ORIGINAL ARTICLE

Evaluation of the biological effectiveness of a novel Schiff base against protoscolices of *Echinococcus granulosus* in laboratory albino rats compared to the effectiveness of Albendazole using an electron microscope

[1]ABDUL–JALEEL AZIZ KARIM ALQARAGHLI, MANSOOR, JADAAN ALI ALKHALED[2]
[1] Department of Biology, College of Education, University of Al-Qadisiyah, Iraq
[2]Department of Microbiology, College of Vet. Medicine, University of Al-Qadisiyah, Iraq
Correspondence to Abdul–Jaleel Aziz Karim Alqaraghli Email: abduljalil.aziz@qu.edu.iq ; mansoor.ali@qu.edu.iq

ABSTRACT

This study included the preparation and spectroscopy of the new Schiff base, as a two-step Schiff base was prepared, the first of the reaction of 4-nitro-acetophenone with terephthalaldehyde. The second step is the reaction of 2-aminothiazole with the product of the first reaction. The compound was diagnosed using spectroscopic methods that included an IR Spectra, ^{1}H-NMRspectra and a mass spectra, and the results were practically identical to expectations.

Lethal dose was determined to half (LD_{50}) for Schiff base after they were dissolved with DMSO, and then the safe therapeutic dose was calculated, which was 0.18 g/ kg.

This compound was dosed orally as a therapeutic dose for a group of male rats, after they were infected with hydatid cysts. To find out the therapeutic efficacy of it on the hydatid cysts, and to compare it with a group that was infected with the same disease and treated with Albendazole, the dosing period lasted 30 days for each of the above groups.

The results of the Field Emission Scanning Electron Microscope (FE-SEM)study of the hydatid cysts of the positive control group showed that the hydatid cyst contains aGerminal Layer (GL) and contains large numbers of Protoscolices. Also, in the infected group and treated with the lip base, the presence of cracks and very few Protoscolices in the germinal layer compared to the positive control group, as well as the presence of wrinkles in the hydatid cyst.

Keywords: Echinococcusgranulosus, Schiff base, Chalcone, Electron microscope

INTRODUCTION

The adult *E.granulosus* is one of the smallest tapeworms. They often have only two to four Proglottids a few millimeters (2-11 mm) in length[1,2]. In contrast, Its larval stages (Hydatid cysts) are among the largest known tapeworms and can grow to a diameter of 50 cm or more[1].

*E. granulosus*requires two mammalian hosts to complete its life cycle, a final host (usually dogs or other canids) and intermediate host (wild or livestock mammals). While, a human is an accidental host[3].

Symptoms in humans depend on the affected organ. In the liver, these symptoms are represented by enlargement of the liver in the form of palpable or imperceptible masses when palpable, with liver abscesses, abdominal pain, vomiting, and nausea, as well as an increase in hepatic blood pressure, secondary fibrosis in the bile ducts, and shrinking Inferior vena cava. Due to its large size, a hydatid cyst creates great pressure on the diaphragm when it attaches to it, causing it to breach and expel the contents of the cyst in the chest[4]. In the lung, the clinical symptoms depend on the size of the cyst and its condition whether it is healthy or torn, and the presence of the cyst causes pressure within the affected lobe 5][, with chest pain of varying severity and coughing as well as shortness of breath and Haemoptysis in the lungs. Inside the alveoli, it leads to pneumonia and thus lung damage[6].

The Schiff bases are named after the Italian German chemist Hugo Schiff (1834 - 1951) who prepared the first compound of this group, which contains the Azomethine group (-HC=N-) or the Imine group (>C=N-).) In 1864, and he could not predict the effect of these molecules on modern chemistry[7].

Schiff's bases show a wide range of biological activities, including antifungal, antibacterial, antimalarial, antiproliferative, anti-inflammatory, antiviral, antipyretic and pharmacological properties 8][. The imine or azomethene groups are found in many natural, naturally derived and abnormal compounds, and the imine group present in such compounds has been shown to be important for their biological activities[9].

MATERIAL AND METHODS

All chemicals were purchased from BDH, and used without further purifications. FT-IR spectra were recorded in KBr, Shimadzu spectrophotometer in the range of 4000-200 cm^{-1} used KBr disc to the two ligands. Melting points were measured with an electro thermalsturat apparatus, model SMP30. ^{1}HNMR spectra were recorded on a Bruker DRX System AL500 (500 MHz) spectrometer in DMSO, chemical shift in ppm relative to internal TMS. The micro analysis (C, H, N) of ligands were carried out by using CHNS-O PerkinElmer model 2400-11-Mass spectra are recorded with Agilent technologies 5975 mass spectrometer.

Synthesis of Schiff base: This compound was prepared in two steps as follows:

The first step: 0.06 mol (9.909 g) of 4-nitro-acetophenone were dissolved In 300 ml of ethanol alcohol, then 0.012 mol (1.68 g) of KOH were added. The reaction was stirred for

an hour in temperature 0-5℃, then 0.06 mol (8.0478 g) of Terephthalaldehyde were added, and the reaction was stirred at room temperature for 24 hours, then 350 ml of distilled water added to it, then offset by hydrochloric acid concentration of 10%, then nominated output was collected sludge[10].

The second step: Putting 0.02 mol (2.002 g) of dissolved 2-aminothiazole in 50 ml of ethanol alcohol with 0.02 mol (5.6254 g) of first reaction product into a 100 ml reaction flask equipped with a condenser, the mixture was acidified with a few drops of acetic acid After that, the mixture was heated back to reflux for five hours. The reaction was continued using TLC. After it had ended, the mixture was left to cool down, then the sediment formed was filtered through a filter paper and left to dry. Then it was re-crystallized using absolute ethanol 11][. Physical properties and analytical data were recorded in Table 1.

Table 1: Physical properties and analytical data for the synthesized Schiff base

Molecular formula	M. Wt	m.p C°	Color	Yield%	PH
$C_{22}H_{32}N_2OS$	39.363	189 – 191	Dark orange	89	3.6

Determination of median lethal dose: Male rats were divided into random groups dosed orally with the prepared compound to determine the LD_{50} using a stomach tube, after dissolved by Dimethyl sulfoxide (DMSO). The rats were monitored for 72h and weakness, unstable walking, loss of balance and death was checked during this period. Injection started with low dose then continued to high dosages based on Litchfield and Wilcoxon (1949) equation[12]:

$$LD_{50} = \text{highest dosage} - \frac{\sum ab}{n}$$

Where, LD_{50}, Highest dosage 100% of rats, a the amount of difference between among dosages,b total dead animals for each dose is (previous dose + second dose/ 2) , ∑ a b the sum of a × b, and n , the number of animals used for each dose.

Parasite study: Hydatid cyst samples were obtained from infected sheep from the Najaf massacre in Najaf province. Those samples were transferred directly to the Department of Biology/ College of Education/ University of Al-Qadisiyah after placing them in plastic containers and placing ice in those containers. To preserve Protoscolices inside the cyst[13].

Isolate of Protoscolices: Protoscolices was obtained from cysts in the laboratory by putting the liver of infected sheep containing these cysts in a sterile dish and sterilize the surface mediated by a piece of cotton soaked with alcohol ethyl concentration of 70%, then make a hole by medical syringe capacity of 10 ml,To withdraw as much liquid of the cyst as possible to reduce the pressure inside.

The cysts were then opened with a scalpel, and the secondary cysts and germinal layer were extracted; It contains the largest number of Protoscolices. Thereafter, the caplets suspension was kept in 4 ml of Kerb's Ringer solution per 1 ml of suspension; to estimate the vitality of the primary heads and calculate their number[14].

Injection of rats with viable protoscolices: Male laboratory rats were injected after knowing the appropriate number of live protoscolices in a volume of 0.7 ml of protoscolices suspension and Phosphate Buffered Saline (PBS). After dividing the laboratory animals into random groups, with 10 rats per group, the injection site was sterilized with 70% ethyl alcohol then rats were injected with 2×10^3 live protoscolices. In the intraperitoneal (IP) cavity[15].

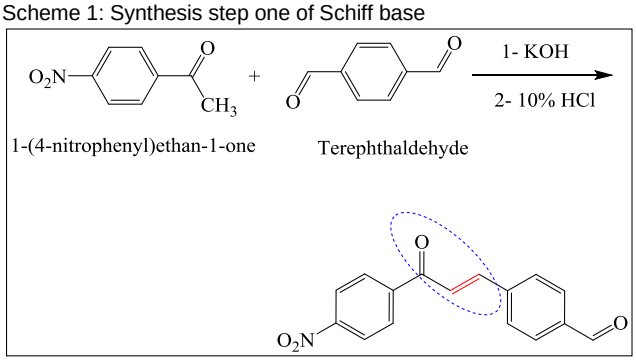
Treatment of the infected rats: Rats infected with hydatid cysts were treated 6 months after injection, as a group of oral administration was dosed with a Schiff base that was prepared at a dose of 0.18 g/ kg (after dissolving it with 0.1 ml of DMSO), which is the therapeutic dose of the compound, and the dose was at a rate of once daily for a month, the second group was dosed with Albendazole at a dose of 0.5 mg/ ml/ rat at a rate of once daily for a period of a month. The third group was infected and was not treated as a positive control group. The fourth group was not infected and treated, and it was used as a negative control group.

Field Emission Scanning Electron Microscope (FE-SEM): FE-SEM was used; for revealing the external shape of the protoscolices and layers of the hydatid cyst. The slides were prepared according to the method used by[16]

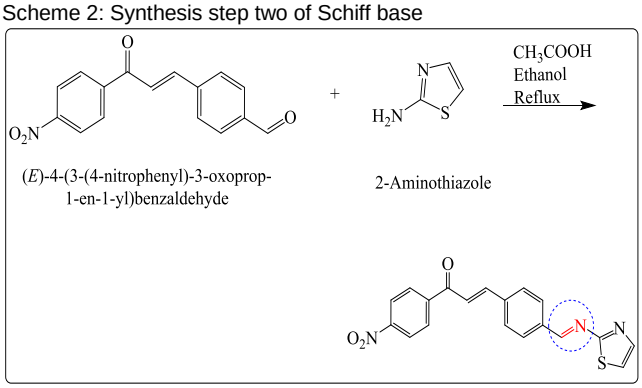
RESULTS

The novel Schiff base in a good yield as follows Scheme1, 2

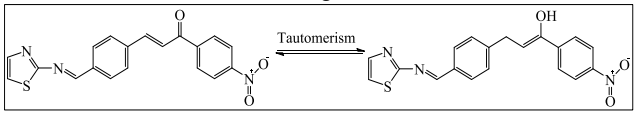
Scheme 1: Synthesis step one of Schiff base



Scheme 2: Synthesis step two of Schiff base



^{1}H-NMR spectra: The ^{1}H-NMR spectra of the Schiff base was characterized by the emergence of a singlet with a chemical shift of 9.14 ppm with a 1H integral belonging to the proton of the azomethinegroup HC=N–, this signal is an important evidence for the success of the compound preparation, as the spectrum for this compound was characterized by the emergence of multi singlet at a chemical shift of 8.66-7.61 ppm it refers to the protons of the aromatic ring of benzene as well as of the thiazole ring. On the other hand, the singlet appeared that belonged to the proton of the olefin group (C=CH) at 7.05 ppm, which belonged to the chalcone group. The spectrum also showed a singlet that belongs to the proton of the OH group, which is formed as a result of the keto-enol tautomerism at a chemical shift of 10.06 ppm, and this phenomenon is one of the important phenomena that characterizes the chalcone. Fig.1.



IR Spectra: IR Spectra for the prepared compound showed a group of beams, using KBr disk. The prepared compound showed five main absorption beams due to the elastic vibrations of the groups.
[Ar–H, C=CH, C=O, C=N (thiazole), C=N and C=C] within the ranges: [3068-3106, 1698, 1655, 1601, 1458 cm^{-1}] respectively .

The emergence of the amplitude oscillation beam of the azomethinegroup C=N at 1601cm^{-1}, with the emergence of a new absorption beam in the imine compounds (Schiff base) at 1655 cm^{-1} due to the amplitude oscillation beams of the C=N group of the thiazol ring associated with the chalcone compound and this clear evidence of the binding of 2-aminothiazole with the chalcone derivative, as well as confirming that the amplitude oscillation beams of the amine group associated with the thiazole did not appear at site 2 in the substrate on the formation of the azomethinegroup, which belongs to the prepared Schiff base.Table 2 and Fig. 2 illustrate this.

Mass Spectra: It appears in the mass spectra of the Schiff base molecular ion signal detection ($M^{+\cdot}$) at m/ z = to 363, as shown in Fig. 3, as it showed a pattern of break - ups for the prepared compound accurately be composite as a way to be fragments indicating the health of the structures proposed compound[17].

Fig. 1: ^{1}H-NMR spectra of Schiff base

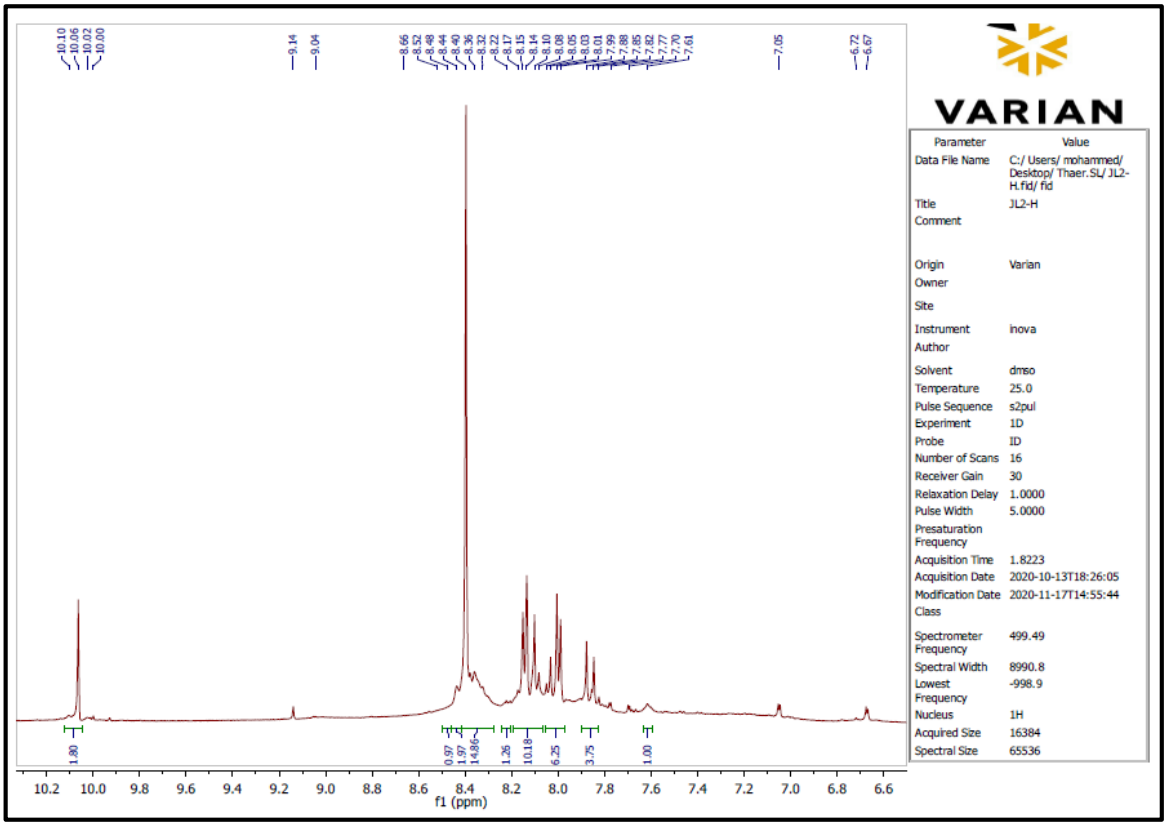


Table 2 : Selected Bands of Diagnostic Importance from the IR Spectrafor the prepared compound

Compound	Ar–H and C=CH Str.	C=O Str.	C=N (thiazole) Str.	C=N Str.	C=C Str.
$C_{19}H_{13}N_3O_3S$	3106-3068m	16981s	1655 s	1601s	14581 s
Str. = Stretching , m= medium , s=strong , w=weak					

Fig. 2: IR Spectra of the prepared Schiff base

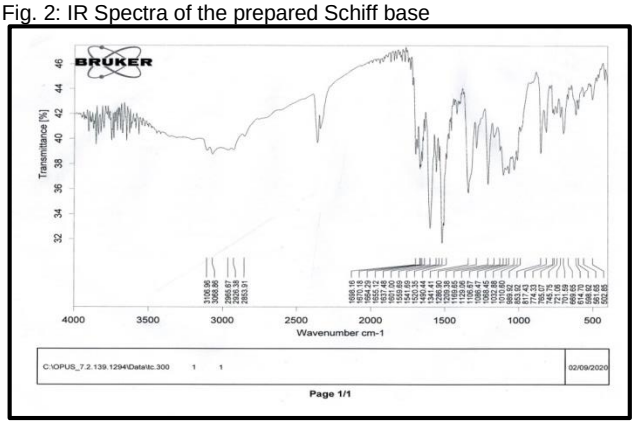
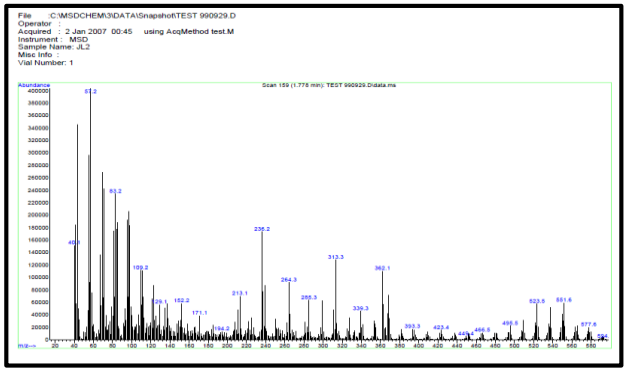


Fig. 3: mass spectra for the Schiff base



Determination of LD_{50} The LD_{50} of the compound was determined to the half by dosing elevated concentrations of this compound in rats by the oral administration, after dissolving it with DMSO. The death of rats was watched with increasing dose concentrations, and the concentration of the dose that killed all rats was determined, and then the value of the safe therapeutic dose was calculated for it. In order to administer it to male white rats, as shown in Table 3.

Table 3: value LD_{50} for compound applied tomale rats

Dose G / kg	No of animals	No. of dead animals	a	b	axb
0.50	10	0	–	–	
0.75	10	0	0.25	0	0
1	10	2	0.25	1	0.25
1.5	10	4	0.5	3	1.5
2	10	6	0.5	5	2.5
5.2	10	8	0.5	7	3.5
3	10	10	0.5	9	4.5
$\sum ab = 12.25$					

$$LD_{50} = 3 - \frac{12.25}{10}$$

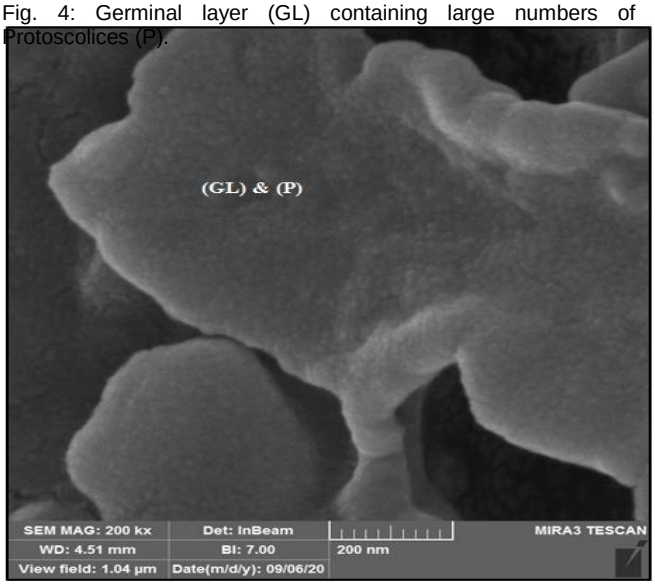
$$= 3 - 1.225 = 1.775 \approx 1.8\ g/Kg$$

The LD_{50} was then divided by 10, so that the safe therapeutic dose was 0.18 g/ kg.

Field Emission Scanning Electron Microscope (FE-SEM): After dissecting the animals and sending the hydatid cyst samples to the University of Tehran/ Iran. The results for the different groups showed fine details, whether it was phenotypic details of the hydatid cyst composition of the positive control group in which the rats were infected with *E. granulosus*without treatment, or their combination in the albendazole treated groups, and the prepared compound.

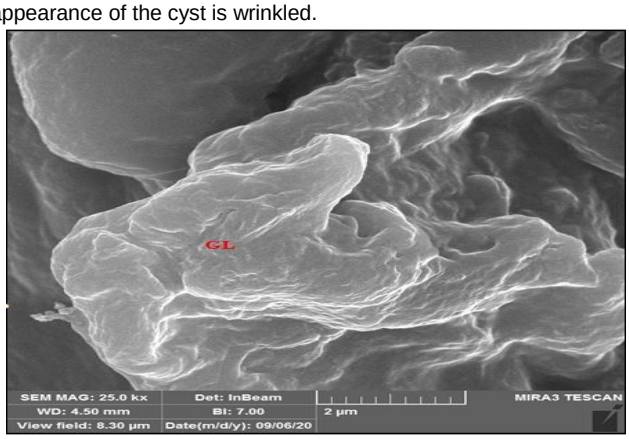
In the positive control group, the hydatid cyst sections demonstrated the GL, which contained large numbers of Protoscolices . Fig. 4illustrate this.

Fig. 4: Germinal layer (GL) containing large numbers of Protoscolices (P).



The second group infected and treated with Albendazole had very few Protoscolices in the GL, and the general appearance of the cyst was wrinkled. Fig. 5 illustrates this.

Fig. 5: Protoscolices in the germinal layer are few, and the general appearance of the cyst is wrinkled.



The group infected with cysts and treated with a Schiff base showed cracks, and very few Protoscolices were observed in the GL compared to the positive control group, and wrinkles were observed in the hydatid cyst when using higher zoom power. Fig. 6 and 7. illustrate this.

Fig. 6: The presence of Cracks(C) fissures in the hydatid cyst, and the presence of a few number of Protoscolices (P) in the Germinal layer (GL).

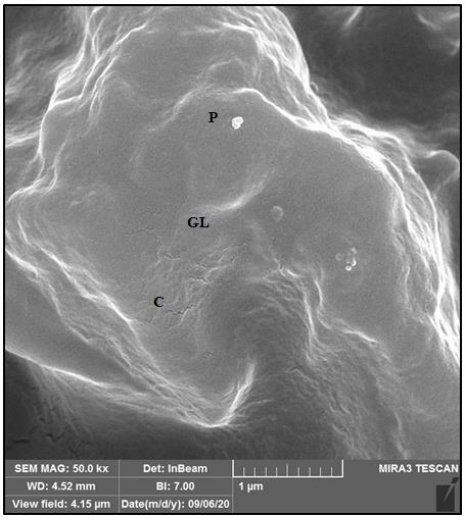
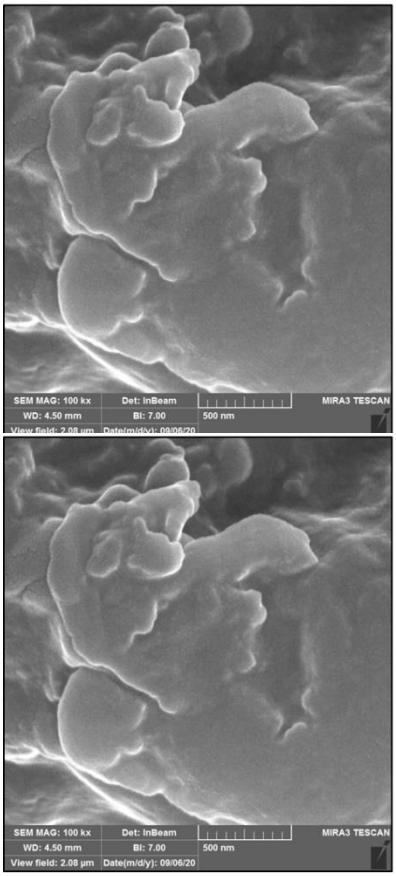


Fig. 7: The presence of wrinkles in the layers of the hydatid cyst



DISCUSSION

Currently, treatment of echinococcosis depends on surgery and/ or chemotherapy, depending on various factors such as cyst size and location, vitality, interaction between the cyst and adjacent tissues of the host, bacterial and fungal infection accompanying the infection, and possible complications such as cyst rupture and spillage of its contents from the Protoscolices[18].

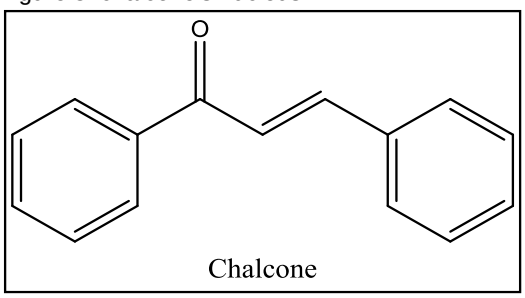
For non-operative cases, chemotherapy with albendazole, a broad-spectrum anthihelminthic anthelmintic, mebendazole and praziquantel is the only option[19].

In our current study, the results of the electron microscopy of the Schiff base showed a greater effect than the effect that resulted from the use of Albendazole, which is represented by the cracks caused by the two compounds prepared in the hydatid cyst.

Schiff bases form an important class of the most widely used organic compounds and have a wide range of applications in many fields including analytical, biological, and inorganic chemistry. Schiff bases have gained importance in the medical and pharmacological fields as a result of a wide range of biological activities such as the E.granulosus20][and the anthelmintic 21][. The nitrogen atom in the azomethene group can participate in the formation of a hydrogen bond with the active centers of cellular components and interfere with the normal processes of the cell[23,22].

Chalcones are an important class of natural products belonging to the family of flavonoids (bioactive secondary metabolites of plants). There is widespread interest in galactone-based nucleation compounds (Fig.8) due to their unique chemical composition and broad spectrum of pharmacological properties including antitubercular[24], antimicrobial[25], antioxidant 26][, and antimitotic[27], antimalarial and antileishmanial[28], anti-inflammatory[29], anticancer[30] and modulation of P-glycoprotein-mediated multidrug resistance[31].

Figure 8: chalcone's nucleus[32]



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