.17 b	1.00b
80	4.86a	2.18a	0.31a

Means in a segment of a given column followed by the same letter are not significantly different at P > 0.05 using the new Duncan's multiple range test. Key: CTRL/DRVT=citrulline derivative; CMTG/FRCT= chromatographic fractions; EtOH/CRD; ethanol crude extract; CBFN= carbofuran

Discussion

The present study has demonstrated that root-knot nematodes are very sensitive to derivatized citrulline, chromatographic fractions from crude extracts (of water melon rind) and crude extracts of water melon rind. The sensitivity was directly proportional to treatment concentration. Action of the treatment materials is related to the presence of diverse organic compounds in each material. The infra-red spectroscopy result of the fraction revealed the presence of functional groups like hydroxyl, carboxyl and amino. This is in agreement with the report of Lakshmipathy and Sarada (2013), on the presence of these functional groups in water melon rind extracts; it is possible that the hydroxyl groups in some cases bond with various cations and anions in the soil forming complexes that are probably potent to nematode survival depending on the soil pH. It is conceivable that soil chemical properties may have affected nematodes when the constituent organic compounds of the treatment material form complex with exchangeable soil trace metals like potassium, calcium and magnesium. This can create a synergy mechanism for enhanced toxicity on plant parasitic nematode. Oxidation to carbonic or carboxylic acids at higher soil pH may make the water film in the soil to be toxic to plant parasitic nematodes. Carboxylic acid from *Eichornia crassipes* has been found effective against *M. incognita* on banana in the field (Waele and Romulo 1998). A strong nematicidal activity of 3,4 dihydroxybenzoic acid against *M. incognita* juveniles and eggs was reported by Nguyen et al. (2013); 94.2% mortality at 1.0 mg/mL was observed. The GC/MS analysis revealed the presence of vaccenic acid, erucic acid, 4-hydroxybenzoic acid, chlorogenic acid, coumaric acid, p-anisic acid, hydroxycinnamic acid and cinnamic acid. This is in consonance with the findings of Al-Sayed and Ahmed (2013). Reports by Rizvi and Shahina (2014) established erucic and vaccenic acid as part of the constituents of *Citrullus colocynthis,* while stating that the methanolic extract of seed, pulp and fruit exhibited nematicidal activity after 72 hours of exposure with 100, 60 and 80% mortality respectively. Mahajan et al. (1985), stated the effectiveness of phenolic compounds like trans cinnamic acid, chlorogenic acid and caffeic acid in suppressing egg hatch of *M. incognita*. Chlorogenic and caffeic acids are however reported to be involved in the defence mechanisms operating in plant against nematodes (Gebel 1982). Compounds with long alkyl chains and flavonoid glycosides (^{1}H-NMR upfield signals) have been proved to increase permeation into nematodes with a resultant increase in toxicity (Dijan et al. 1994; Atolani et al. 2014b); this however explains the mortality rate observed in the laboratory and the reduction in nematode population seen at the end of the experiment in the screenhouse.

Conclusion

Derivatized citrulline, from crude ethanolic extract of *C. lanatus,* chromatographic fractions as well as the crude ethanolic extract of water melon exhibited immense nematicidal potential thereby contributing to the management of *M. incognita* populations on *C. olitorius*. Further study is in progress to establish the efficacy of these substances under field conditions including utilization of the rind waste directly as soil amendment.

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