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Molecular Diagnosis of Hymenolepisnanain Experimentally Infected Swiss Mice and Study the Effect of the Hot Aqueous Extract of Syzygiumaromaticum (clove) on the Worms

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Abstract.

The present work was initiated to provide a molecular diagnostic based evidence of the Hymenolepisnana experimental infection in 50 Swiss mice and to study the hypothetical effect of the clove (Syzygiumaromaticum) seed hot aqueous extract (CSE) on the worms in 25 experimentally infected mice. The mice were assigned randomly according to the type of treatment given to the mice into five even groups, CSE at 5mg/kg (5G), CSE at 10mg/kg (10G), CSE at 15mg/kg (15G), mebendazole at 40mg/kg (40G), and distilled water (DW) as a control group (CG), in which all groups were given treatments twice daily for 15 days. Here, the ITS-1 gene was handled as a genetic target for the parasitic diagnosis. The results unveiled that the incidence rate of H.nana was8% (4 out of 50mice). For the CSE effect on H. nana, no eggs were detected in the feces of the infected mice in the 15G on day 11thof treatment; however, for the mebendazole, this was only shown in 40G on day 13th of the treatment. For the rest of the groups, there were continuous detections of the eggs in the feces of the mice belonged to5G, 10G, and CG for the whole period of the treatment. Due analyses performed in this study, the CSE contained both the saponins, flavonoids, and terpenoids. In addition, the lethal and therapeutic doses of the extracted oil were also calculated as 225 and 2.25mg/kg, respectively. The current trial provides important treatment proofs for the treatment of infections caused by H. nana using the clove (Syzygiumaromaticum) seed hot aqueous extract.

Keywords. Aqueous Extract, Hymenolepisnana, Mebendazole, PCR, Syzygiumaromaticum.

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INTRODUCTION

Hymenolepis nana (Dwarf tapeworm)is one of the common infectious worms of highly public concerns. It is mostly common in countries of hot tropical and sub-tropical areas of the world [1], providing a suitable environment for the growth and development of the ova and embryo reaching the infective stage of the worm [2]. It is also widely spread in densely populated areas due to increases in direct exposure to the eggs that shorten the period of lifecycle[1].

The worm causes a few pathological changes due to its growth and development of cysticercoids in the intestinal villi. In the case of severe infection, the symptoms appear as a topical inflammation caused by the presence of the hooks in the scolex of the worm [1]**,** diarrhea, weight loss, and sometimes neurological symptoms[3]. The adult worm may remain in the intestinal ileum or migrate to the bile and pancreatic ducts [4].

Recently, it has been focused on the use of medicinal plants to treat many diseases because they contain effective substances with small concentrations that can interact with each other affecting the infective worms without leaving side effects compared with those induced by regular medicines[5]. This can include the anthelmintic activities of cold and hot aqueous and alcoholic extract of pumpkin (*Cucurbitapepo L.*) [5] and the anticestodal effects of aqueous extract of *C. pepo* on *H.nana* in mice[6].

Syzygiumaromaticum (clove) has many antiseptic properties that help in eliminating and preventing growth of germs, parasites, and intestinal worms. It has also been used in an oil form globally to ease toothache and inflammationdue to its vital constituent eugenol, an analgesic and anti-inflammatory agent [7].

MATERIALS AND METHODS

1) Lab animals

In the present study occurred between September, 2018 to June, 2019, Fifty healthy female mice (10-12 weeks old and 25-30gms of weight) were obtained from the College of Science, University of Al-Kufa City,Al-Najaf province, Iraq. The mice were transferred to the College of Science, University of Al-Qadisiyah, Diwaniyah City, Al-Diwaniyah province, Iraq. The animals were placed in plastic cages provided with food and water as ad libitum in healthy housing requirements (ventilation, light, temperature, and humidity). The animals spent 15 days of an acclimation period before launching the experiment. Examination of animal feces was done using formal-ether sedimentation methods (explained in details in a later section) to make sure the animals are parasite-egg free.

2) Source of H.nanaeggs

H.nana eggs were isolated from excreted fecal pellets (2gm) of seven naturally infected female mice. The pellets were exposed to the air at 27 to 28°C with relative humidity of 35 to 40% for 24hrs. The eggs were then

washed three times with the physiological saline solution and centrifuged at 1500rpm. The eggs were magnetic-stirred with one-millimeter-glass beads for10mins to remove the egg shells[8].

3) Experimental infection

After starving for 5hrs, the mice were oral-fed with a solution contained *H .nana* eggs (50 eggs/0.4ml) using a stomach tube connected to a 1-ml syringe[8]. The mice were left for three weeks followed by detecting the eggs of *H. nana* in the feces of those mice.

4) Diagnosis

- *Microscopic diagnosis*

The formal-ether sedimentation method included the use of 0.5gm of feces thoroughly mixed with 10ml of saline solution. Then, the emulsion was filtered by a layer of gauze into tubes which were centrifuged at 2000rpm for 10mins. The supernatant was removed, and the sediment was washed with 10ml of saline solution and centrifuged again. This washing step was repeated several times until getting a clear supernatant. After that, 10ml of 10% formalin was added to the sediment, mixed thoroughly, and left for 5mins for fixation purposes.Later,1-2ml of ethyl acetate was added, and the tubes were sealed up. The tubes were exposed to a strong mixing and centrifugation steps at 1500rpm for 10mins. Following that, four layers were built up (from top to bottom); ethyl acetate, debris, formalin, and sediment. The first three layers were completely got rid of keeping the sediment layer untouched which was later mixed with the rest of the liquid remained in the tube. Then, a drop of the precipitate was transferred by a pipette and mixed with a drop of saline solution and a drop of iodine solution, and the mixed-drop was placed on a glass slide, covered with a covers lip, and examined under alight microscope at 10X and 40X[9].

- *Molecular diagnosis*

A) DNA extraction

Extraction of the DNA from50fecal samples of the experimentally infected mice with *H. nana* was conducted using the Stool Genomic DNA Extraction Kit and its accompanied protocol (Bioneer, Korea).

B) Conventional polymerase chain reaction technique

A polymerase chain reaction (PCR) technique was performed using ITS-1 gene as a molecular target. The ITS-1 primers were F: 5- CGGAAGGGATACTTACACGTTC -3andR: 5-GCTCGACTCTTCATCGATCCACG-3 to amplify a region at 646bp.Using the Primer 3plus software, the primers were designed and purchasedfrom Pioneer Company, Korea.

C) Preparation of PCR master mix

PCR reaction mixture was prepared using the AccuPower® PCR Master Mix kit supplied by the Korean company Bioneer and according to the company instructions as shown in table (1).

Table 1. PCR master mix components

PCR master mix	Volume
DNA template	5µL
Forward primer 10pmol	1.5µL
Reverse primer 10pmol	1.5µL
PCR water	12µL
Total	20µL

Table 2. PCR Thermo cycler conditions

PCR Step	Repeat cycle	Temperature	Time
Initial denaturation	1	95 C	5 min
Denaturation	30	95 C	5 sec.
Annealing		58 C	30 sec
Extension		72 C	3 min
Final extension	1	72 C	10 min
Hold	-	4 C	Forever

5) Preparation of hot aqueous extract of S.aromaticum (clove) seeds

The preparation of CSE included grinding the clove seeds with an electric grinder followed by taking 10gm of the resulted dry powder and mixing it with 200ml of boiled DW. Then, the mixture was left for 24hrs for better extracting the active ingredients of the clove seed powder. After that, the solution was filtered with a filter paper (Wattman No.2) using a Buchner connector connected toan electric discharge device. Later, the solution was centrifuged at 300rpm for 15mins for obtaining a clear solution. Then, the solution was dried out using a rotary evaporator device under pressure at 45˚C followed by a complete drying process of the extract placed in a glass flask (known as weight) in the oven at 50˚C[10].

6) Effect of the hot aqueous extract of S.aromaticum (clove) seeds on H. nanain experimentally infected mice

After confirming that the mice were infected with *H. nana*(25mice), the infected mice were assigned randomly according to the type of treatment given to the mice into five even groups, CSE at 5mg/kg (5G), CSE at 10mg/kg (10G), CSE at 15mg/kg (15G), mebendazole at 40mg/kg (40G), and distilled water (DW) as a control group (CG), in which all groups were given treatments twice daily for 15 days.

7) Chemical detection of active substances in the hot aqueous extract of S.aromaticum(cloves) seeds

The chemical detection of some active substances in the CSE was carried out according to the method described in [11] as following:

A) Detection of tannins

The CSE at 0.5gm was placed in a tube and washed three times with petroleum ether and dissolved in 10ml of a hot saline solution. The solution was divided into two equal parts into two tubes, 2-3 drops of ferric chloride were added to one tube, and 2-3 drops of gelatin salts reagent were added to the other tube. The appearance of a dark blue color in one tube and turbidity in the other tube indicated the presence of tannins.

B) Detection of saponins

The CSE at 0.5gm was placed in a clean tube, and then 10ml of DW was added to this tube followed by well-shaking the tube for 30s. After that, the tube was left to settle for a while until foam formation that lasted for less than an hour. The appearance of the foam indicated the presence of saponins.

C) Detection of flavonoids

The CSE at 0.5gmwas washed three times with petroleum ether and dissolved in 30ml of 80% ethanol. The solution was filtered and used in the following tests:

1) The filter solution at 3mlwas put in a test tube followed by adding 1ml of 1% aluminum chloride solution into the tube. The appearance of a yellow color indicated the presence of flavones.
2) The filter solution at 2mlwas put in a test tube followed by adding 0.5ml of magnesium into the tube. The appearance of a pink or red color indicated the presence of flavones.

D) Detection of alkaloids

The CSE at 0.5gm was heated up with 5ml of 2N HCL in a water bath. The solution was shaken for 10mins followed by cooling down the solution. Then, the solution was drained and divided into two parts into two tubes, in which a few drops of the Meyer reagent were added into one tube, and a few drops of the Valsers reagent were added into the other tube. The appearance of a slight turbidity or a heavy deposit in any of the tubes indicated the presence of alkaloids.

E) Detection of Terpenes

The CSE at 0.5gm was washed three times with petroleum ether and dissolved in 10ml of chloroform. Then, 5ml of the solution were taken, and 0.5ml of acetic anhydride was mixed with it in a separate tube. Following that, three drops of sulfuric acid were added. The appearance of a pink to purple color indicated the presence of terpenes.

F) Detection of Glycosides

The CSE at 0.5gmwas boiled with 10ml of (0.5N)KOH that contained 1ml of 3% hydrogen peroxide solution. Then, it was extracted by mixing it with 10ml of benzene and then by mixing 5ml of the resulted solution with 3ml of 10% ammonium hydroxide solution. The solution was left to separate into two layers. The appearance of a pink or pink to red color in either layer indicated the presence of glycosides.

8) Isolation of volatile oil from S. aromaticum (clove) seeds

The extraction of the volatile oil included the use of 7gm of floral buds mixed with750ml of DWin a two-liter glass flask. The oil was prepared by a steam-distillation process. Then, the oil was collected and separated from the water using a separation funnel with n-hexane solvent. Later, the solvent was evaporated using a rotary evaporator at 40˚C and kept in the refrigerator at 4˚C until needed for the next analyses[11,12].

9) Determination of the lethal dose (LD_{50}) and the therapeutic dose of the volatile oil extracted from S.aromaticum (clove) seeds

The lethal dose (LD_{50}) and the therapeutic doses were calculated using 35 mice that were randomly divided into seven even groups of which six groups were given one of the following ascending concentrations (50,100,150,200,250, and 300 mg /kg)of thevolatile oil extracted from clove seeds. Thegroup No.7 was given DW as a control group[13]. A dose of 0.2ml/20gm/twice daily/for two days was given to the mice using a gastric tube,the LD_{50} andthe therapeutic doses were extracted according to the following formula:

LD_{50}= the dose that begins with killing all the animals in the group - total (difference between doses * the average of mortality between each consecutive group) / the number of animals in each group[14].

Therapeutic dose= 0.1 of lethal dose.

10) Statistical analysis

Data were analyzed using the statistical analysis of variance F-test under a probability level of$p \leq 0.05$ [15].

RESULTS AND DISCUSSION

1) Microscopic diagnosis

The results of the microscopic examination by the sedimentation method of the fifty experimentally infected mice showed that the number of infected mice was 25 (50%) mice. Figure (1) shows the presence of worm eggs that appear in a wheel-like shape in the fecal samples of those mice.

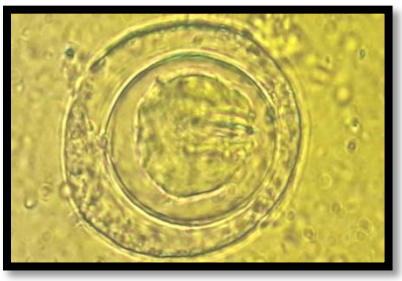

Figure 1. Egg of H. nana

2) Molecular diagnosis (Conventional-PCR)

The molecular methods are preferable over the serological methods in the diagnosis of living organisms because of the high sensitivity and specificity of those methods[16].The results of the molecular examination using the PCR for the fifty fecal samples of the experimentally infected mice indicated that the presence of the ITS-1 gene(646bp)was in 4(8%)samples as revealed in figure (2).

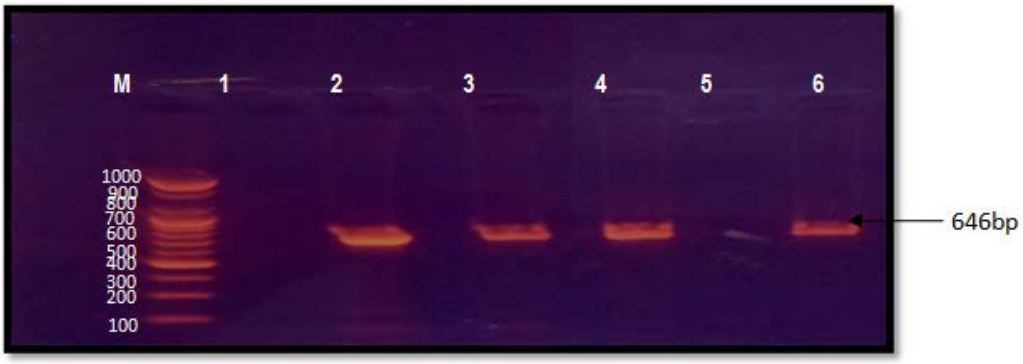

Figure 2. PCR-electrophoresis image. Columns 2,3,4, and 6 represent positive mouse fecal samples of the ITS-1 gene at 646bp. Column M represents the ladder at 1000-100bp.

In this study, it was found that the molecular detection rate, 8%, was lower compared to that, 50%,using the microscopic detection. This may be due to phenotypic similarity between *H. nana* and *H.diminuta* [17]or as a result of inaccurate microscopic diagnosis. The results of the current study do not agree with the findings in the study of [18] who showed that the PCR products of the mitochondrial C oxidase gene of *H.nana* (a rat isolate),*H. diminuta* (a ratisolate), and *H. nana*(a human isolate) were 700,702, and 715bp, respectively. Moreover, our data do not match up the results of study [19] that uncovered the sequences of *Cox1* (776-793bp) and *Pnad5* (816-846bp) genes of *H.nana* in rats and mice using a PCR technique and a sequencing method. This findings of the current study demonstrated that there were no differences between *H. nana* isolates from mice and rats.

3) Effect of a hot aqueous extract of S.aromaticum (clove) seedsonH. nanain experimentally infected mice

For the CSE effect on *H. nana*, no eggs were detected in the feces of the infected mice in the 15G on day 11thof treatment; however, for the mebendazole, this was only shown in 40G on day 13th of the treatment. For the

rest of the groups, there were continuous detections of the eggs in the feces of the mice belonged to 5G, 10G, and CG for the whole period of the treatment, table (3). This may be due to the presence of substances in the seeds of the clove such as eugenol substances that have a repellent effect against the worms [20].

Table 3. Shows the effect of the hot aqueous extract of S.aromaticum(clove) seeds and mebendazoleonH. nanain the experimentally infected Swiss mice.

Days / Type and concentration of treatments	Number of mice infected with *H. nana*							
	1st day	3rd day	5th day	7th day	9th day	11th day	13th day	15th day
5 mg / kg	5	5	5	5	5	5	5	4
10mg / kg	5	5	5	5	5	4	4	3
15 mg / kg	5	5	5	5	3	0	0	0
Mebendazole40 mg / kg	5	5	5	5	4	2	0	0
Distilled water	5	5	5	5	5	5	5	5
Total	25							

F-Calculated: 11. 8*F-F tabular: 9.4 *Significant at $P \leq 0.05$.

Eugenol has broad antimicrobial activities against fungi, acid-fact bacteria, as well as Gram-negative and Gram-positive bacteria. In addition, Asian cloves are considered as a drug that treats a number of infections such as cholera, tuberculosis, malaria, and scabies. In America, cloveis used for eliminating viruses, candida, bacteria, worms, and protozoa due to the presence of eugenol which is as an essential oil has been recognized to inhibit the growth of *Giardia*[21, 22]. It has been unveiled that the hot aqueous extract of *S.aromaticum* flowers inhibited bacterial species such as *Streptococcus spp., Staphylococcus sp., Escherichia coli, Pseudomonas sp., Proteus spp,* and *Klebsiellaspp.*[23].

However, the alcoholic extract of *S. aromaticum* was revealed to be able of killing the protoscoleces of *Echinococcusgranulosus* at a concentration of 1500µg/ml after 60mins [24].

For mebendazole, it inhibits the formation of microtubules in worms and decreases the absorption of glucose. This leads to depletion the parasite storage of collagen which reduces the energy levels in the form of ATP and eventually causing death of the parasite [25,26].

4) Chemical detection of active substances in the hot aqueous extract of S.aromaticum (clove) seeds.

The results of chemical detection indicated that CSE contained of saponins, terpenoids, and Flavonoids while did not have tannins, glycosides, and al-kaloids, table (4).

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Table 4. Chemical detection of active substances in the hot aqueous extractofcloves

Compounds	Result of tests
Tannins	-
Glycosides	-
Alkaloids	-
Flavonoids	+
Saponins	+
Terpenoids	+

The results of our study were different from those observed by other studies which indicated the presence of terpenoids, trigly cerides, and phenolic compounds in oils extracted from cloves [27]. Moreover, the presence of tripeptinoids, triterpens, and phenolic compounds in oils extracted from cloves was confirmed disagreeing with the current study findings[28].

5) Determination of the lethal dose (LD_{50}) and the therapeutic dose of the volatile oil extracted from S. aromaticum (clove) seeds

The results, table (5), showed the number of deaths in mice after giving different incremental concentrations of the volatile oil extracted from the cloves. The LD_{50}detected in the present work was225mg/kg of body weight, while the therapeutic dose was 2.25mg/kg of body weight.

Table 5. Determination of the LD50andthe therapeutic dose of the volatile oil extracted from cloves

Concentration (mg/kg)	No. of animals	No.of deaths within 48hrs	(Difference between concentrations)A	(Mean mortality between each consecutive group)B	B*A	Total B*A
50	5	0	50	0	0	375
100	5	0	50	0	0	
150	5	1	50	1+0/2=0.5	0.5*50=25	
200	5	1	50	1+1/2=1	1*50=50	
250	5	3	50	1+3/2=2	2*50=100	
300	5	5	50	3+5/2=4	4*50=200	
Distilled water	5	0	0	0	0	
Total	35					

LD_{50}=300mg/kg-375/5=225mg/kg.

The therapeutic dose = 2.25mg/kg.

CONCLUSION

In the present experimental study, we were successful in detecting *H. nana* in the experimentally infected Swiss mice using theITS-1 gene. Interestingly, there is an important effect of the hot aqueous extract of *S.aromaticum*(clove)seeds on *H. nana* in the experimentally infected Swiss mice. Moreover, the clove seeds have the volatile oil, flavonoids, saponins, and terpenoids in their components. Importantly, the $LD_{50}$of the volatile oilis 225mg/kg while the therapeutic dose is 2.25mg/kg.

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