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Bio-Nematicide compositions and activities of neem and wild sunflower leaf extracts on root-knot nematode eggs and juveniles

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Abstract
In the laboratory, phyto-chemical analysis were carried out on neem, *Azadirachta indica* and wild sunflower, *Tithonia diversifolia* leaf extracts and were also separately assessed on root-knot nematode, *Meloidogyne incognita* eggs and second-stage juveniles to measure their respective effects on egg hatch inhibition and juvenile mortality levels. The treatment were 25% stock solution neem leaf, 50% stock solution neem leaf, 75% stock solution of neem leaf, 100% (stock) solution neem leaf, 25% stock solution wild sunflower leaf, 50% stock solution wild sunflower leaf, 75% stock solution wild sunflower leaf, 100% (stock) solution wild sunflower leaf, and Distilled water (Control). I ml. of each treatment were dispensed separately into 1 ml suspension containing approximately 100 *M. incognita* eggs and aliquot of 1ml of *M. incognita* juveniles suspension containing approximately 100 freshly hatched *M. incognita* juveniles. The experiment was a completely randomized design with nine treatments replicated four times. The results show that the mortality of the root-knot nematode juveniles and egg hatch inhibition increased per exposure time and as plant extract concentration rate increases The results of the phyto-chemical analysis revealed the presence Alkaloids, Polyphenols, Tannins, Sterols, Flavonoids, Saponins, Terpenoids, Glycosides while Quinones, Anthocyanins, Anthraquinones were absent in both neem and wild sunflower leaf extracts. Coumarins was present in Neem but absent in Siam weed leaf extracts.
Keywords: Root-knot nematode, Neem, Wild sunflower, Phyto-chemical, Control.
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1. Introduction

Plant-parasitic nematodes cause 12.6% of global crop yield loss which is equivalent to an estimated 157 billion dollars per year (Nicol et al. 2011; Das et al. 2021; Marin-Bruzos et al. 2021). In particular, root-knot nematodes (*Meloidogyne* spp.) have a large host plant spectrum and cause low crop yields. They rapidly reproduce under favourable conditions, and the increment of the population causes low crop yields (Perry et al. 2009). Over the years, researchers have developed a variety of cultural, mechanical, and chemical applications to control root-knot nematodes (Sharma et al. 2020). Nematicides can be effectively used to prevent crop losses from plant-parasitic nematodes. Chemical nematicides are still the most effective means of plant-parasitic nematode control. However, intensive nematicide use destroys beneficial microorganisms in the soil; therefore, nematicide usage is neither a healthy nor an economical solution for growers. In order to overcome these problems, it is crucial to adopt effective and environmentally friendly methods to control nematodes (Kasapoğlu, 2024). One such practice would be the use of plant extracts that have nematicidal properties (Aydınlı and Mennan 2014). Neem leaf extracts at different concentrations have showed remarkable root-knot nematode control with almost 87% control .The mortality of *Meloidogyne* spp. was showed to be nearly 80% after 48 hours of incubation (Duong et al., 2014).

The root knot nematode, *Meloidogyne incognita*, is sedentary endo-parasite and is among the most damaging agricultural pests, attacking a wide range of crops (Katooli et al. 2010). This pest is responsible for approximately 50% of overall damage (Abbasi et al., 2008). Root-knot nematode attacks over 2000 plant species causing stunted growth and finally low yield. For this reason, a few nematicides should be applied at each cropping season. Currently, research on alternative methods to nematicide is primarily focused on plant extracts and their components. The effect of plant extracts and essential oils in controlling plant-parasitic nematodes is well documented (D'Addabbo et al. 2020). However, few studies have been published on the control of *M.*

incognita using plant extracts. This study focused on the bio-nematicide compositions and activities of neem and wild sunflower leaves on root-knot nematode eggs and juveniles.

2. Materials and Methods

2.1 Experimental site: The experiment was carried out t at the Crop Prptection Laboratory, Ladoke Akintola University of Technology (LAUTECH), Ogbomosho, Oyo State, Nigeria. Neem and wild sunflower leaves were collected within the premises of Teaching and Research Farms of the university and was air dried at room temperature for 4 weeks after which it was blended into powdered form. The powdered neem and wild sunflower leaves were used for subsequent experiments.

2.2 Qualitative phytochemical screening of neem and wild sunflower leaves:

The phytochemical components of leaf extracts of neem and wild sunflower were carried out in the laboratory following a standardized protocol as described by Shaikh and Patil (2020).

Test for Alkaloids: Mayer's and Wagner's reagent test: To 2ml of plant extract in different tubes, 2 ml of 2% hydrochloride acid was added. Then 3 drops of Mayer's and Wagner's reagent were added. The presence of a creamy white/yellow precipitate was observed for Mayer's and a brown/reddish precipitate for Wagner's signifies the presence of alkaloids.

Test for Tannins: Braymer's test: To 2ml of plant extract, 2 mL of water was added with 3 drops of (10%) ferric chloride. The formation of a Blue-green colour precipitate indicates the presence of Tannins

Test for Saponins: Frothing Test
To 2ml of plant extract, 2mL of distilled water was added and shaken in a graduated cylindrical for 15 minutes lengthwise. Formation of a 1cm layer of foam is observed which shows the presence of Saponin.

Test for Flavonoids: Alkaline reagent test: To 2ml of plant extract, 2ml of 2% sodium hydroxide was added, and a few drops of diluted hydrochloride acid. An intense yellow color becomes colorless with the addition of diluted acid showing the presence of flavonoids

Test for Quinones: Sulphuric acid test: To 2ml of extract, 2ml of isopropyl alcohol was added, and 2ml concentrated sulphuric acid. A red colour was formed and indicates the presence of Quinones.

Test for Glycosides: Sulphuric acid test: To 2ml of plant extract, 2ml 50% of sulphuric acid was added. The mixture was heated in boiling water for 15minutes. Fehling solution was added and the resulting mixture was heated to boiling. A brick red precipitate indicates the presence of glycosides.

Test for Terpenoids: To 0.5 ml of extract, 2mL of chloroform was added and concentrated sulphuric acid was added carefully. Formation of a red-brown color at the interface indicates the presence of terpenoids.

Test for Phenols: Ferric chloride test: To 2 ml of the extract, 2 ml of distilled water followed by a few drops of 10% ferric chloride was added. Formation of a Dark green/bluish-black color indicates the presence of phenols.

Test for Coumarin: Sodium hydroxide test: To 2ml of extract, 2ml of 10% NaOH was added. The formation of yellow colour indicates the presence of coumarin.

Test for Steroids: Salkowski test
2ml of plant extract was mixed with 2ml of chloroform, followed by the addition of 10 drops of acetic anhydride and 3 drops of concentrated sulphuric acids. The emergence of a red-rose colour indicates the presence of steroids.

Test for Anthocyanins: HCI test
To 2 ml of extract, 2 mL of 2NHCl and 3 drops of ammonia solution were added. A pink-red
solution that turns blue-violet after the addition of ammonia indicates the presence of anthocyanin.

Test for Anthraquinones: Borntrager's test: To 2ml of plant extract 2mL drops of 10% ammonia was added and

then shaken vigorously for 30 seconds. A pink, violet, or red-coloured solution indicates the absence of anthraquine.

2.3 Quantitative phytochemical screening of neem and wild sunflower leaves:

Determination of total alkaloid contents: The alkaloid content was determined using the method described by Van Tans (2018). A 5 g sample was mixed with 200ml of 10% acetic acid in ethanol in 250ml beaker. The mixture was filtered for 4 hours, and the resulting extract was then concentrated in awater bath to approximately 25% of its initial volume. Concentrated ammonium hydroxide was gradually added until precipitation occurred. The precipitate was then treated with a diluted ammonium hydroxide solution and filtered. The final product was obtained as a weighted and dried alkaloid.

Determination of total saponin contents: The methodology described by Adepoju et al (2024) was followed precisely. A 20g sample of each macerated with 100cm^3 of 20% ethanol. The combined extracts were reduced to 40ml using a 90ºC water bath, followed by the addition of 20ml diethyl ether. The aqueous layer was separated and washed twice 10ml of 5% aqueous sodium chloride. The remaining solution was heated to a constant weight, and saponin content was determined.
Determination of total phenolic contents: Siddiqui et al., (2017) described a method for determining the total phenolic content in plant extract using spectrophotometric analysis with the Folin-Ciocalteu test. The procedure involves preparing a reaction mixture in a 25ml volumetric flask by combining 1ml of plant extract, 1ml of Folin–Ciocalteu reagent, and 9ml of distilled water. After a 5-minute reaction time , 10ml of a 7% sodium carbonate are added. Gallic acid standards (20-100µg/ml) are prepared using the same procedure. The absorbance of the test and standard solutions is then measured at 550nm using a UV/ Visible spectrophotometer after a 90-minute incubation period at room temperature. The total phenol content is expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

2.4 Effect of Neem and wild sunflower leaf extracts on the survival of root knot nematode, *Meloidogyne incognita* second stage juveniles. Aliquots of 1ml of *M. incognita* second stage juveniles suspension containing a count of 100 freshly hatched juveniles were pipette into each of the transparent glass petri-dishes containing: 25% stock solution neem leaf, 50% stock solution neem leaf, 75% stock solution of neem leaf, 100% (stock) solution neem leaf, 25% stock solution wild sunflower leaf, 50% stock solution wild sunflower leaf, 75% stock solution wild sunflower leaf, 100% (stock) solution wild sunflower leaf, and Distilled water (Control). The glass Petri-dishes were labeled accordingly and arranged randomly on the bench at the Crop Protection Laboratory, Bee House, Ladoke Akintola University of Technology, Ogbomosho, Nigeria. The experiment was completely randomized design with 9 treatments in four replicates. Percentages of M. incognita that were dead were put on record at every 24 hours for 7days
.
2.5 Extraction of root-knot nematode eggs: Galled roots were collected from *Celosia argentea* on which a pure culture of *Meloidogyne incognita* was initially raised. The galled roots were washed properly under running tap in order to get rid of attached soil. The roots were cut into small pieces and shaken with 0.5% sodium hypochlorite solution for five minutes in a Kilner's jar as suggested by Hussey and Barker (1973) in order to digest the gelatinous matrix encasing the eggs. The content was sieved through a 200 mesh sieve nested over a 500 mesh sieve. The 500 mesh sieve containing the eggs was placed under a running water tap in order to remove residual Sodium Hypochlorite solution. Root knot nematode eggs were collected in the distilled water inside 500 ml sized beaker for subsequent experimental use. The number of eggs in 1 ml egg suspension was standardized so that 1 ml egg suspension contained approximately 100 fresh *Meloidogyne incognita* eggs.

2.6 Root knot nematode, *Meloidogyne incognita* egg hatch Experiment: Aliquots of 1 ml of *Meloidogyne incognita* egg suspension containing approximately an hundred (100) fresh *M. incognita* eggs were dispensed into each of the transparent glass petri-dish containing 10 ml of different plant extracts and control. An hundred (100) fresh M. incognita eggs dispensed into distilled water served as control. The experiment was incubated at 27$\pm$3ºC temperature. The glass Petri-dishes were covered with glass covers to prevent evaporation. The experiment was a completely randomized design with 10 treatments, replicated 5 times. Counts of un-hatched eggs made at every 24 hours for 7 days under the stereomicroscope

3. Results

3.1 Qualitative phytochemical screening of Neem and Wild sunflower leaf extracts: It was evident that alkaloids, polyphenols were highly present in wild sunflower leaf. Tannins, Sterols, Flavonoids, Saponins, Terpenoids, Glycosides were moderately present. while Quinones, Anthocyanins, Anthraquinones and Coumarins were absent. Neem leaf has high quantity of Alkaloids, Flavonoids, Sterols, Coumarins, Terpenoids, while Saponins, Polyphenols, Glycosides, and Tannins were moderately present. Anthraquinones, Anthocyanins and Quinines were absent in Neem leaf (Table 1).

Table 1: Qualitative analysis of Neem and Wild sunflower leaf extracts

Phytochemicals	Wild sunflower leaf	Neem leaf
Alkaloids	+++	+++
Flavonoids	++	+++
Saponins	++	++
Polyphenols	+++	++
Sterols	++	+++
Coumarins	-	+++
Terpenoids	++	+++
Glycosides	++	++
Anthraquinones	-	-
Anthocyanins	-	-
Tannins	++	++
Quinones	-	-

Key: + slightly present, ++ moderately present, +++ Highly present and – absent

Table 2 shows that the quantity of Saponin was 3.37±0.16%, Alkaloid 13.02±0.17% and Phenol 25.52±0.07 GAE/mg/g, in Wild sunflower leaf extract.
In neem leaf extract, the quantity of Saponin was 2.4± 0.12% , Alkaloid 10.3 ± 0.18%, Phenol 65.55±0.07 GAE/mg/g (Table 2)

Table 2: Quantitative analysis of Neem and Wild sunflower leaf extracts

Sample	% Saponin	% Alkaloid (GAE/mg/g)	Phenol
Wild sunflower Leaf	3.37± 0.16	13.02± 0.17	25.52±0.07
Neem Leaf	2.4± 0.12	10.3 ± 0.18	65.55 ± 0.07

Data expressed as mean ± SD (n=3); GAE= garlic acid equivalent; QE = quercetin equivalent.

3.2 Effects of neem and wild sunflower leaf extracts on the hatchability of root- knot nematode eggs:

Table 3 elicits the inhibitory effect of aqueous extract of neem and wild sunflower leaves on the hatchability of root-knot nematode eggs. It was evident that the percentage egg hatch was 61% in the control (0%) at day 1, 90% at day 2, while it was 100% at day 3 till day 7. In 25% aqueous extract of neem leaf, there was 18% egg hatch. Egg hatchability was totally inhibited from day 2 to day 7. At 50%, 75%, 100% there was no egg hatch from day 1 to day 7. This shows that 50%, 75%, and 100% concentrations of the aqueous extract of Siam weed, resulted to 100% egg hatch inhibition.

Table 3 also shows that in the aqueous extract of Wild sunflower, egg hatch were 26% (Day 1), 100% total egg hatch inhibition at (Day 2 - 7) and between day 3 and 7. There was no egg hatch for day 7.
The lowest egg hatch inhibition was observed in 0%, while the highest was recorded at 100% in both samples.

Table 3: Aqueous extract of neem and wild sunflower leaves on the hatchability of root- knot nematode eggs

Plant part	Concentration	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Neem leaf	0%	61c	90b	100b	100b	100b	100b	100b
	25%	18b	0a	0a	0a	0a	0a	0a
	50%	0a	0a	0a	0a	0a	0a	0a
	75%	0a	0a	0a	0a	0a	0a	0a
	100%	0a	0a	0a	0a	0a	0a	0a
Wild Sunflower leaf	25%	26b	0a	0a	0a	0a	0a	0a
	50%	0a	0a	0a	0a	0a	0a	0a
	75%	0a	0a	0a	0a	0a	0a	0a
	100%	0a	0a	0a	0a	0a	0a	0a

Foot note: Figures under each day in the Table 4 were the number of nematode eggs that have been hatched into juveniles.

Same superscript (alphabet) along same column indicates no significant difference

Table 4 shows the effect of aqueous extract of neem and Wild sunflower leaves on the root-knot nematode juveniles' mortality. The results show that in the control (0%) there were 100 live nematodes on day 1 and 2. On day 3, 4, 5, 6, 7 the number reduced to 92, 86, 61, 60 and 42 respectively. It was observed that there was low mortalities at no and low aqueous extract of Neem leaf concentrations. At 25% concentration of Neem leaf extract, there were 39 live nmatode juveniles on day 1, by day 2, 17 live nematodes were left. From day 3 – 7, 100% mortalities were observed. The higher the concentration of the aqueous extract of Neem leaf extract, the higher the mortality. At 50%, 75%, 100% concentrations, it was observed that there was total juvenile mortality throughout the exposure time (1 -7 days).

Table 4 revealed that Wild sunflower at 25% concentration has 41 live nematodes on day 1 which significantly reduced to 16 on day 2. From day 3 to day 7, mortality rates were significantly increased causing total nematode mortality. At the higher concentrations of 50%, 75%, 100%, the total nematode mortality was recorded during exposure days (1 – 7 days).

Table 4: Aqueous extract of neem and wild sunflower leaves on the root - knot nematode juveniles' mortality.

Plant part	Concentration	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Neem leaf	0%	100c	100c	92b	86b	61b	60b	42b
	25%	39b	17b	0a	0a	0a	0a	0a
	50%	0a	0a	0a	0a	0a	0a	0a
	75%	0a	0a	0a	0a	0a	0a	0a
	100%	0a	0a	0a	0a	0a	0a	0a
Wild Sunflower leaf	25%	41b	16b	0a	0a	0a	0a	0a
	50%	0a	0a	0a	0a	0a	0a	0a
	75%	0a	0a	0a	0a	0a	0a	0a
	100%	0a	0a	0a	0a	0a	0a	0a

Foot note: Figures under each day in the Table 3 were the number of live nematodes

Same superscript (alphabet) along same column indicates no significant difference

4. Discussion

Preliminary qualitative phytochemical analysis performed in this study revealed the presence of Alkaloids, Terpenoids and Saponin in the leaf extracts of Neem and Wild sunflower. This finding corroborates the earlier research work of Mishra (2018) who indicated that Neem and Wild sunflower formulations contains phenols, amino acids, aldehydes and fatty acids were antagonistic to root-knot nematodes and other soil-borne pest. The extracts and metabolites of these plants have been found to exhibit various bioactivities including, nematicidal properties, antimicrobial, insecticidal, phytotoxic among others (Susmitha et al., 2013). Mariana et al. (2018) reported that the most characteristic metabolites in Neem are called limonoids which has considerable interest

due to its broad biological activity and fascinating structural diversity. Mariana et al. (2018) indicated that flavonoids are purified from neem fresh leaves which confirms earlier findings by Dash et al. (2018) that extracts from neem are rich in secondary metabolites and Phytoconstituents such as saponins and flavonoid and reducing sugar. Investigation by Phytochemical studies by Rahman et al. (2018) on wild sunflower plants indicated that plants of this genus had led to the isolation of compounds including lignins, flavonoids, terpenoids, steroids and Nitro group containing compounds.

The results of this study support the survey of Sturtz et al. (2018) on the nematicidal activity of neem, and wild sunflower compounds based on 24-h LD50 values and reported that there is potent nematicidal activity of these extracts against the root-knot nematodes egg hatch. Similar findings were reported by Wahome et al. (2018) who observed that extracts from these plants increased mortality percentage of juveniles and inhibited nematode egg hatch when compared with control. Resha et al. (2018) evaluated the efficacy of extracts from Neem on their activity against the root-knot nematodes and reported that there was highest decrease in the number of 53 root-knot nematodes J2 mortality and mobility inhibition when juveniles were exposed to the extract at higher concentration of 100 ml/l. The results of this study support the report of Lambert et al. (2018) that aldehydes, fatty acids, phenols, amino acids are released from botanical extracts and are antagonistic to root-knot nematodes. It was observed that the rate of juvenile mortality was directly proportional to the concentration of extracts and exposure period. Findings of Abdul et al. (2018) who indicated that extract of leaves from Neem, Wild sunflower are toxic against the second stage juveniles of root knot nematode support our findings. The findings are in agreement with those by Odeyemi et al. (2018) who showed the inhibitory effects from leaf extracts from Wild sunflower. The nematicidal effect of Neem, Wild sunflower extracts may possibly be attributed to their high contents of certain oxygenated compounds which are characterized by their lipophilic properties that enable them to dissolve the cytoplasmic membrane of nematode cells and their functional groups interfering with the enzyme protein structure.

5. Conclusion

This study provides information on the usage of plant-based extracts against root-knot nematodes. Leaf extracts from neem and wild sunflower appear to have a higher potential in the control of *M. incognita* and have shown to be favorable alternatives to synthetic nematicides against root-knot nematodes. The application of organic amendments has in the past been recognized in the improvement of soil health and management of parasitic nematodes. Due to environmental benefits associated with plant-based extracts, they have been considered in integrated nematode management. Although many research have shown reduced parasitic nematode populations with the use of organic amendments, others have shown increased populations with the effectiveness of these amendments (Renco, 2018).

Introduction of plant-based extracts as an option in the management of parasitic nematodes has become a major component in productivity and sustainable management of soil health. Various botanical have variable effects on the biological activities in soil and more so against pathogens .Soil nematodes are affected by botanicals added in the form of extracts, compost and other soil amendments. Different plant-based extracts used in this study showed varying bio-nematicidal variables.

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