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Evaluation of the Therapeutic Efficacy of Alcoholic Extract of Tannins (Before and After Acidosis) on Laboratory Mice Experimentally Infected with the Parasite *Cryptosporidium parvum*

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ABSTRACT

Plants extracts are the one of the most important sources that have been used in both ancient and recent to many facture variants, types of medical drugs and that was the aim of the current study to find safe, effective and available natural alternatives by measuring the therapeutic effect of the alcoholic extract of tannins before and after the acid hydrolysis of the extract, in laboratory mice experimentally infected with *C. parvum* and using three therapeutic concentrations (2, 1.3, 1) mg / ml, and the alcoholic extract before the acid hydrolysis proved its therapeutic efficacy with a noticeable decrease in the number of Oocyst excreted with the feces, as the Oocyst were no longer excreted from the ninth day of the treatment. Parasite Oocyst on the fifth day of treatment were treated by oral administration of the extract in infected mice, in the other hand the current study recorded that Cryptosporidiais as a common disease in the study area, with infection rate of 26%.

Keywords- *Cryptosporidium parvum*, *Cypresuss semperverins*, Alcoholic Extract, Acidosis, Metronidazole.

I. INTRODUCTION

Infection with intestinal parasites is one of the most important problems that human societies suffer from[1] especially children, as it causes them to have diarrhea, malnutrition, delays in mental and physical development, weak cognitive functions, and digestive disorders whose severity varies according to the type of parasite that causes the disease, the age of the person and his immune status. And infection is more common among children under the age of 5 years, as the number of cases diagnosed with diarrhea in children is estimated at approximately 1.7 billion cases, and 525,000 children die annually under the age of five [2]

Cryptosporidium parvum (*C.Parvum*) is a pathogenic protozoan parasite belonging to the phylum Apicomplexan that causes Cryptosporidiosis characterized by severe watery diarrhea[3]. It is a parasite that infects intestinal epithelial cells and causes malabsorption leading to severe diarrhea. It is considered the second leading cause of diarrheal diseases transmitted by water and food worldwide and ranks second after rotavirus.[4] Due to the breadth of the host and the environmental stability of this parasite, species have been identified, and of these species, Cryptosporidium hominis and *Cryptosporidium parvum* are responsible for most human infections[5] , and are the main cause of death for children with diarrhea, with 48,000 deaths due to acute infection in 2016.[6] [7] [8]

C. parvum proliferates within intestinal epithelial cells of the small intestine, impairing intestinal barrier function, resulting in progressive atrophy of the villi, malabsorption of nutrients, and severe diarrhea. Impaired intestinal epithelial barrier function and increased permeability are commonly associated with diarrheal diseases caused by intestinal infections[9] that the genomes of microorganisms - including fungi, viruses and bacteria in addition to the primary parasites - called the microbiome, act as a mucosal barrier in the intestine that prevents the invasion of pathogenic

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microorganisms and has a positive effect on host metabolism and the development of the immune system. Any defect in this barrier will negatively affect therapeutic responses and drug resistance[10]

C. parvum oocysts, which are shed by the infected organism (human or animal), are difficult to eradicate because they are resistant to most chemical disinfectants as well as commonly used water treatments such as chlorine [5][11]. and due to its small size (4-6 microns in diameter) that cannot be removed by typical filtration systems, in addition to its ability to cause infection, as 10 bags can contract the disease[7] and transmission of infection occurs through fecal-oral through Ingestion of food or drink contaminated with Oocysts of the parasite, or direct contact with animals or people who have the disease [12]

As for the treatment of *C. parvum* infection, it does not have an effective treatment directly, except for Nitazoxanide, which is proven by the World Health Organization, but it is not effective for people with immunodeficiency, and many anti-Cryptosporidiosis compounds have been used, but they did not give effective positive results and did not work On killing Oocyst permanently in a record period, including Metronidazole Azithromycin, Paromomycin[13], and from recent studies Nullscript was identified as a compound that has a growth inhibitory effect, and was less toxic to host cells, as Nullscript was able to significantly reduce Oocyst secretion in Mice infected with *C. parvum*[14]

Medical plants

Medicinal plants are defined as those plants that possess active constituents with medicinal and curative properties, and they are secondary metabolites. Medicinal plants are considered a rich source of compounds used in the development of drug production[15]. Use the whole plant or even parts of it that contain Active ingredients that are produced and stored inside the plant and possess the properties of therapeutic effects[16].

The natural extracts derived from medicinal plants - which were used as traditional medicines in the past - have encouraged research studies and new drug discoveries, which have a promising role in medical treatments such as cardiovascular diseases, some types of cancer, Alzheimer's, asthma, arthritis, through The role that antioxidants play in curbing free radicals and thus reversing oxidative stress, which is why it has become extremely important to find natural sources of antioxidants to promote health through drug development. They are also recommended as nutritional supplements after modifying and treating bioactive compounds to reach the proper drug standard [17]

General description of the plant

Cupresuss semperverins is an evergreen tree 35-30 m high with conical crown branches, dark green leaves 2-5 mm long, oval or oblong green cones at the beginning of its growth, turning after 20-24 months to yellowish-brown in color [18] and is considered a pioneering species at the level of the family because of its long history in the Mediterranean region and its distribution in the form of vast forests. mm annually. It also prefers well-ventilated, deep, moist, non-clay soils. It has the ability to grow in dry, rocky soils and can live for more than 100 years[19][20]

It is one of the well-known trees in the Mediterranean and is also spread in the southern coasts of the Caspian Sea, Syria, Iran, Lebanon, Iraq, Cyprus and some Greek islands. It also extends between the Himalayas to China. It is also planted in gardens and fields because of its aesthetic value and can play the role of prevention as it represents a windbreak[21]

Active Compounds in Cypress

Chemical analyzes revealed the primary components of the plant as it contains major components represented by saponins 1.9% - alkaloids 0.7% - flavonoids 0.22% - tannins 0.31% and phenolic compounds 0.067% in addition to essential oils[18]

II. MATERIALS AND METHODS

Samples collection

220 faeces samples were collected from children under the age of 5 years of both sexes who suffer from intestinal colic and persistent diarrhea, who have been admitted to Ibn Al-Atheer Hospital, Mosul General Hospital and Al-Salam Hospital in Nineveh Governorate in the period from the beginning of October 2021 - At the Epril 2022, the families of the children were given clean plastic bottles, on which were recorded the sample data (date of sample collection, gender, age, area of residence).

The first group: samples were preserved in a potassium dichromate solution at a concentration of 2.5%. Two volumes of the solution were added to each volume of the fecal sample, and the samples were kept in a refrigerator at a temperature of 4 C.[22]

The second group: samples kept in the refrigerator without any addition at a temperature of 4°C until the isolation process for the parasite.

After collecting and storing the samples, the study was conducted on them from three axes figure (1):

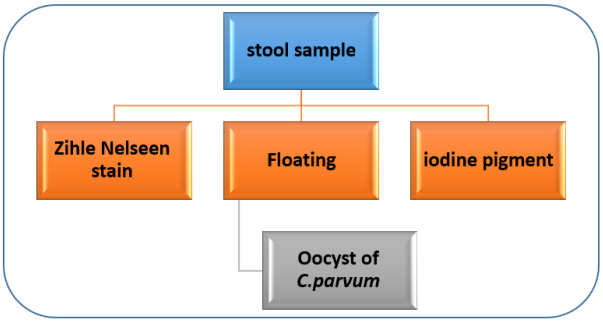

Figure 1: Scheme showing the dyeing with the modified Zihle Nelseen

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Laboratory mice

Laboratory mice were used Swiss Albino Mice Balb-C strain, as they were dose with a suspension containing *cryptosporidium parvum* cysts in order to induce the experimental infection. Mice were obtained and bred in the animal house of the College of Veterinary Medicine / University of Mosul, where the room was equipped with drawers, lighting, and means of cooling and heating, as the temperature was recorded between 20-30 degrees Celsius, and the mice were placed in special cages with 11 plastic cages provided With an iron cover that contains a place to put the feed and a place to put a special water bottle containing a thin tube at the end of it to drink water, the feed ration consists of 10% animal protein, 20% soybean meal, 45% yellow maize forage, 24% rice bran, 0.5% lime o.5% table salt, and rat stool samples were examined before use to ensure that they were free of any other parasitic infestation.

Collection of plants used for study

The nut of the local tannins *Cupresuss semperverins* was used in the current study, the picture of which is shown below Figure(2)

Preparation of the alcoholic extract

- 200 gm of tannins were ground and the entire quantity was taken and placed in a 1000 ml beaker
- 500 ml of petroleum ether was added to isolate the tannins oil and it was extracted according to the Grand method

The remaining filtrate was taken from the petroleum ether extraction and 300 ml of ethanol at a concentration of 70% was added (ethanol was diluted with distilled water by 70 ml ethanol-30 ml distilled water).

- Depending on the Grande method of extraction, the beaker was placed containing the starrier for 72 hours with Paramagnetic placed inside the beaker without the use of the thermostat at all, and the beaker was covered with a piece of Para film
- The product is filtered through filter paper and the alcoholic extract is collected in a clean container
- Take the product to the Rotary Evaporator Figure (3) to condense the material and get rid of the ethanol and get a concentrated Crude raw material

Separation of phenolic compounds from plant extract

Most of the phenolic compounds are found inside the plant linked with sugar by glycoside bonds to form glycosides. Therefore, the acid hydrolysis method was used to purify and diagnose phenolic compounds by breaking the glycosidic bonds and obtaining free and pure phenolic compounds by taking (20) ml of alcoholic extract of tannins. Each separately, and 25 ml of HCL acid (13% diluted) was added to it. It was placed in a flask for the Mentail thermal apparatus with the addition of the boiling stone. The heat escalation was conducted for one hour at a temperature of 100 °C, then left the flask until it cooled and the solution was taken and placed in the separating funnel and added to it. 25 ml) Ethyl Acetate, after shaking the separating funnel with its contents, it is fixed on the metal clamp and left until it settles and two layers are clearly visible. The lower layer is heavy because it contains sugar. It was disposed of, and the product of the upper layer consisting of phenols was collected. One of them was placed in sealed dark bottles and placed in the refrigerator until it was diagnosed by HPLC device[23] and the second part was placed in a Rotary Evaporator device to get rid of ethyl acetate for the purpose of trafficking.

Diagnosis of active phenolic compounds

After conducting the acid decomposition of the sample, the active phenolic compounds of tannins were diagnosed using (HPLC) High Performance Liquid Chromatography figure(4)**,** device in the laboratories of the Ministry of Science and Technology / Baghdad. The device was used of the German type (SYKAM) with a flow rate of 1 ml / min, such as the mobile phase (A, B).

A=Methanol: D.W. :acetic avid (85:13:2)
B=Methanol: D.W. :acetic avid (25:70:5)

III. RESULTS AND DISCUSSION

Examination and diagnosis of oocyst sacs isolated from humans

The results of microscopic examination of faeces samples collected from children under 5 years of age and of both sexes using modified Zeehel-Nielsen dye showed that the Oocyst of *C.parvum* The results are in agreement with what was also found by [24][25][26] in the fact that the parasite oocyst are spherical in shape, red to pink, surrounded by a transparent halo. Figure(5)

While the Oocysts appeared after using the Flotation Method with a Sheathers solution, in a transparent circular shape with distinctive walls containing clear, dark-colored dots representing the spores as in Figure (3), which is similar to the description that came with it [27] These studies showed that oocyst appear spherical and transparent with dark walls containing dark spots.

Diagnosis of the active substances in the tannins plant

The results of the chemical analysis of the alcoholic extract before and after the acid decomposition of tannins using HPLC technology showed that the plant contains 5 compounds of the active substances that are considered phenolic compounds [18] which are Gallic acid, Tannic acid, Ferulic acid, Hydrobenzoic acid, Chlorogenic acid in both alcoholic and aqueous extracts with a difference in the concentration of the active substances that were calculated according to Behbahani's equation [28] which showed that Tannic acid had the highest concentration within the alcoholic extract.

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Table 1: The number of Oocyst from mice infected with *C.parvum* parasite during the treatment period with alcoholic extract of tannins before acidosis

Dosage type	Dosage concentration (MI/Mg)	Oocysts during the day 1	Oocysts during the day 5	Oocysts during the day 9	Oocysts during the day 11	Oocysts during the day 13
Negative control	1/ml distilled water	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0
Positive control	1/ml hanging of C.P	101.00 ± 2.58 f	108.66 ± 3.29 h	±110 1.66 h	113 ± 2.39 e	93.50 ± 2.75 c
Alcohol extract	2	83.35 ± 4.86 de	64.66 ± 2.95 d	.66 ±0 .42 b0	.00 ±0 .00 a0	.00 ±0 .00 a0
	1.3	56.00 ± 2.19 cd	40.66 ± 5.85 b	9.33 ± 2.23 bc	.66 ±0 .42 a0	.00 ±0 .00 a0
	1	71.00 ± cd4.21	69.00 ± 1.34 def	22.00 ± 4.47 de	4.00 ± 1.78 ab	.00 ±0 .00 a0

Table 2: Fecal weight of mice infected with *C.parvum* parasite during treatment with alcoholic extract of tannins before acidosis

Dosage type	Dosage concentration (MI/Mg)	Stool weight during the day 1	Stool weight during the day 5	Stool weight during the day 9	Stool weight during the day 11	Stool weight during the day 13
Negative control	1/ml distilled water	.163+-0 .005 a0	.223±0 .051 bc	.191±0 .006 cd	.180±0 .005 fg	.161±0 .006 b
Positive control	1/ml hanging of C.P	.526±0 0.22 d	.480±0 .007 f0	.583±0 .007 e0	.536±0 .004 h0	.336±0 .019 f0
Alcohol extract	2	.550±0 .018 d0	.180±0 .012 ab0	.133±0 .005 a0	.166±0 .002def0	.120±0 .003 a0
	1.3	.450±0 .015 c0	.130±0 .010 a0	.136±0 .005 a0	.173±0 .005efg0	.170±0 .004 bc0
	1	.536±0 .202 d0	.220±0 .004 bc0	.158±0 .005 ab0	.150±0 .008bcd0	.190±0 .000 cd0

Table 3: Body weight of mice infected with *C.parvum* parasite during treatment with alcoholic extract of tannins before acidosis

Dosage type	Dosage concentration (MI/Mg)	Body weight during the day 1	Body weight during the day 5	Body weight during the day 9	Body weight during the day 11	Body weight during the day 13
Negative control	1/ml distilled water	26.80± 2.03abcd	28.86± 3.90 d	28.63± 1.49 c	29.90± 1.40 d	29.01± .45 e
Positive control	1/ml hanging of C.P	23.03± 1.38 a	19.95± .50 a0	21.30± 1.11 a	20.83± 1.19 a	.00±20 .67 a0
Alcohol extract	2	28.50± .56 bcd0	26.30± .55 cd0	26.81± 0.50 b	27.81± .22 cd0	26.70± .40 de0
	1.3	29.13± .68 cd0	24.43+- 1.74 b	27.15± .34bc0	26.90± .313bc	25.10± .64bcd0
	1	29.68± 1.66 d	26.30± 2.95 cd	25.10± .49abc0	25.30± .53abc0	25.60± .31 cd0

Table 4: The number of Oocyst from mice infected with *C.parvum* parasite during the treatment period with alcoholic extract of tannins after acidosis

Dosage type	Dosage concentration (MI/Mg)	Oocysts during the day 1	Oocysts during the day 5	Oocysts during the day 9	Oocysts during the day 11	Oocysts during the day 13
Negative control	1/ml distilled water	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0	.000 ±0 .000 a0
Positive control	1/ml hanging of C.P	102.33 ± 2.95 g	102.33 ± 2.04 f	111.16 ± 3.91e	116 ± 8.64 c	106.83 ± 3.85 b

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Alcohol extract	**2**	**72 ± 1.93 cd**	**0.66 ± .42 b0**	**.000 ±0 .000 a0**	**.000 ±0 .000 a0**	**.000 ±0 .000 a0**
	1.3	**91.33 ± 2.23 ef**	**18.66 ± 6.21 c**	**.000 ±0 0.000 a**	**.000 ±0 .000 a0**	**0.000 ± .000 a0**
	1	**65.33 ± 1.83 bc**	**22 ± 4.56 c**	**0.66 ± .42 a0**	**.000 ±0 .000 a0**	**.000 ±0 .000 a0**

Table 5: Fecal weight of mice infected with *C.parvum* parasite during treatment with alcoholic extract of tannins after Acidosis

Dosage type	Dosage concentration (MI/Mg)	Stool weight during the day 1	Stool weight during the day 5	Stool weight during the day 9	Stool weight during the day 11	Stool weight during the day 13
Negative control	**1/ml distilled water**	**.163 ±0 .005 a0**	**.223 ±0 .051 bc0**	**.191 ±0 .006 cd0**	**.180 ±0 .005 fg0**	**.161 ±0 .006 b0**
Positive control	**1/ml hanging of C.P**	**.526 ±0 0.22 e**	**.480 ±0 .007 f0**	**.583 ±0 .007 h0**	**.536 ±0 .004 h0**	**.336 ±0 .019 f0**
Alcohol extract	**2**	**.183 ±0 .010 a0**	**.166 ±0 .004 c0**	**.125 ±0 .002 ab0**	**.125 ±0 .006 a0**	**.145 ±0 .006 c0**
	1.3	**.230 ±0 .007 b0**	**.156 ±0 .018 bc0**	**.120 ±0 .000 a0**	**.145 ±0 .002 bcd0**	**.130 ±0 .000abc0**
	1	**.363 ±0 .011 c0**	**.130 ±0 .009 a0**	**.140 ±0 0.003 c**	**.165 ±0 .002 ef0**	**.165 ±0 .002 d0**

Table 6: Body weight of mice infected with *C.parvum* parasite during treatment with alcoholic extract of tannins after acidosis

Dosage type	Dosage concentration (MI/Mg)	Body weight during the day 1	Body weight during the day 5	Body weight during the day 9	Body weight during the day 11	Body weight during the day 13
Negative control	**1/ml distilled water**	**30.03 ± 2.19 a**	**28.06 ± 1.71 b**	**29.26 ± 1.72 c**	**29.80 ± 1.77 c**	**30.20 ± 1.77 c**
Positive control	**1/ml hanging of C.P**	**27.36 ± 1.71 a**	**21.76 ± .34 ab0**	**20.11 ± .18 a0**	**19.85 ± .15 a0**	**19.67 ± .16 bc0**
Alcohol extract	**2**	**25.36 ± 3.49 a**	**25.40 ± 3.23 ab**	**23.23 ± 1.22 ab**	**26.56 ± 2.30 bc**	**27.71 ± 2.02 bc**
	1.3	**29.90 ± 1.35 a**	**26.80 ± 1.72 ab**	**26.35 ± 1.40 bc**	**26.55 ± 1.31 bc**	**27.15 ± 1.27 bc**
	1	**25.76 ± 2.25 a**	**22.80 ± 2.79 ab**	**23.50 ± 2.67 abc**	**25.85 ± 2.61 abc**	**26.45 ± 2.43 bc**

Therapeutic efficacy of alcoholic extract before and after acidosis of tannins

During the current therapeutic study that was conducted, special criteria were relied on, represented by counting the number of *C.prvum* Oocysts in the excreted stool of laboratory mice experimentally infected with parasite Oocysts, in addition to the weight of the excreted stool resulting from infection and measuring body weight on this basis and by applying the equation Arithmetic to find the therapeutic efficacy of each extract (before and after acidosis) during the treatment follow-up days, the following was reached:

The results of the therapeutic study showed the different effect of treatment with the extracts in mice experimentally infected with the parasite *C.parvum* on the number of Oocyst, the amount of excreted faeces and body weight during the first treatment days, taking the number of Oocyst and the amount of excreted feces to decrease as we progress in the treatment and according to the concentrations used in addition to a gradual increase in Body weight in conjunction with the improvement of the symptoms of the disease of diarrhea, lethargy and shedding of fur.

The results of treatment with alcoholic extract at a concentration of 2 mg/ml before the acidosis procedure showed that the shedding of parasite Oocyst stopped on the ninth day of treatment with a therapeutic efficiency of 98.7% to reach 100% on the thirteenth day of treatment, while the results were better with treatment with alcoholic extract after acidosis through The excretion of parasitoid cysts was stopped on the fifth day of treatment at a concentration of 2 mg / ml and with a therapeutic efficiency of 99.9% to reach 100% on the ninth day of treatment, followed by a decrease in the weight of excreted stools and an increase in body weight in conjunction with the stopping of excretion of parasitoid cysts.

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At the same time, the efficiency of the alcoholic extract was recorded as 99.8% at a concentration of 1.3 mg/ml before the acid hydrolysis procedure, to reach 100% on the thirteenth day of treatment, while the efficiency of treatment with the alcoholic extract at a concentration of 1 mg/ml was recorded as 100% on the thirteenth day of treatment.

While the results treated with the alcoholic extract after the acid hydrolysis procedure showed more positive for the suspension of Oocyst expulsion since the fifth day at a concentration of 2 mg / ml. As for the efficiency of the treatment with the alcoholic extract at a concentration of 1.3 mg / ml after the hydrolysis, it was recorded 99.9% on the seventh day of treatment to reach 100% on the ninth day.

While the results of treatment with a concentration of 1 mg/ml recorded a therapeutic efficiency of 99.9% on the ninth day, reaching 100% on the eleventh day of treatment.

From here, we conclude that the efficiency of the alcoholic extract from tannins after the acid decomposition gave more important results and faster treatment than the alcoholic extract before carrying out the acid decomposition and separating the phenolic compounds from each other, in addition to that the higher the concentration of the extract, the higher its therapeutic efficiency as soon as possible.

Figure 2: *Cupresuss semperverins* cone

Figure 3: Apparatus Rotary Evaporator

Figure 4: Apparatus HPLC

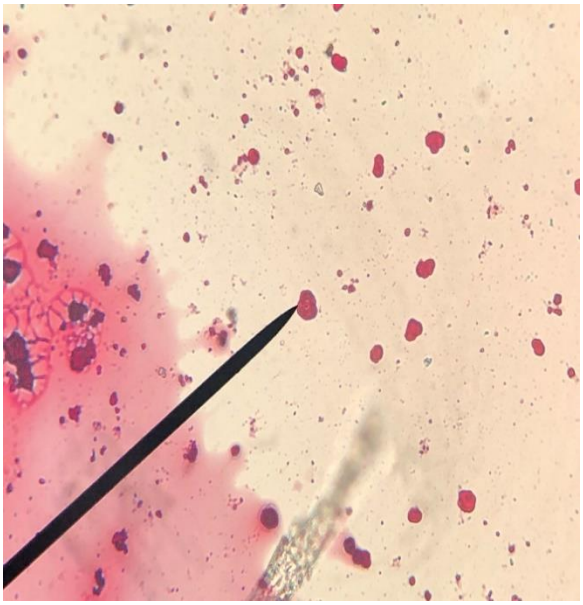

Figure 5: Oocyst of parasite *C.parvum*

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