International Journal of Agriculture and Plant Science
www.agriculturejournal.in
Online ISSN: 2664-7664, Print ISSN: 2664-7656
Received: 15-07-2022, Accepted: 02-08-2022, Published: 18-08-2022
Volume 4, Issue 2, 2022, Page No. 1-7

Melia azedarach L. extracts and their activities on common bacteria and *Caenorhabditis elegans*

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Abstract
Objective: To explore the antibacterial and nematode resistant activities of *Melia azedarach* fruits and *Melia azedarach* barks.
Methods: The crude extracts were prepared by Soxhlet extraction with 70% ethanol as solvent. Then the purified samples were dynamically adsorbed by S-8 macroporous resin; The antibacterial effect of the crude extracts of *Melia azedarach* fruits and *Melia azedarach* barks and their purified samples were determined by drilling method, and the MIC value of sensitive strains was determined by double dilution method; The half lethal concentration of *Melia azedarach* purified samples was determined by 96 well plate method. The results showed that the crude extract and purified sample of *Melia azedarach* barks had good antibacterial effect. The antibacterial effect of purified sample of *Melia azedarach* barks on the tested strains: *Enterobacter cloacae* > *Salmonella enteritidis* > *Escherichia coli* > *Enterobacter aerogenes* > *Pseudomonas aeruginosa* > *Xanthomonas oryzae pv.oryzae* > *Pseudomonas solanacarum*. The antibacterial effect of the crude extract of *Melia azedarach* barks was *Escherichia coli* > *Enterobacter aerogenes* > *Salmonella enteritidis*. The purified samples of *Melia azedarach* fruits and *Melia azedarach* barks had good killing effect on *Caenorhabditis elegans*, and the half lethal concentrations were 7 and 2.12 mg/ml respectively.

Keywords: *Melia azedarach L*., toosendanin, antibacterial activity, nematicidal activity

Introduction
Melia azedaeach L is a Melia plant of Meliaceae, which is extremely rich in plant resources. It is distributed in Hebei in the north, Yunnan and Guangxi in the south, and Sichuan in the West. It is mainly produced in Shanxi, Gansu, Shandong, Jiangsu, Zhejiang, Hunan, Guangdong, Guangxi, Yunnan, Hubei, Guizhou and other places, and this species has many local names, such as *Melia azedarach*, forest tree, purple flower tree, golden umbellate, emerald tree, fire twist tree, etc [1]. Both Shennong materia medica and tujing materia medica contain neem, which is bitter and cold in nature and belongs to the liver, spleen and stomach meridians. It is commonly used in the folk for heartache, colic, vermin and other diseases, and externally for the treatment of acne, scabies and epilepsy [2]. The main organic molecules of Melia azedarach fruits include terpenoids, flavonoids, steroids, acids, anthraquinones, alkaloids, saponins and tannins [3]. Among the various active ingredients of *Melia azedarach*, Azadirachtin is the most important active ingredient. It has toxic and killing effects on a variety of pests, mainly including repelling insects, resisting food, inhibiting the growth and development of insects, contact killing, internal inhalation and other toxic effects [4]. In addition, Melia azedarach also has the effects of relieving pain and killing insects. It is clinically used to treat abdominal and flank pain, colic, vermiform abdominal pain and chilblain [5]. At present, a number of studies at home and abroad have shown that *Melia azedarach* fruits have antibacterial and insecticidal effects.

Materials and Methods
1. Materials
1.1 Biomaterials
(1) Plant materials: *Melia azedarach* fruits was collected from Mianyang City, Sichuan Province, China. *Melia azedarach* barks was purchased from deshantang, a Qianjin Yaofang, Anhui Province, China.
(2) The tested strains: *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Staphylococcus aureus*, *Xanthomonas Campestris pv.citri*, *Xanthomonas oryzae pv.oryzae*, *Pseudomonas solanacarum*, *Salmonella enteritidis* and *Pseudomonas aeruginosa* are provided by instructors.
(3) Caenorhabditis elegans is provided by Caenorhabditis genetics Center (CGC).

1.2 Standards
Toosendanin standard was purchased from Chengdu ruifensi Biotechnology Co., Ltd. (batch No.: rdd-c01602108025), with a purity of 98.0%.

1.3 Main reagents
Anhydrous ethanol (Chengdu synthetic industrial chemical Co., Ltd., batch No. 20201206, content ≥ 99.7%), methanol (Chengdu Jinshan Chemical Reagent Co., Ltd., batch No. 20210222, content ≥ 99.5%), thiazole blue (RON reagent, batch No. rh359996, purity 98%), cholesterol (Shanghai Lanji biology, content ≥ 95%), LB broth (Shanghai Bo microbiology Co., Ltd., batch No. 20210828). Beef extract, peptone and tryptone were purchased from Beijing aoboxing Biotechnology Co., Ltd. Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate, anhydrous calcium chloride, magnesium sulfate, PBS buffer. S-8 macroporous resin was purchased from Mianyang Yaoxin reagent.

1.4 Main instruments
1/10000 electronic balance (saidoris Scientific Instruments Co., Ltd., BSA224S-CW), intelligent temperature controller (Tianjin Tianfen analytical instrument factory, SB-2), box type resistance furnace (Tianjin taist Instrument Co., Ltd., SX-5-12), thin layer chromatograph (Tianjin Tianfen analytical instrument factory, SB-2), rotary evaporator (Shanghai Yarong biochemical instrument factory, RE-52AA) Dual ternary / two-dimensional liquid chromatograph (Thermo Fisher, ultimate 3000DGLC), single person double-sided purification workbench (Suzhou purification equipment Co., Ltd., SW-CJ-1F), constant temperature incubator (Shanghai Yiheng Scientific Instruments Co., Ltd., BPH-9162), high-pressure steam sterilizer (Sanyo Electric Co., Ltd., MLS-3780).

2. Methods
2.1 Pretreatment of *Melia azedarach* material
The *Melia azedarach* fruits and *Melia azedarach* barks are crushed in a pulverizer, then passed through a 60 mesh screen and sealed for storage. Melia azedarach fruits were dried in oven at 60℃ for 48h.

2.2 Preparation of crude extract of *Melia azedarach*
Refer to the method of Zhenggang Wang *et al* [6]. Soxhlet extraction was used, 70% ethanol was used as solvent, and the ratio of material to liquid was 1:15. The crude extracts were obtained by heating and reflux extraction for 6 hours and volatilizing the solvent.

2.3 Purification of crude extract of *Melia azedarach* by macroporous resin
Refer to the method of Zhenggang Wang *et al* [6]. The crude extract obtained by the method under "2.2.2" is dissolved in water and then filtered. The pretreated S-8 macroporous resin is used for purification through dynamic adsorption. Take 70% ethanol as eluent, start collecting when the outflow liquid shows clear blood red, stop collecting when it shows reddish or yellowish, and volatilize the solvent to obtain the purified sample.

2.4 Determination of toosendanin in purified samples by HPLC
Preparation of toosendanin reference solution: accurately weigh the toosendanin reference solution and add methanol solvent to prepare 1 mg/ml solution.
Preparation of purified sample solution: accurately weigh the purified sample of Melia azedarach obtained by the method under "2.2.3" and add methanol to prepare 20 mg/ml solution.
Chromatographic analysis conditions: refer to the detection method of toosendanin standard of Chengdu ruifensidan Biotechnology Co., Ltd. The chromatographic column is welchc18 column (250 mm × 4.6 mm, 5.0 μm) The mobile phase is acetonitrile water (45:55), the column temperature is 30℃, the detection wavelength is 210 nm, and the injection volume is 10 μl.

2.5 Antibacterial activity of Melia azedarach extract and purified samples
2.5.1 Bacterial activation
The bacteria preserved in the laboratory were inoculated into beef extract peptone liquid medium and cultured at 37℃ for 24 hours. Dilute the bacterial concentration to $5\times(10^7\sim10^8)$CFU/ml with sterile water by Michaelis Turbidimetry [8].

2.5.2 Sample preparation
The crude extracts of Melia azedarach barks and Melia azedarach fruits prepared under the method of "2.2.2" were prepared into 20 mg/ml solution with purified water as solvent, and the purified samples of Melia azedarach barks and Melia azedarach fruits obtained under the method of "2.2.3" were prepared into solutions with concentrations of 30, 25, 20, 15, 10, 5, 2.5, 1.25 mg/ml with purified water as solvent. These samples were also used for the determination of anti nematode activity.

2.5.3 Determination of antibacterial activity
Use the punching method, pour the plate, and after cooling, absorb 120 μL of E. coli solution or Staphylococcus aureus solution is evenly coated on the culture medium. Drill five 6 mm holes at an equal distance on each plate, three of which are filled with 20 μL of the crude extracts of *Melia azedarach* barks and *Melia azedarach* fruits with a concentration of 20 mg / ml and the samples purified by macroporous resin. Add 20 μL to the other two holes respectively purified water and 50 μg/ml neomycin was used as negative and positive control respectively.

Each strain was repeated 3 times. After 24 hours of incubation at 37℃, the size of bacteriostatic circle was measured and the bacteriostatic effects of four samples were compared.

2.5.4 Determination of antibacterial spectrum

The sample with a concentration of 20 mg/ml that has a good antibacterial effect on Escherichia coli or Staphylococcus aureus is subjected to the same operation as "2.2.5.3" for Enterobacter aerogenes and other 7 kinds of bacteria, and the antibacterial circle is determined to screen out sensitive strains.

2.5.5 Determination of minimum inhibitory concentration (MIC)

Refer to Zhong Xiwen's method and make some changes [7]. Take 8 test tubes, add 1ml LB liquid culture medium to each tube, add 1ml of 20 mg/ml sample to the first tube, shake well, suck 1ml and move to the next tube. Use the same operation until the 7th tube. Suck 1 ml from the seventh tube and remove the solution after mixing. Tube 8 is blank control. The drug concentrations in each test tube were 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0.000 mg/ml respectively. Finally, add 20 μL to each tube And shake well. Each strain is set up for 3 repetitions, cultured in a 37 ℃ incubator for 24 hours, and then added with 20 μL of MTT (5 mg/ml), continue to culture for 1 hour and observe the color change. The minimum concentration of MTT without discoloration is the minimum inhibitory concentration.

2.6 Anti nematode activity of purified samples of *Melia azedarach*

2.6.1 Nematode culture

Take activated Escherichia coli (op50) 80 μL apply it to NGM medium and culture it in 22℃ medium for 24h [8]. Use the tweezers after burning and cooling to draw 1 × 1cm square pieces from the purchased medium containing a large amount of Caenorhabditis elegans, picked out into NGM medium containing Escherichia coli. When placing the square block, closely fit the wired worm surface with the E. coli surface of NGM medium. When placing the square block, closely fit the wired worm surface with the Escherichia coli surface of NGM medium. Incubate at 22 ℃. Rinse the NGM medium containing nematodes with sterile water, and dilute the washed nematode solution to 50 μL contains 20~60 nematodes.

2.6.2 Determination of anti nematode activity

Add 50μL of nematode liquid to 96 well plate and record the number of live insects under the microscope, and then add 50μL purified samples of *Melia azedarach* were cultured in a 22℃ incubator for 24 hours, and the number of dead nematodes was observed and recorded under the microscope. At 50μL purified water is the blank control. Repeat each group three times. Calculate the mortality or adjusted mortality according to the following formula. Repeat the above method with different concentrations of *Melia azedarach* purified samples to determine the half lethal concentration IC50. The mortality of blank control was less than 5%, and no correction was needed.

$$\text{Nematode mortality} = \frac{\text{Nematode death}}{\textit{The total nematodes}} \times 100\%$$

$$\text{Nematode corrected mortality} = \frac{\text{Handing nematode mortality} - \text{Control nematode mortality}}{1 - \textit{Control nematode mortality}} \times 100\%$$

Results and analysis

1. Determination results of toosendanin in purified Melia azedarach samples by HPLC

The results showed that under this chromatographic condition, toosendanin in the purified samples of Melia azedarach barks and Melia azedarach fruits was well separated. Because toosendanin contains isomers, it contains two peaks with retention times of about 7.85min and 9.58min respectively. The determination results of the purified sample of Melia azedarach barks showed that it contained two peaks, and the retention time was basically the same as that of the standard, while the purified sample of Melia azedarach fruits macroporous resin contained only one peak, and the retention time was about 7.8min, which was basically the same as that of the first peak of the standard. Therefore, the purified samples of Melia azedarach barks and Melia azedarach fruits contain toosendanin. The measurement results are shown in Figure 1.

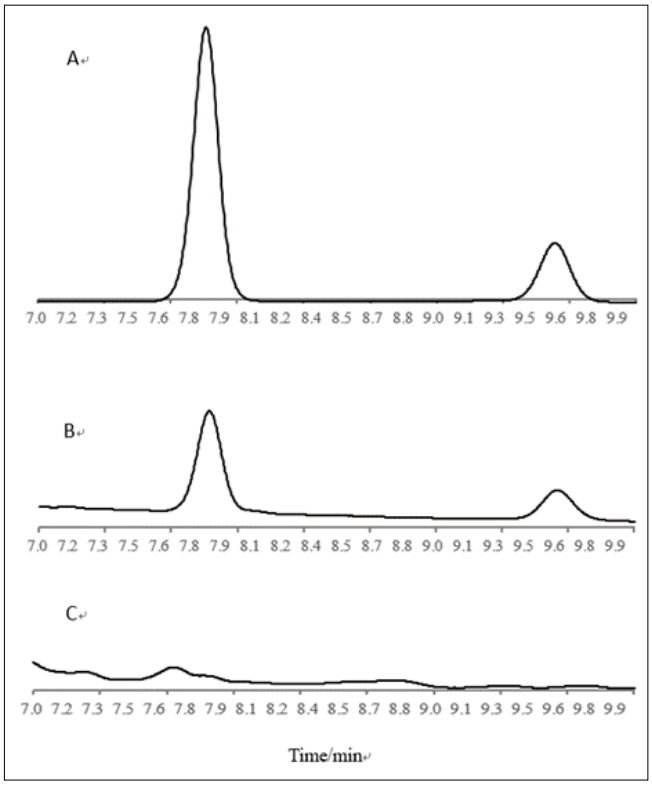

Fig 1: chromatographic peak of toosendanin in Melia azedarach purified sample

A. Toosendanin standard; B. Purified samples of Melia azedarach barks with macroporous resin; C. Purification samples of Melia azedarach fruits by macroporous resin

2. Antibacterial activity of Melia azedarach fruits and Melia azedarach barks

2.1. Antibacterial activity of Melia azedarach against Escherichia coli and Staphylococcus aureus

The antibacterial activity of the crude extracts and purified samples of *Melia azedarach* fruits and *Melia azedarach* barks against Escherichia coli and Staphylococcus aureus was determined by drilling method, and the extracts with antibacterial activity were screened out. The results are shown in Table 1. With purified water and 50 μg/ml neomycin was used as the negative and positive control. When the test concentration was 20mg/ml, only the crude extract of *Melia azedarach* barks and the purified sample had a certain antibacterial effect on Escherichia coli, and the diameter of its antibacterial circle was 13.96 and 13.43mm respectively, while the extract of *Melia azedarach* fruits had no antibacterial effect on both bacteria. Therefore, the extract of *Melia azedarach* barks was screened to have antibacterial activity.

Table 1: inhibitory effect of Melia azedarach barks and Melia azedarach fruits extracts on Escherichia coli and Staphylococcus aureus

Sample	Bacteriostatic circle diameter (mm)	
	Escherichia coli	**Staphylococcus aureus** $^{+}$
Crude extract of Melia azedarach barks	13.96 ± 0.39 b	—
Melia azedarach barks purified sample	13.43 ± 2.70 b	—
Crude extract of Melia azedarach fruits	—	—
Purified samples of Melia azedarach fruits	—	—
Neomycin（50 μg/mL）	24.92 ± 1.73 a	21.12 ± 1.41
Purified water	—	—

Note: $^{+}$ indicates Gram-positive bacteria; The data is the mean ± SD of three repeated determinations; Lowercase letters indicate the significant difference between treatments (LSD, $P < 0.05$), and the same lowercase letters in the same column indicate that there is no significant difference between treatments; - Indicates that there is no bacteriostatic circle.

2.2 Antibacterial spectrum of Melia azedarach

The antibacterial effect of the crude extract of Melia azedarach barks and purified samples on other bacteria is shown in Table 2. When the final concentration for the test is 20mg/ml, the crude extract of Melia azedarach barks has antibacterial effect only on Salmonella enteritidis and Enterobacter aerogenes, and the diameter of the antibacterial circle is 11.11 and 11.85mm respectively. The purified sample has antibacterial effect on other common bacteria except *Xanthomonas Campestris pv.citri*, and the antibacterial effect on Enterobacter cloacae is

the most obvious. The diameter of the antibacterial circle is 15.85mm, and the antibacterial effect on *Pseudomonas solanacarum* is the worst, with the diameter of the antibacterial circle being 11.43mm.

Table 2 antibacterial activity of crude extract and purified samples of Melia azedarach barks

Tested bacteria	Bacteriostatic circle diameter (mm)			
	Crude extract of Melia azedarach barks	Melia azedarach barks purified sample	Neomycin	Purified water
Xanthomonas oryzae pv.oryzae	—	12.34 ± 0.78 ab	25.29 ± 3.91	—
Xanthomonas Campestris pv.citri	—	—	22.40 ± 2.16	—
Pseudomonas solanacarum	—	11.43 ± 0.64 b	24.90 ± 0.35	—
Salmonella enteritidis	11.11 ± 0.36	13.84 ± 0.54 ab	23.92 ± 2.90	—
Pseudomonas aeruginosa	—	12.34 ± 0.06 ab	21.54 ± 1.34	—
Enterobacter aerogenes $^{+}$	11.85 ± 0.53	12.66 ± 0.62 ab	22.85 ± 1.35	—
Enterobacter cloacae	—	15.85 ± 3.10 a	25.08 ± 2.18	—

Note: $^{+}$ indicates Gram-positive bacteria; The data is the mean ± SD of three repeated determinations; Lowercase letters indicate the significant difference between treatments (LSD, $P < 0.05$), and the same lowercase letters in the same column indicate that there is no significant difference between treatments; - Indicates that there is no bacteriostatic circle.

2.3 Minimum inhibitory concentration of Melia azedarach extract and purified samples

The MIC of the screened sensitive strains was determined by double dilution method. The results are shown in Table 3. The MIC of the crude extract of Melia azedarach barks to Salmonella enteritidis was 5mg/ml, while the minimum inhibitory concentration (MIC) to Escherichia coli and Enterobacter aerogenes were 2.5 and 1.25mg/ml, respectively. The purified samples of Melia azedarach barks showed good inhibitory effect on sensitive strains, and the MIC of *Xanthomonas oryzae pv.oryzae* was 0.625mg/ml, which was the lowest.

Table 3 minimum inhibitory concentration of Melia azedarach extract on sensitive bacteria

Tested bacteria	MIC (mg/mL)	
	Crude extract of Melia azedarach barks	Melia azedarach barks purified sample
Xanthomonas oryzae pv.oryzae	>10	0.625
Pseudomonas solanacarum	>10	1.25
Salmonella enteritidis	5	2.5
Pseudomonas aeruginosa	>10	2.5
Escherichia coli	2.5	2.5
Enterobacter aerogenes	1.25	1.25
Enterobacter cloacae	>10	2.5

3. Anti nematode activity of Melia azedarach fruits and Melia azedarach barks

The lethal effect of purified samples of Melia azedarach at different concentrations on Caenorhabditis elegans is shown in Figure 2 and Figure 3. The linear regression equation of lethal effect of Melia azedarach barks sample is $y=32.432\ln(x)+25.649$, and the half lethal concentration (IC50) is 2.12mg/ml. The linear regression equation of the lethal effect of Melia azedarach fruits sample is $y=57.096\ln(x)-61.065$, and the half lethal concentration (IC50) is 7.00mg/ml.

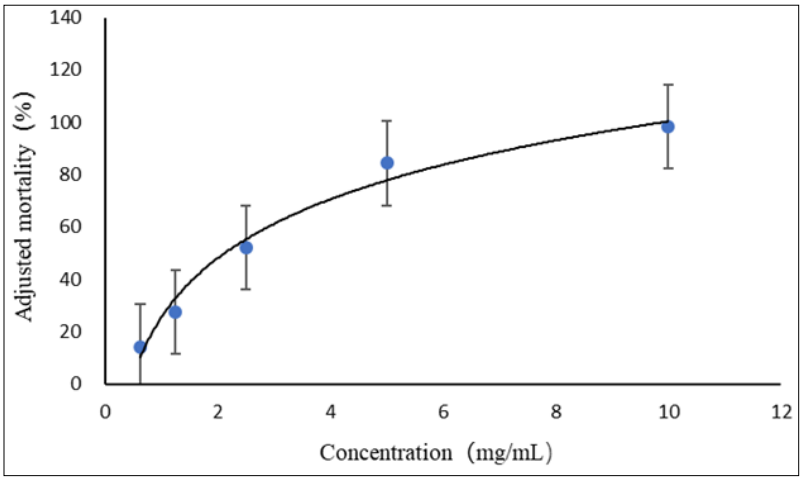

Fig 2: lethal rate of Caenorhabditis elegans treated with different concentrations of Melia azedarach barks purified samples

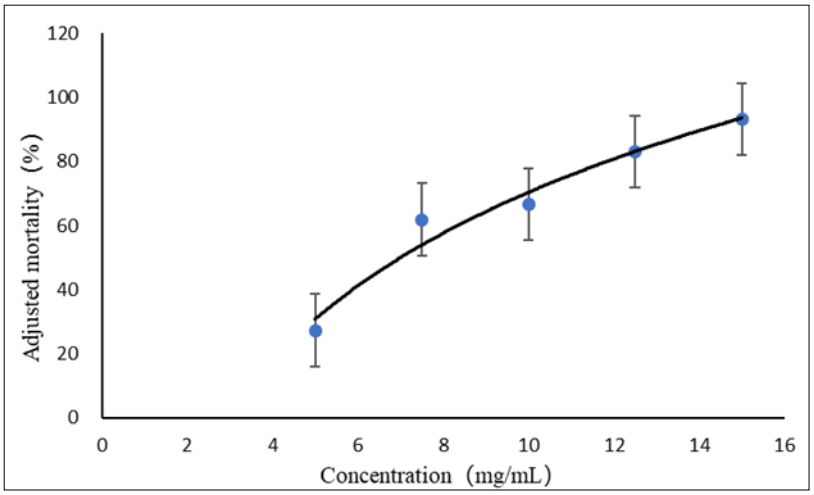

Fig 3: lethal rate of Caenorhabditis elegans treated with different concentrations of Melia azedarach fruits purified samples

Discussion

Toosendanin has a semi acetal structure, and there are always two tautomers [9]. There is only one peak in the HPLC chromatogram of toosendanin in the purified sample of Melia azedarach fruits, which may be caused by the low content of toosendanin in the material, partial degradation during extraction, and poor purification effect. Studies have shown that the content of toosendanin is related to the origin, freshness and harvest time of materials. The content of toosendanin in Melia azedarach fruits from different sources varies greatly [10], and the fresher the Melia azedarach fruits is, the higher the content is [11], and the content of toosendanin is the highest when harvested between January and February. After February, the fruits falls off, and the content of toosendanin decreases [12]. The materials used in the experiment are picked up, stored for a long time, and the content of toosendanin is low. Solvent, temperature, pH value, light, ascorbic acid addition, common metal ions and other factors can affect the structure of toosendanin, leading to its decomposition [13].

The antibacterial test results showed that the extract of Melia azedarach fruits had no inhibitory effect on Escherichia coli and Staphylococcus aureus at the concentration of 20mg/ml. However, studies have shown that 0.25g/ml water extract of Melia azedarach fruits has a good antibacterial effect on gram-negative, positive bacteria and fungi, and its antibacterial effect on bacteria is better than fungi as a whole [14]. The main reason for this result is the degradation of effective components in Melia azedarach fruits or different extraction solvents. However, the antibacterial experiment of Melia azedarach extracted with different solvents showed that the antibacterial spectrum extracted with ethanol was broader and the antibacterial effect was better [15]. Toosendanin in Melia azedarach is the main antibacterial component, but hydroxycoumarin isolated from Melia azedarach fruits can enhance the antibacterial effect [16].

At present, nearly 100 species of plants in more than 40 families have been reported to have nematicidal activity, and there are more than 100 plant compounds with nematicidal activity in more than 10 categories [17]. As a plant insecticide, Melia azedarach has a strong ability to kill Caenorhabditis elegans. The half lethal concentration of the purified sample of Melia azedarach barks is only 2.3mg/ml. And studies have shown that Melia azedarach fruits and Melia azedarach barks also have obvious killing effect on pine wood nematodes. After ethanol extraction, they are prepared into a concentration of 100mg/ml, and the death rate of pine wood nematodes is as high as 90% [18]. Therefore, Melia azedarach has a good effect of killing nematodes.

Conclusion

Melia azedarach barks and its samples purified by macroporous resin have good antibacterial effect, but the antibacterial spectrum of Melia azedarach barks purified samples is broader. The diameter of the antibacterial circle of the crude extract of Melia azedarach barks is 11.11-13.96mm, and it is the most sensitive to Escherichia coli. The diameter of the bacteriostatic circle of the purified sample of Melia azedarach barks was 11.43-15.85mm, and it was the most sensitive to Enterobacter cloacae. The purified samples of Melia azedarach fruits and Melia azedarach barks have good killing effect on Caenorhabditis elegans, and the half lethal concentrations are 7 and 2.12 mg/ml respectively. The anti nematode activity of the purified samples of Melia azedarach barks is higher than that of the purified samples of Melia azedarach fruits.

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