

## Effectiveness of *Azadirachta Indica* (Neem) Plant Extract Compared with Albendazole on the Vitality of Hydatid Cyst *Protoscolices* (In Vitro)

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### Abstract

The cestode worm *Echinococcus granulosus* larval stage parasitically infects humans and animals' livers and lungs, causing cystic echinococcosis. Surgery with scolicidal agents to inactivate live protoscolices is the main treatment for cystic echinococcosis (CE). An extract of neem tree leaves was chosen to treat this parasite that causes this disease, and its effects were laboratory tested on the vitality of the primary fungi, which is infective to humans, taken from the livers of infected sheep in the holy governorate of Karbala. This study used four concentrations of alcoholic neem tree leaf extract (1.250, 2.500, 5.000, and 10.000) ppm to count protoscolices in 5 microliter volumes before and after treatment and compare them to albendazole. All concentrations of Neem leaves extract were effective, but 10,000 ppm was the most effective, as the percentage of mortality reached 100% after 30 minutes. The lower concentration of 1,250 ppm caused 90.62 % after 240 minutes. The above shows that Neem plant extracts inhibited protoscolices growth in all treatments. Due to its wide range of chemicals and biologically active compounds, which affect parasite cell mitochondrial membranes and cause apoptosis, many scholars call it the tree of the future.

### Introduction

A major parasitic disease is echinococcosis, or hydatidosis. Taeniid tapeworm metacestodes cause human echinococcosis, a zoonotic infection (Totomoch-Serra *et al.*, 2020). Echinococcosis was listed by (WHO) the World Health Organization (WHO) among the most neglected diseases because it causes devastating health, economic, and social consequences to billions of people (WHO, 2022). The parasite impacted numerous animal species, including both wildlife and livestock, and it also led to various zoonotic implications (Rialch *et al.*, 2018).

There are many variations related to this genus at the taxonomic level of species according to the term's morphology, modes of transmission, host specificity, antigenicity, and developmental phases (Lymbery and Thompson 2012). The genus *Echinococcus* was contained at least 10 valid species, each of them having different genotypes and strains, namely *E. multilocularis*, *E. vogeli*, *E. felidis*, *E. canadensis*, *E. equinus*, *E. shiquicus*, *E. ooligarchus* and *E. oligarhtus*, (Nakao *et al.*, 2013).

Every species needed an intermediate and definitive host. Carnivores like dogs carry this parasite in their small intestines. Domesticated and wild ruminants, humans, and camels are intermediate hosts for *Echinococcus* species. Adult worms are 2–11 mm long with 2–7 proglottid segments. A proglottid, or segment, has one genital opening and matures into the penultimate segment. Worm scolex has two rows of hooks (Jenkins *et al.*, 2005; Elham *et al.*, 2014).

The treatment of hydatid cysts includes two therapeutic options: chemotherapy and surgery (invasive or percutaneous) (Brunetti & Vuitton, 2010). Mebendazole and albendazole were chemotherapeutic agents for hydatid cysts. These drugs have a wide range of side effects, such as abdominal pain, abnormalities in liver function, diarrhea, dizziness, nausea, teratogenic effects, of headache (Maggiore *etal.*,2012)

Scolicidal agents can be used in surgery to minimize the impact of possible dissemination and spillage of the protoscolices. The scolicidal agent effectivity is characterized by testing at low concentrations for a short exposure time and should maintain the stability after mixing with cyst fluid (diluted), low cost, non-toxic, non-toxic and easily available (Anthony *et al.*,2005& Norouzi *et al.*,2020)

Currently, there is a herbal renaissance around the world to treat many diseases and alleviate the side effects resulting from expensive synthetic drugs (Raghvendra and Balsaraf, 2014). Medicinal plants are used as alternatives to Western medicine in many developing countries to treat many medical conditions. According to a survey conducted by the World Health Organization (WHO), 80% of the population uses traditional medical treatments and medicines to meet their health needs (World, 2003). due to little cost, fewer side effects, and wide availability. Between these medical plants is indica, or *Azadirachta indica* or neem tree, which belongs to the family Meliaceae. It is considered one of the most important species in all countries of the world (Dallaqua *etal.*, 2013). Among the famous names of the Neem tree in Europe and Asian countries are the Indian Lilac, Margosa tree, Al-Shareesh, the village pharmacy, and the natural medicine store, which It means treating and preventing most diseases (Islas *etal.* , 2020).

This study aimed to assess the in vitro scolicidal effects of the methanolic extract from the leaves of *Azadirachta indica* on the viability of hydatid cyst protoscolices.

## 2. Methods and Materials

**2.1. Plant collection:** Fresh (*Azadirachta indica*) (Neem) trees were obtained from Hilla Agricultural Nursery.

### 2.2. Preparation of *Azadirachta indica* Plant Extract

To remove all dust, neem leaf samples were washed with tap water and rinsed with distilled water. These leaf samples were dried in the shade for 10 days at room temperature before being ground into powder in a cylindrical grinder. China (Mainland) FZ-102 TCEP machine at 1400 r/min (Abed *etal.*, 2021).



Picture (1) *Azadirachta Indica* Leaves

### 2.3. Extraction of *Azadirachta Indica* Leaves

To extract neem leaves, 600 ml methanol was added to 60 g ground dried leaves. A shaking machine shook the mixture for 3 hours a day for 3 days to ensure homogeneity. The mixture was gravity-filtered into methanol. To remove solvent residue, a rotary evaporator at 50°C and 4.5 bar concentrated filtrate. The plant extract was concentrated and stored in an evaporating dish until use (Para gas *etal.*, 2018).

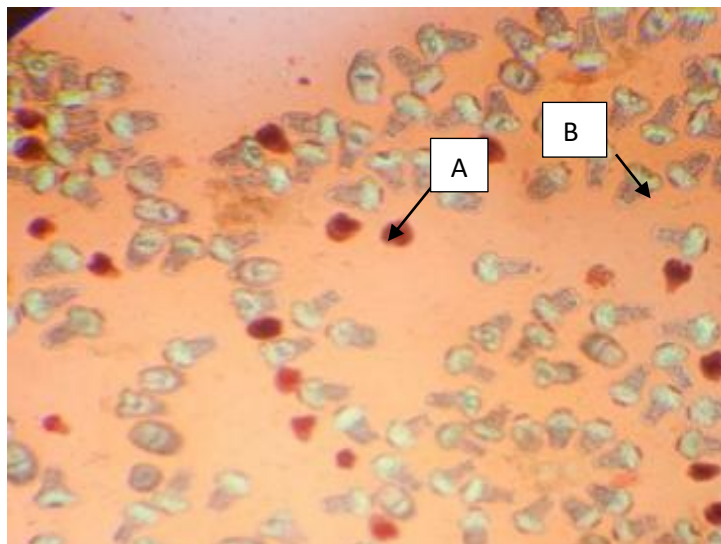
### 2.4. Collection of Protoscolices

A sample of protoscolices was collected from the livers of sheep that were infected with hydatid cysts and had been slaughtered in the Karbala Governorate. The sample was placed in a clean container and dispatched to the labs of the Environmental Health Department at the University of Karbala in cooled containers to ensure the protoscolices remained alive. Immediate action was required, beginning with the sterilization of the outer surface of the infected organ using an alcoholic iodine solution. An external incision was then made in the hydatid cyst, followed by washing the cyst to extract the liquid using a 10 ml syringe with a 21 G needle. The cystic fluid was further withdrawn from the inside with a Pasteur dropper, injecting it multiple times to maximize the collection of protoscolices attached to the germ layer. All liquid was then withdrawn and transferred to a glass beaker, where it was allowed to sit for about 10 minutes for the protoscolices to settle at the bottom. The excess liquid was subsequently discarded to concentrate the protoscolices to the desired volume (Smyth, 1985).

### 2.5. The Vitality Test of Protoscolices:

A constant volume transfer method was employed to assess the viability of protoscolices. 5 microliters of the protoscolices suspension were extracted and placed onto a microscope slide. An equal volume of aqueous eosin dye at a concentration of 0.1% was applied to the slide, and it was examined under 400x magnification. The dead protoscolices were counted, and the dye that penetrated their membranes turned them red (picture2). The living protoscolices, in contrast, appeared green. After determining its quantity in (5) microliters to convert and calculate it in one milliliter, multiply the

number by 100. The total number of protoscolices was calculated based on an average of three repeats (Landa-Garacia *et al.*, 1997). The penetration of aqueous eosin dye is a physical process, meaning that any physiological abnormality that arises is one of the factors contributing to increased permeability, which allows the dye to enter while the live protoscolices maintain their typical greenish color (Dehkordi *et al.*, 2019).



Picture( 2): Microscopic image of *Echinococcus granulosus* Protoscolices; A-live (non-stained) with 0.1% eosin stain (green) and B-dead (stained) red after neem plant extract treatment.

## 2.6. Preparing the Concentration of *Azadirachta Indica* (Neem) Plant Extract for this Study

The stock solution was prepared by dissolving 10 microliters of neem plant extract that was used in the study in 90 microliters of methane solvent; thus, we have prepared the stock solution at a concentration of 100,000 PPM, and from this stock solution were prepared concentrations (1,250, 2,500, 5,000, 10,000) PPM during the parasite treatment. Then, if the desired concentrations are obtained by diluting the stock solution with the alkaline liquid of granular forks, this is done by taking (10) microliters of the stock solution left over and (90) microliters of hydatid cyst liquid; thus, we obtained a concentration of (10,000) PPM. To obtain a concentration of (5.000) PPM, we add (5) microliters of neem extract to the (95) microliters of hydatid cyst fluid, as well as for the rest of the concentrations if the volume of plant extract is reduced and the volume of hydatid cyst liquid is increased (Roach and Tickner, 2004).

## 2.7. The Albendazole Drug

Albendazole was bought from a nearby pharmacy and came in an emulsified form. Four different concentrations were used in the neem plant extracts by using the dilution equation.

$$C1 * V1 = C2 * V2$$

$C1 = 100 \text{ mg}$  (this is the drug).

$C2 = 10 \text{ mg}$ , which is equivalent to 10,000 PPM.

$V2 = 1 \text{ ml}$  of hydatid cyst liquid volume.

$(V1) = 10.000 \text{ PPM}$ . (The desired volume was taken from the drug to prepare the concentration.)

Applying the equation results in the addition of (0.1) ml of the drug to (0.9) ml of hydatid cyst liquid. Thus, we got the concentration (10.000 PPM) as well as for the rest of the concentrations.

### **2.8. Effects of *Azadirachta Indica* (Neem) on the Vitality of Protoscolices:**

After the protoscolices were isolated from the infected organ (liver) and placed in a small centrifuge tubes the size is according to the desired concentration, and then the tubes were taught to ensure that they do not mix with each other in the water bath the water should be placed at a temperature of  $37 \text{ }^\circ\text{C}$  to maintain the vitality of the protoscolices and to be a temperature of the environment is similar to the temperature of the host, the time was fixed the moment the extracts was added to be directly performed calculation at time zero immediately after the treatment, a third withdrawal was made with the micropipette and a volume of (5) microliters in each time to represent a third replicate and placed on a glass slide and add the same volume of aqueous eosin formula at a concentration of (0.01) and then examined by compound microscopy and under a magnification power of (10 x) the red dead were recorded for the third time. This process was repeated for certain time periods.

### **2.9. Statistical Analysis**

The statistical program SPSS was used to perform statistical analyses (Morgan *et al.*, 2010).

## **3. Results and Discussions :-**

Surgery is the best treatment for many hydatid cyst infections, with varying degrees of success. Most medicines' metabolites, like benzimidazole, mebendazole, and albendazole, may be fatal (Hayajneh *et al.*, 2014). Store-bought albendazole is poorly absorbed by the digestive tract. The liver is the organ most affected by this parasite infection, so low liver concentration makes it less effective. Albendazole can cause allergic purpura, influenza-like illness, encephalitis syndrome, and drug rash (Karakay , 2000). This study examined neem extract's effect on hydatid cyst protoscolices.

Neem leaves extract's in vitro lethal effect on *Echinococcus granulosus protoscolex* was valuable and led to the discovery of a new side-effect-free drug. This study suggests that the extract's natural and bioactive compounds can kill protoscolices medicinally.

**3.1 Effect of *Azadirachta Indica* (Neem) Leaves Extract on the Vitality of Protozoa**

The table (3-1) shows that the percentages of living protozoa increased significantly over time when compared to the control, which only reached 42% after 240 minutes. The percentages reached 100% at 10,000 ppm after 120 minutes, followed by a high of 99.86% at 5,000 ppm after 240 minutes. The lowest percentages were at 1,250 ppm (90.62%) and 2,500 ppm (94.76%), respectively. Here, the effective effect of neem plant extract in getting rid of protozoa and in all concentrations was used. We see big differences in the concentration, which was 10,000 ppm right after the plant extract treatment and after 60 minutes when we compare the results to the L.S.D. at the probability level ( $P > 0.05$ ). There were also significant differences at the concentration of 5,000 ppm and after 120 min, as well as for the rest of the concentrations (1.250 and 2.50 ppm), as the plant extract had an effect with significant differences after 240 min.

Table (3-1) Effect of *Azadirachta indica* (Neem) leaves extract on vitality of protozoa of *E. granulosus*.

Concentrations	mortality percentage %				
	Immediately	30 min	60 min	120 min	240 min
0 PPM (control)	12.72	20.11	28.59	33.21	48.00
1.250 PPM	20.68	28.96	40.62	63.11	90.62
2.500 PPM	24.16	33.83	49.41	64.22	99.01
5.000 PPM	43.65	60.01	82.44	100.00	
10.000 PPM	52.87	100.00			
L.S.D.	41.22				

Aqueous and alcoholic plant extracts showed great effectiveness against many parasitic species, The aqueous and methanolic extracts of some herbs, including Amber Hyacinth, showed remarkable activity against the parasite *Cryptocaryon irritans* (Goto *et al.*, 2015).

The results achieved from our study are a result of the lethal effect of the plant extract of the Neem tree leaves on *Echinococcus granulosus*, and its ability to induce programmed cell death in protozoa cells, which reduces cell vitality and causes damage to the cell infrastructure. In addition, the essential oils from the green bean tree *Pistacia khinjuk* starch showed anti-worm activity against protozoa (Ozdikmenli and Zorba, 2014).

The study was conducted by (Saleem *et al.*,2018) indicated that Neem has a huge number of biological effects when used. Neem extracts are insecticidal, antibacterial, anti-inflammatory, and antiparasitic. In addition, some parts of neem can treat many diseases due to the therapeutic properties that this plant has. Such as antioxidants, antifungals, antimalarials, anticancers, and anti-inflammatory agents, it has antioxidants and anti-tumor properties, in addition to its role in treat many skin diseases (Ghosh *et al* 2016). The researchers (Aslam *et al*, 2010) mentioned that neem leaves possess many secondary receptors, such as calcium compounds, alkaloids, saponins, and alkaloids, which contribute to preventing the growth and development of microbes. Also, the study conducted by Udeinya *et al.*, (2008) showed that alcoholic and watery leaf extracts and neem bark are effective agents in combating

malaria, as some studies have highlighted shedding light on the preventive capabilities of the neem plant. Ashok Yadav *et al.*, (2017) pointed out the importance of neem as an effective antimalarial.

Also due to the presence of many components that had the ability to penetrate parasite membranes and lead to the occurrence of changes in the physiological state of parasites and destroy them at high concentrations (Dehkordi *et al.*, 2019).

Protoscolices' viability is strongly influenced by alkaloids. This depends on concentration variance (Delorenzi *et al.*, 2001). Alkaloids can damage cell organelles, destroy nuclei, and kill cells (Gidado *et al.*, 2007). The phenols activate the acetylcholine esterase enzyme, which controls cell membrane flexibility and permeability, causing the membrane to lose its permeable properties, allowing toxic molecules to enter and kill the parasite (Barzin *et al.*, 2019).

Medicinal plants contain a wide range of active substances with different pharmacological properties. These substances include biologically active compounds such as steroids, alkaloids, flavonoids, polyphenolics, saponins, and terpenoids, which were isolated from different plant species (Zubairu Sadiq *et al.*,2020). The quantitative and qualitative composition of bioactive substances in medicinal plants can vary depending on the type of plant and the specific part of the plant used (Kumar and Tewari, 2018).

Many plant extracts containing phenols have the ability to alter enzyme actions and denature proteins that are responsible for basic metabolic activities and might cause microorganisms to lose their ability to be living (Marizel *et al.*, 2012). They also have effects in reducing the proportion of vitality of protoscolices (Zainab Alaa *et al.*,2024).

**3.2. Effect of Albendazole drug on the vitality of protoscolices of *E. granulosus***

From the results that showed in table (3-2), one can notice that Albendazole treatment showed a minor effect immediately after treatment and up to 30 minutes of treatment at the low concentrations of 2.500 and 1.250 ppm when the mortality rate reached (20.93 and 18.56) % respectively. while the mortality rate increased significantly when reached (41.77) % at the concentration of 10.000 ppm and (34.07) % at the concentration of 5.000 ppm. After 120 minutes, the highest mortality percentage of protoscolices was at the concentration of 10,000 PPM when it reached (100) %, and also at the concentration of 5,000 PPM it reached (98.91)%, while the percentages reached (80.13) % at the concentration of 2,500 PPM and the percentage was (67.38) % at the lowest concentration of 1.250 PPM.

The lethal effect of albendazole treatment results from the changes caused in the cell structure of the parasite as it eliminates the stellate organization of the cells, creating many bubbles in the membrane, as well as the loss of fine capillaries, and increases the sediments of fatty cells inside the cells and depletes their glycogen reserves (Mahmoodi *et al.*, 2023).

Table (3-2) Effect of Albendazole drug on vitality of protoscolices of *E. granulosus*

Concentrations	mortality percentage				
	Immediately	30 min	60 min	120 min	240min
0 PPM (control)	15.72	20.11	29.59	33.21	42.51
1.250 PPM	10.34	18.56	23.96	45.64	67.38
2.500 PPM	15.16	20.93	37.20	64.12	80.13

5.000 PPM	20.45	34.07	59.38	68.95	98.91
10.000 PPM	28.82	41.77	72.91	100.00	
L.S.D.	38.69				

When comparing the results of the alcoholic extract of neem tree leaves in this study with the effect of Albendazole-treated tables (3-1) and (3-2), we find that the concentration of 10,000 PPM gave a 100% mortality result after 120 minutes, and it is a long time when compared with the concentration of 10,000 PPM of the plant extract of the neem tree leaves when it reached 100% after only 30 minutes. This leads us to think and research to tone down the possibility that plant extracts are an alternative to chemotherapy for ureteral cysts and other diseases in the future.

**Conclusions**

The plant extract of neem tree leaves has a significant and highly effective effect against the protoscolices parasite *Echinococcus granulosus* in vitro. However, further investigations of its efficacy in vivo are required.

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