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Nematicidal activity of fruit citrus peels against root-knot nematode

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Abstract

The objective of this research is to evaluate the effect of different citrus fruit against root-knot nematode. The nematode is roundworm that are commonly seen in the environment of the earth. While some species are toxic parasite, others play an important role in cycle and research area.Root-knot nematode are damaging to nearly all plant, including fruit, veggies and some cereal crops. The main signs of the root-knot nematode infection are knot or gall that are generated on the root. The phytochemical in citrus fruit contains flavonoids, carotenoids, essential oil, mucilage, tannins. The main health benefits of citrus are anti-inflammatory, antifungal, antiviral anticarcinogenic, hyperglycaemia. The extract of fresh peels of orange, lemon, showed nematostatic effect against Meloidogyne spp. The nematicidal activity in all the extract of fresh peels but was greatly enhanced in the extract of lemon peel against adult nematode, orange peel against juveniles and egg hatching were completely stops in orange peel extract when compare to lemon peel. The methods were used as extraction of nematode using Baermann funnel method, mounting, staining of root materials, effect of citrus peel extract on egg hatching, juveniles and adult nematode. The citrus peels controlling root knot nematode which is more beneficial than using chemical fertilizer causes pollution, natural ways gave benefits for environment.

Keywords: Citrus peels, Meloidogyne spp, Baermann funnel method, mounting, staining of root materials, infectivity.

Introduction

Roundworms known as nematodes can be found in all types of environments on earth. While some species are parasites that can be dangerous, others are essential to the nutrition cycle and medical research. Field nematode infestation is poly-specific, although depending on the one or two species outnumber the others in terms of agroclimatic conditions. In view of this, control measures have been taken to either prevent nematode invasion, limit its population, lessen its impact on the crops, or employ a combination of these concepts. According to the literature, root

knot nematodes (*Meloidogyne incognita*) are damaging to nearly all plants, including fruits, veggies, and some cereals crops. The species *M. javanica, M. incognita, M. graminicola,* and *M.exigua* are significant economically in India. The main signs of the root knot nematode infection *(M. incognita)* are knots or galls that are generated on the roots **(Garca *et* al., 2015).**

Every year, plant parasitic nematodes cause crop losses totaling 125 billion dollars worldwide (Chitwood, 2003). The root- knot nematodes, or *Meloidogyne* species, are mostly to blame for most losses. Globally, root-knot nematodes have been reported to be able to infect more than 2000 different species of plants. Poor development, reduced quality and yield, and increased susceptibility to other infections are all effects of the root-knot nematode **(Back *et al*., 2002; Castello *et al*., 2003).** Infested regions have been managed using Phyto nematodes using a variety of tactics, including organic additives, biological management, and chemical nematicides. Nematicides made of synthetic chemicals, however, pose environmental risks, are scarce in many developing nations, and have been phased out of use by many due to their expensive costs.

Organic amendments are frequently used to increase crop yield and soil fertility, but they also have a suppressive effect on plant pathogenic fungi and nematodes. A number of weedy plants are reported to contain nematostatic and nematicidal potential *A. mexicana* and *A. aspera* being the highly toxic against root-knot nematode, *Meloidogyne incognita* **(Khan *et al*., 2017),** in form of inhibition of egg hatching and mortality of J2 juveniles, presumably due to the occurrence of alkaloids, flavonoids, tannins, saponins, phytosterols, mucilage gum in the aqueous extracts of these weeds. Many plant species from 57 families have been found to contain nematicidal compounds.

*Meloidogyne spp*are the most damaging plant-parasitic nematodes (PPNs), agricultural and horticultural crops. *M. incognita* is one of 98 species in the genus, and it causes root galls in nearly all vascular plants**.** Because of their rapid generation time, high reproduction rate, entophytic and sedentary nature, *Meloidogyne spp* are extremely difficult to control **(Engelbrecht *et al*.,2018).**

Chemical nematicides are being scrutinized due to growing awareness of their negative effects on soil, water, the environment, and non-targeted organisms. Furthermore, many of these nematicides have developed resistance in *Meloidogyne spp*. As a result, there is a lot of emphasis on finding a long-term alternative to chemical nematicides for *Meloidogyne* control.

Scientific classification

Kingdom: *Animalia*
Phylum: *Nematoda*
Class: *Secernentea*
Order: *Tylenchida*
Family: *Heteroderidae*
Genus: *Meloidogyne*

All nematodes go through four juvenile stages (J1-J4) before reaching adulthood. *Meloidogyne* parasites hatch as vermiform, second-stage juveniles (J2) from eggs, the first mount occurring within the egg. Newly hatched juveniles spend a brief period of time in the soil, in the rhizosphere of the host plants. They may re-invade their parent's host plants or migrate through the soil in search of a new host root. During the free-living stage, J2 larvae do not feed and instead rely on lipids stored in the gut. Using *Arabidopsis thaliana* as a model host, an appropriate model system for studying the parasitic habits of plant-parasitic nematodes has been evolved. *Arabidopsis* roots are initially small and transparent, allowing every detail to be observed.

The J2s go through morphological alterations and develop saccate after receiving additional nutrition. They mount three times if they aren't fed more, and finally mature into adults. Feeding resumes and the reproductive system develops in females, who are almost spherical **(*Eisenbacket al*., 1991).** An adult female can live for three months and lay hundreds of eggs during that time. After harvesting the plant's aerial components,

females can continue to produce eggs, and the egg is typically where the plant survives between crops.

Temperature affects how long a life cycle lasts **(Trudgill *et al.*, 1994).** Although it's likely that the individual stages of the root-knot nematode life cycle's component parts, the link between rate of development and temperature is linear over much of the optima for various stages of the life cycle, such as egg formation, host root invasion, or growth, varies slightly. The optimal temperature varies across the species of the genus *Meloidogyne*. Development takes place in *M.javanica* between 13 and 34 °C, with optimal development taking place at around29 °C.

Citrus fruit

The *Rutaceae* family of evergreen shrubs and small trees includes citrus, which includes Oranges, Mandarins, Tangerines, Limes, Grapefruits, Lemons, and Citrons as well as numerous hybrids and varieties**(Waleed*et al.*,2010)** Citrus is grown in tropical, subtropical, and temperate climates. In the Northern Hemisphere, fruitsespecially oranges and grapefruitmature between mid-December and early April. Fruit is generally available all year long.

Due to its useful and health-related components, such as vitamin C, carotenoids, flavonoids, pectin, calcium, and potassium, citrus fruit is one of the most popular fruits in the world. Citrus fruits are valued as a precious source of soluble and insoluble fibre, and they have a number of health advantages, including the removal of toxins from the body **(*Pragasam et al.*,2013).** Fiber increases gastric adsorption in the small intestine, slows down the energy absorption process, and keeps the liver and bile duct functioning properly. Citrus fruit has higher concentrations of flavonoids, terpenes, phytonutrients, a variety of phenolic compounds, vitamins, and carotenoids than other fruits.

Phytochemical in citrus fruit

Citrus phytochemicals are found in numerous portions of fruits, and they have cholesterol-lowering effects as well as antibacterial, antiviral, antifungal, anti-carcinogenic, antithrombotic, or anti-inflammatory characteristics. These active substances, which have antioxidative potential and are recogonised as health promoters due to their wide range in the human body, include phenols, carotenoids, phytoestrogen, and sulphides **(kuo S *et al*., 2006).**

Flavonoids

Citrus fruit is a high source of flavonoids, which have numerous physiological characteristics involved in regulating antiviral, anti-microbial action and activity. They exist as glycosides or a glycose, particularly in citrus juices as glycosyl derivatives (flavonoid glycosides, FGs), which may have positive effects on human health **(Loizzo MR *et al*.,2012)**.

Carotenoids

Sweet oranges, Mandarins, and Grapefruits are rich sources of carotenoids.

The main function of carotenoids pigment compounds is to protect against the various diseases of the human body and control-health-related elements.

Citrus fruits are rich in lutein and zeaxanthin, which are good for the body's immune system and eyes.

Cara navel oranges and mandarin fruits are an abundant source of carotenoids analysis of the important carotenoids found in citrus fruits shows that Mandarins have greater values than Grapefruit, Oranges are next, and Lemons have no carotenoids in them.

Essential oil

Citrus species are known to be abundant sources of aromatic compounds; In example, citrus fruit peels contain 400 volatile and nonvolatile chemicals. Essential oils have been utilized for medicinal and aromatic purposes since antiquity, and they are still used today for their antispasmodic and antibacterial properties in a variety of cosmetic and allied businesses, as well as in different pharmacies **(Ono E, Inoue J, *et al*., 2011)**

Mucilage

Citrus fruit seeds, peels, and pulp all contain Mucilage, which is a fiber-like substance that when combined with water produces a gel-like structure. Citrus seeds contain psyllium, which helps to improve digestion and speed up the removal of cholesterol. Citrus fruits also have a higher ratio of soluble and insoluble fibre, which lowers cholesterol levels **(Jung UJ *et al*., 2004)**.

Tannins

When compared to other citrus fruits, grapefruit, lemon, and lime have a higher content of tannins compounds. These tannins are crucial in stopping diarrhoea, reducing bleeding, and controlling other extreme bodily secretions **(Matsuda H *et al*., 1991).**

Some health benefits of citrus

Citrus fruits contain a variety of biological functions that support body wellness (Table 3). Moreover, lemon and sweet orange fruits have a wide variety of bioactive chemicals that have been shown to decrease liver disorders by 60–70% **(Sidana J *et al*., 2013).**

However, citrus juice, particularly grapefruit juice, contains enzymes, especially P-45 enzyme, which is thought of as a natural aid for controlling obesity and contains a variety of distinct proteins that burn human lipids.

More than 200 bioactive chemicals that affect human body regulators can be found in lemon juice, which also contains a number of bioactive compounds in its fruit. Lemon and Sweet Orange juices, on the other hand, have a variety of bioactive substances that can manage lipids and prevent 60 to 70% of liver illnesses.

Main health benefits of citrus

Anti-carcinogenic qualities: Citrus flavonoids have anti-tumour and anti-carcinogenic capabilities.

Cardiovascular properties: Citrus flavonoids inhibit the adhesion and clumping of red blood cells.

Hyperglycaemia: Citrus flavonoids have significant anti-hyperglycaemic effects, in part via binding to starch, boosting hepatic glycolysis and glycogen levels, and decreasing hepatic gluconeogenesis.

Anti-inflammatory, Antiallergic, and Analgesic Activity: Citrus flavonoids, including hesperidin, diosmin, quercetin, and other et flavonoids, have demonstrated dose-dependent anti-inflammatory activity via affecting the metabolism of arachidonic acid and histamine release.

Antifungal and antiviral activity: Is one of the flavonoids' features that correlates with their physiological action in plants.

Materials and Methods

Collection of samples

The soil and roots for collection of nematodes from the Agricultural field, Tirukovilur, Tamil Nadu. Matured fruits of orange (Citrus sinensis) lemon (Citrus limon) were purchased from the local supermarket, Tirukovilur.

Sterilization of fruit peel

The fruit were cut into small pieces. The peels were first washed with running tap water for 30 minutes. There after the peels were disinfected with detergent solution (Tween 20) for 10 minutes. And again, washed with distilled water and then washed with 80% of ethanol for 30 seconds followed by distilled water and then surface sterilized with 0.1% mercuric chloride solution for 10 minutes. Then the peels were washed 5 times with sterile distilled water to remove the sterilant.

Preparation of fruit citrus peel powder

The sterilized citrus peel were dried for a week in the shade. Using a kitchen blender, the dried peels were turned into a fine powder. Peel powder was made from various citrus fruits and kept in air tight container or jar.

Preparation of different solvent extract

The Citrus sinensis (orange) and Citrus limon (lemon) powder was extracted with different solvent such as methanol, chloroform and water. 10g of peel powder of each fruit was suspended in 100 ml of solvent.

Extraction of nematode from root material

Isolation of nematode by baermann funnel method

The Baermann funnel is used for extraction of active nematode from plant materials and soil. The sample size depends on the funnel diameter and the types of material. If extraction is from soil, the final suspension is dirty.

Its original version, the sample was wrapped in a tissue cloth and almost fully incubated in water resulting in very low nematode recovery

Modified version use a wire basket plus filter to spread the sample over a larger area. In addition, the sample is only immersed half-way into the water.

Procedure

1. Carefully wash the sample and cut into 1 cm sized pieces. Take a sub-sample of particular size and wrap it in a piece of cheese cloth, forming a loose ball.
2. Make sure the funnel is clean. Place with water until it reaches up to 1 cm below the rim.
3. Be careful to avoid formation of air bubbles. Make sure that the clip closes well and that the rubber tube does not leak. Tap some water when an air bubble has formed.
4. Hang the cheesecloth with the sample in the funnel so that the sample is totally submerged, without touching the bottom of the funnel. Nematode will crawl out of the material into the water and settle.
5. After a period of 16-72 hours the nematode suspension can be tapped by opening the squeezer clip.
6. Regularly tapping and adding with increase nematode vitality.
7. Instead of wrapping the sample in cheese cloth, it can also be spread out on a sieve, which is just touching the water level.
8. The extraction surface is larger compared to the cheese cloth and extraction efficiency a little higher.
9. Make sure the sample never becomes dry.

Mounting

Nematode mounts can be made on ordinary glass slides or on Cobb slides (aluminium double cover slip slides'). When carrying out routine analysis of nematode communities, it is common to use mass mounts enclosing hundreds of nematodes on one large (76 x 50 mm) glass slide **(Bongers *et al.*, 1989).**

Temporary mounts

Mount in water

Living nematodes can be easily identified and studied in a water mount. In this type of mount, a number of structures like the spear, the

esophageal lumen, and the excretory pore can be seen more easily than in dead and fixed nematodes. Water mounts are usually made on common glass slides, with a paraffin ring. They are sealed with nail varnish or candle wax.

Procedure

1. Place a droplet of water on a glass slide
2. Put the nematode(s) with a handling needle in the center of the water droplet. Make sure that they do not float, but remain on the bottom
3. Apply the cover slip with a pair of forceps. Remove excess water with a filter paper and seal the cover slip with nail varnish or candle wax (fix it first at three points, and then apply the whole ring). Let the ring dry well (10 minutes) before placing the mount under a microscope.

Staining of root material

Staining simplifies microscope dissection of nematode infection. Staining agents which stain nematode are used. Plant tissues are not, or only slightly stained. Examples are cotton blue, acid fuchsin and iodine.

Sodium hypochlorite acid-fuschin method (Byrd et al., 1983).

Clearing root tissue

1. Wash roots with water and place them in a 150 ml beaker. Large root systems may be cut into sections for staining.
2. Add 50 ml of tap water and an appropriate amount of chlorine bleach (5.25% NaOCI) to clear the root tissue Soak the roots for 4 minutes in the NaOCl solution and agitate occasionally.

Staining nematodes in root

1. Rinse the roots with running tap water for about 45 seconds and then immerse them in water for 15 minutes to remove any residual NaOCl which may affect staining with acid fuchsin.
2. Drain the water and transfer the roots to a glass beaker with 30-50 ml of tap water
3. Add 1 ml of stock acid-fuchsin stain solution to the water and boil for about 30 seconds on a hotplate or in a microwave oven.
4. To prepare stock acid-fuchsin solution, dissolve 3.5 g acid fuchsin in 250 ml acetic acid and 750 ml distilled water
5. Cool the solution to room temperature, drain the stain solution, and rinse the roots in running tap water

Destaining the roots

1. Destain the roots by boiling in 20-30 ml of glycerin acidified with a few drops of 5 N HCI
2. Distribute the roots in a small amount of glycerin on a Petri dish cover, gently press against the cover with a Petri-dish bottom and observe under a dissecting microscope. A pair of glass plates may be used instead of Petri dishes.
3. Roots may be stored in acidified glycerin with little loss of contrast between nematodes and roots

Effect of citrus peel extracts on egg hatching

Each BP1 dish received an aliquot of 0.3 ml of extracts. There were ten egg masses picked into the extract. In place of the extracts for the control, distilled water was used. After being placed in a small Petri dish (5.7 diam.) and being sealed with parafilm, the BPI dish was incubated at 28°C and monitored after 24 and 72 hours. The number of nematodes that hatched throughout the treatment periods was tallied, and the egg masses for the reversibility tests were transferred from the extracts into distilled water at 24 -72 hours following treatment. The egg masses were transferred to other BPI dishes to continue hatching after the number of nematodes that hatched in the control during the 24 h and 72 h treatment periods was counted. Once the extracts were removed, the number of nematodes that hatched was recorded. The number of juveniles hatched in the extract during the treatment period was divided by the number hatched in the control

to determine the percentage of egg hatch inhibition by the extracts. By dividing the number of juveniles that hatched after being transferred from the extracts into distilled water by the number that hatched in the control for the same amount of time, the percentage of inhibition of egg hatch after the removal of the extracts was calculated. The fresh peel extracts underwent testing. Each treatment had four replications. Two times the experiment was conducted.

Effect of citrus peel extract of juvinels

For the test, aliquots of 6 ml of the filtrates and 0.2 ml of the nematode suspension were pipetted into a little Petri dish (5.4 cm in diameter). The Petri plates were covered with Parafilm a dissecting microscope. Nematodes that responded to touch but did not die were categorized as paralyzed. To verify the paralyzation effect, the immobile worms were transferred to distilled water and the live and dead nematodes were counted after 24 hours. The filtrates of the fresh peels underwent the testing. In place of the extracts, distilled water was utilized for the control. Each treatment received four replicates, and the experiment was run twice.

Figure 1

Nematode soil and root

The fruit peel were collected and sterilized and made into powder. **(Figure 3, 4, 5, 6)**

Effect of citrus peel extract on infectivity

After adding 30 ml of fruit peel extract to 150 g of sterile sands in a plastic cup (175 ml), an aliquot of 1 ml of nematode suspension (1000 nematodes/ml) was added. In place of the extracts, tap water was utilized as the control. To stop evaporation, the cups were then fastened with a rubber band and saran wrap. They were kept in a growth room at 28 °C for two days.

Results

Citrus is an important plant used in Ayurvedha and Siddha system of medicine. The antinematicidal study of citrus peel has been summarized here.

Collection of nematode sample and peels

The soil and roots were collected from the agricultural field, Tiruvannamalai district Tamil Nadu. **(Figure 1, 2)**

Figure 2

Nematode infected root

Figure 3
Orange peel

Figure 4
Lemon peel

Figure 5
Orange peel powder

Figure 6
Lemon peel powder

Preparation of different solvent for extraction of fruit peel

The citrus sinensis (orange) ang citrus limon (lemon) peel powder was extracted with different solvent such as methanol, chloroform and water. **(Figure 7, 8, 9)**

Figure 7
Methanol extract

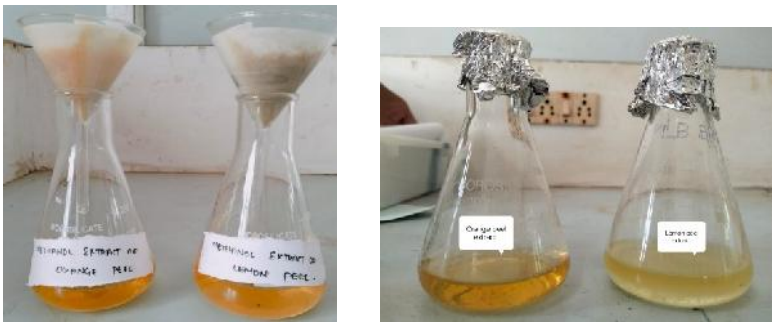


Figure 8
Chloroform extract

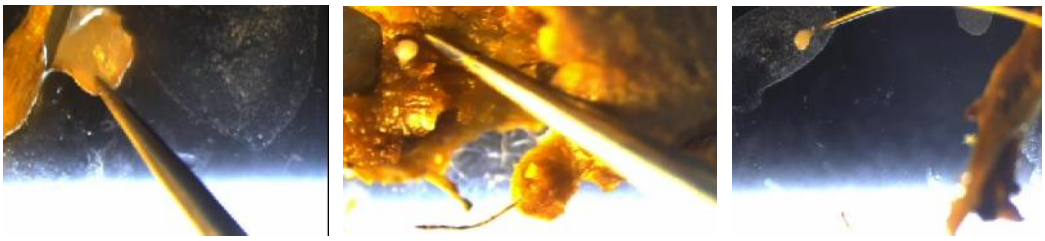
Figure 9
Aqueous extract

Extraction of nematode from plant root materials

In plant root, active stage of nematode was observed under dissecting microscope. **(Figure 10)**

Figure 10

Dissecting nematode



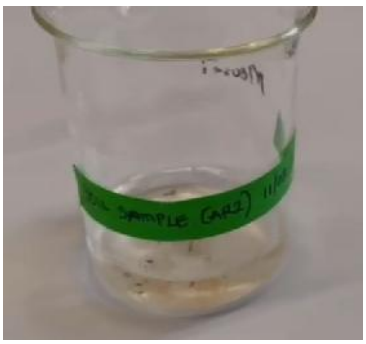
a **b** **c**

a) Dissecting root
b) Female nematode identified
c) Separate the nematode

Isolation of nematode from Baermann funnel method

In this method, nematode containing liquid was obtained. The result were represented in (**Figure 11).**

Figure 11
Baermann funnel method Sample extraction



Mounting

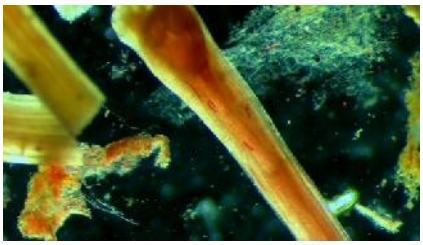
The living nematode were identified by water mount method. It was represented in (**Figure 12).**

Figure 12
Water mount

Acid fuschin staining of root material

The Acid-fuschin stain root material was observed. Nematode gives us non-acid fuschin and it appear in pink colour. It was represented as (**Figure 13).**

Figure 13
Acid fuschin staining root material



Antinematicidal activity of citrus peels against different stages of nematode

Effect of citrus peel extract on egg hatching, juvinels, adult

In hatch of eggs after 24 hours treatment of orange peel gave more death compare to Lemon peel. **(Table 1)**

Table – 1

The effect of extract of fresh citrus peel on egg for 24 hours

Peel	Dead	Paralysed	Active
Lemon peel	22	60	18
Orange peel	15	80	5

The fresh peel extract of Lemon cause 90 % of juveniles was paralyzed after exposure to 24 hours. The fresh peel of orange extract cause 63 % of juveniles paralyzed after exposure to 24 hours. **(Table 2)**

Table - 2
The effect of extract of fresh citrus peel on juveniles for 24 hours

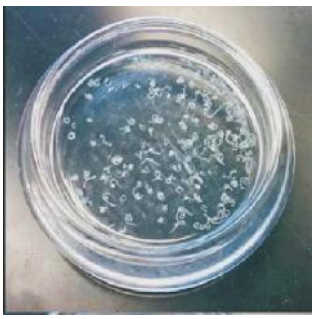
Peel	Dead	Paralysed	Active
Lemon peel	48	42	10
Orange peel	30	63	7

In adult nematode exposure to Orange and Lemon peel for 24 hours to 72 hours, after treatment Lemon extract produce high amount death and paralysis compare to orange extract. **(Table 3)**

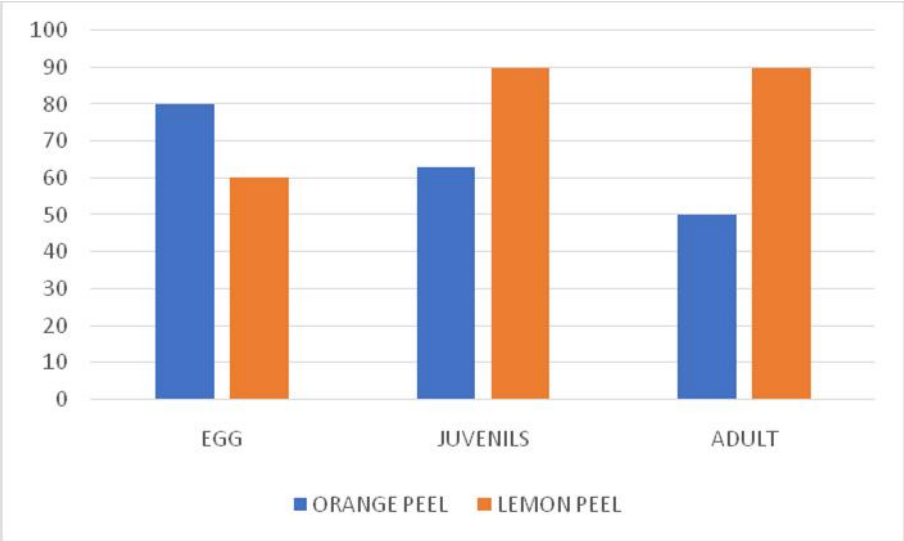
Table - 3
The effect of extract of fresh citrus peel on adult for 24 hours

Peel	Dead	Paralysed	Active
Lemon Peel	45	50	5
Orange Peel	36	50	14

Figure 14
Antinematicidal activity



Graphical representation of effect of lemon and orange peel extract on nematode



Discussion

The results were presented in previous study, provided the information on nematicidal activity of fruit citrus peel against root knot nematodes.

In this present study, the nematode sample were collected from soil and roots of agricultural field. The fruit peels were collected, sterilized and made into powder, then the extraction were prepared using different solvent. This extract used for antinematicidal activity.

Wanli cheng *et al* in 2020, used natural product furfural acetone as nematicide.

In this work, we used Baermann funnel method for isolation of nematodes. The living nematodes were identified by mounting method.

Baermann in 1917 discovered this method for isolation of nematodes. The mounting method to identify the living nematode was discovered by **Jacob and Van Bezooijien in 1984.**

In previous investigation **Bie Yun Tsai** used to stain the nematodes by sodium hypochlorite acid fuchsin method. For this reference we used to stain the root material and observe the non-acid fuchsin nematode as pink in colour.

Gudddadarangavvanahally used citrus peel for antibacterial activity of *Staphylococcus aureus*

Mshari, Ahmed used the citrus peels against plant pathogenic fungi *Alternaria solani*
For this, we used to study the citrus peel against root knot nematodes. This works the extract of citrus peels give more antinematicidal activity against egg hatching, juveniles, adults.

Conclusion

A work was made to nematicidal activity of fruit citrus peels against root knot nematodes. The nematodes were collected from agricultural field, soil and roots. It was identified by Baermann funnel method, mounting and sodium hypochlorite acid fuschin method.

The citrus peel were collected, dried sterilized, powered and made into extract with different solvents. Then the effect of citrus peel extract was on juveniles, hatch of eggs and adult nematodes.

From all the extract, the lemon extract active against juveniles' stage of nematodes. The orange peel extract more effective against eggs hatching. In adult stage lemon extract have more nematicidal activity.

For all citrus peel extract, lemon peel extract had more nematicidal activity. The results obtained from present work was concluded, the lemon peel extract have high nematicidal property. Instead of using chemical nematicides, we use lemon peel extract or lemon peels used to kill the nematodes in agricultural fields, in a very low cost or waste materials. It is more environmentally safe and do not cause any harm to soil.

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