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## **Evaluates the active substances of extracts of date vinegar and Citrus lemon plant fruit on *Enterobius vermicularis* worm in vitro**

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**Abstract---**This work was conduct to account the effects of cold and hot aqueous extracts of Iraqi *Citrus limon* plant fruits and date vinegar in paralysis and die of *Enterobius vermicularis* parasite *in vitro* and determine the most influential plant for extracted secondary compound (alkaloid, terpenoids and phenolic compounds) from it and then determine a most influential secondary compound for analysis by Gas chromatography /mass spectroscopy technical (GC/MS) and extract the most concentrated compound from it to test in paralyze and kill worms. The results appear that cold aqueous extract of *Citrus limon* plant fruits have a high influential especially by 300 mg/ml concentration where its average arithmetic for paralysis and die of worms equal 29.00 and 57.33 minute , respectively, followed by hot aqueous extract of *Citrus limon* plant fruits has effect on paralysis and die of worms in 300 mg/ml concentration where its led to paralysis and die of worms at 67.33 and 104.00 minute , followed by cold aqueous extract of date viengar extract where its average arithmetic for paralysis and die of worms in 300 mg/ml concentration equal 110.00 and 193.00 minute , respectively , followed by hot aqueous extract of date viengar extract where its average arithmetic for paralysis and die of parasite with 300 mg/ml concentration equal 139.00 and 246.00 minute , respectively , with clear significance differences, albendazole drug successful through its high effect on parasite where its average arithmetic for paralysis and die of worms in 300 mg/ml concentration equal to 48.00 and 70.00 minute , respectively. Also a results of secondary compounds of *Citrus limon* plant fruits appears that a high effects of terpenoid compounds extract of *Citrus limon* plant fruits on paralysis and die of worms especially with 300 mg/ml concentration where its led to paraly

and die of worms at 18.00 and 33.00 minute , respectively, while 200 and 100 mg/ml concentrations that led to paralysis and die of worms at 27.00 and 46.67 minute ,respectively for 200 mg/ml concentrations and 38.00 and 65.00 minute ,respectively for 100 mg/ml concentrations, followed by phenolic compounds that led to paralysis and die of worms with 300 mg/ml concentration at 32.33 and 53.00 minute , respectively, as 200 mg/ml concentrations led to paralysis at 38.67 minute and to die at 64.00 minute, also 100 mg/ml concentrations led to paralysis at 70.00 minute and to die at 106.00 minute, followed by alkaloid compounds that led to paralysis and die of worms with 300 mg/ml concentration at 40.00 and 63.00 minute , respectively, also 200 mg/ml concentrations led to paralysis at 62.00 minute and to die at 95.00 minute, also 100 mg/ml concentrations led to paralysis at 75.00 minute and to die at 118.67 minute. As to results of GC/MS technical confirmed that Iraqi *Citrus limon* plant fruits contains limonene compound with retention time 17.651 minute and Peak Area 0.71% , also a results of limonene compound appears that its possess a high efficient in 400  $\mu$  /ml concentration where its average arithmetic for paralysis was 10 minute and for die was 30 minute ,respectively, as well as albendazole drug has a great effect in spite of a little quantity of concentrations on paralyzing and killing of worms with especially in 400  $\mu$  /ml concentration where its led to paralysis at average arithmetic 145.00 minute and to killing at average arithmetic 243.00 minute with significance differences.

**Keywords**--*Enterobius vermicularis*, vinegar, *Citrus limon*, secondary compound.

## Introduction

*Enterobius vermicularis* is a human pathogenic intestinal parasite belonging to the nematodes (Nematoda), Synonyms include “threadworm” and “seatworm” [1]w . Humans are the only natural host of *Enterobius vermicularis*, infection is common in children and it is very related with high population density, socio-economic factors and the habit of fingers sucking [2 ; 3] .The main routes of infection are autoinfection from eggs or larvae deposited on the anus contamination from fomites like bed sheets, pyjamas, door handles, and inhalation of eggs from hands, bed mattresses or dust, eggs are unable to survive in the environment, infections tend to be limited to families and individuals in close proximity like nurseries and boarding schools[4 ;5] *E. vermicularis* spread by fecal-oral rout when patient ingests eggs that initially present in the perianal area of infested patients [6] . Re-infection occurs easily in enterobiasis, and the prevalence of this infection is mainly depend on public health and personal hygiene [7] , retroinfection of the larvae through the anus and then to colon is common [8].The most common symptoms include intense itching ,grinding of teeth and intestinal inflammation[9 ;10],heavy infections may cause sleeplessness, weight loss, hyperactivity, abdominal pain and vomiting , abdominal pain and appendicitis [5; 11] .

Once ingested, the larvae contained inside the eggs are released in the small intestine, and the adult worms diffuse to the caecum and colon , gravid females then migrate at night to the perianal region and to the perineum where they lay eggs [12] .The scotch tape method is very efficient for the diagnosis or detection of *E. vermicularis* eggs [11],the approved antihelminthic agents are mebendazole, albendazole, pyrantel embonate and pyrvinium embonate success rates up to >90% are adulticidal and ovicidal and are therefore considered to be the most effective drugs, for recurrent infections, prolonged treatment for up to 16 weeks is recommended[13;1], treating pinworm infection effectively requires treating all close contacts, mebendazole is a drug of choice[5] .

The medication occurrences many side effects, including headache, nausea, dizziness, metallic taste, and failures in treatment are frequently reported [14] . Along years, medicinal plants are considered as well-established natural sources for the treatment of various diseases [15] , medicinal Plants have a great ability to produce secondary metabolites such as phenolics and polyphenols, alkaloids, terpenoids and essential oils, lectins, and others [16] , secondary metabolites are an important source of new drugs, due to their variety of biomedical activities and chemical components[17]. Plant phenolics are secondary natural metabolites arising biogenetically from either the shikimate pathway producing monomeric and polymeric phenols and polyphenols, which have physiological roles in plants[18] , Phenolic compounds could promote health benefits by reducing the risk of metabolic syndrome and they could contribute to the prevention of disease [19] .

Alkaloids are a huge group of naturally organic compounds which contain nitrogen atom one or more which usually situated in some ring system[20],it's showed a strong biological effects on animal and human in very small doses [20;21] . Several alkaloids have served as drugs and used in pharmacology such as codeine, brucine, morphine, ephedrine, ([16;22]. Terpenoids are the hydrocarbons of plant origin ,their molecules are constructed from two or more of five-carbon isoprene unit ways [23; 24] , Terpenoids are produced in diverse genera of plants, fungi, algae and sponges [25] it can be divided into four groups of compounds that include true terpenes, steroids, saponins, and glycosides[26] . Terpenoids, characterized by valuable pharmaceutical qualities and medical uses [27] .

Vinegar having near about 5% acetic acid incorporated in water, traditionally vinegar implemented in food preservations applications [28]. Acetobacter species are transformation of ethyl alcohol into acetic acid [28;29]. Vinegar contains various nutrients and bioactive components include amino acids, sugars, vitamins, and minerals, acetic acid, gallic acid, catechin and ferulic acid cause antioxidative, antidiabetic, antimicrobial, antitumor, cholesterol-lowering responses[30;29and31]. *Citrus* lemon, it is a flowering medicinal plant belongs to the family Rutaceae [32]. It is cultivated worldwide due to its medicinal importance, have been recognized as some of the most high-consumption fruits in terms of energy, nutrients and health supplements, also used as traditional medicinal herbs to cure diseases in several Asian countries [33] . *Citrus*-derived secondary metabolites, including flavonoids, alkaloids, limonoids, coumarins, carotenoids, phenolic acids and essential oils, are of vital importance to human

health due to their active properties [33;34] which include anti-oxidative, anti-inflammatory, anti-cancer, as well as cardiovascular protective effects, neuroprotective effects[33;35] .

Essential oils are derived from plant material [36], which contain 100 to 250 components, these components include B-caryophyllene, limonene, linalool, and terpiens [37] Essential oils and their components are commonly found in therapeutics, many of which are recommended by physicians for their analgesic, antipruritic and cough suppressant properties[38]. Gas chromatography equipped with mass spectrometry (GC-MS) is a technique that separates complex mixtures into its individual components for identification and quantification [39] this technical is a widely used platform for analyzing volatile complex compounds [40] , GC-MS can offer a quick qualitative function based on the integrity of a compound database and the quantification can be more precise when isotope standards and selected ion mode are used together[40;41].

MS detector provided high sensitivity and good selectivity in samples analysis [40]. Limonene are naturally secondary metabolites found in plant species of the Rutaceae [42;43] , limonene, mainly found as a major component in *Citrus* sp. which possess a valuable potential in synthetic pesticides , food preservatives also as antimicrobial, herbicidal and antioxidant agent [43] , Both are additives in cosmetics, food, industrial solvents and pharmaceuticals because of their fragrant and harmlessness for humans [44;45 and 43].

Aims of study was extracting the active substances from date vinegar and *C. lemon* fruit by using one of the available methods and testing their biological efficacy against the parasite outside the living body 'compared to the Albendazole drug.

## **Methods and Materials**

### **Plant materials Collect**

The fruits of Iraqi *c. limon* plant were collected from the local markets at February 2021 . A sample of plant materials was desiccants in the shade position and then grinding by electric grinder to get on an soft powder which kept in plastic plate and then save in refrigerator.

### **Preparation of an cold and hot aqueous extract**

Cold aqueous extract was attended by take 10 grams from the fruits powder of the *citrus limon* plant and put it with 200 ml of distilled water in the flask 400 ml in size with using a mixer for 30 minute , then put in the test tube in the centrifuge for 10 minutes at 3000 rolls / minute, extract was dried by putting it in the oven at 45 C to obtain on dried extract , keep it in the fridge until use. Hot aqueous extract for this plant was prepare by a same method but use boiling water [46] .

### **Extraction of secondary Plant component**

#### **Extraction of crude alkaloids**

10 g of dehydrated soft powder is extracted by putting it in the filter papers which fixed on thimbles , then adding 200 ml from ethanol alcohol (%99) for 24 hours by soxhlets apparatus .New products were concentrated with rotary evaporator apparatus. It dissolved in 5 ml of ethanol , with added 30 ml of Sulfuric acid (2%) , then using rotary evaporator apparatus to remove an ethanol alcohol . Mayer assay gives white product to ensure an present of alkaloids. Hydroxide ammonium (%10) was putting in separating funnel with putting (10) ml from chloroform, mixing of product was separating into two layers , selected the bottom layer because it contains alkaloids, it was concentrated with rotary evaporator , a new dry product kept in icebox [47] .



Figure 1. Separating funnel apparatus for alkaloids extracted

#### **Extraction of crude phenolics**

Method of [48] was used to extracts phenolics, 20 gm of dried extract put in a glass flask with 400 ml of (%2) acetic acid by using the reflex condenser in (70) degree centigrade water bath for 8 hours. New suspension nominated and put it with N-propanol and sodium chloride substances in the Suppression of separation, been taking the top layer containing phenolic substances, then it was focused with evaporator rotor and dry product keeping in the fridge.



Figure 2. Separating funnel apparatus for phenolics extracted

#### **Extraction of crude terpenoids**

Method [49] has been followed in preparing crude terpenoids compounds extract, that weighs 20 grams of dry matter powder then extracted with chloroform solvent by soxhlet apparatus by using 200ml of chloroform solvents at 45C °

temperature for 24 hour then extracted sample was to focus by rotary evaporators, The sample containing the terpenoids compounds extract was dried up in the electric oven to 45-40C ° degree .The dry material was preserved in sealed glass bottles until use. Reagents were conducted using standard procedures to detect the presence of alkaloids, phenolics and terpenoids substances. Due to the efficiency of a terpenoids compounds, it was chosen to extract effective compounds in it but for difficulty to collect a quantity from it because it contain a high percentage of volatile oils therefor an essential oils are chosen for GC/MS analysis .



Figure 3. Sexehelts apparatus for terpenoid compounds extracted

#### **Extraction of essential oils**

Essential oil was extracted using hydrodistillation and fruits of *C. limon* were properly washed by distal water. The amount of 500 g of fruits were transferred to Clevenger apparatus and 3 L of distilled water was added. The Clevenger apparatus was set at boiling temperature of water for 6 h and distilled fruits essential oil was collected in a dry glass vial. The hydrodistillation was performed thrice .The collected fruits essential oil was treated with anhydrous sodium sulphate that removes the remaining moisture, and stored in a sealed tube at 4°C, for further analysis [50] .

#### **Chemical analysis of essential oils through GC/MS**

Fruits essential oil was analyzed by using Gas chromatography/mass spectroscopy (GC/MS). GC/MS analysis of *Citrus limon* plant fruits essential oil was performed on Agilent Technology (Little Falls, California, USA) 6890 series gas chromatography (GC) system, equipped with 5973 mass spectrometry (MS) detector and a 7683 series auto-injector was used. Compounds were separated on Rtx®-Wax capillary column (30 m . 0.25 mm, film thickness 0.25  $\mu$ m; RESTEK, Pennsylvania, USA). Helium (5N5 grade) was used as carrier gas, with a flow rate of 0.8 mL/min, and the split ratio was 60:1. Sample injection volume was 1  $\mu$ l and the injector temperature was 230°C. The column oven temperature was held at 70°C for 2 min, and then programmed to 130°C at 30°C/min and change the gradient to 230°C with 10°C/ min. Finally, held at 230°C for 6 min and the total run time was 20 min. An electron ionization (EI) system with ionization energy 70 eV was used for detection. The ion source temperature was set at 230°C, the interface temperature was 250°C, detector voltage was 2 kV.

The mass spectrum was acquired in scan mode at a scan rate 0.98 scan/sec within a mass range of 20-800 amu. The measurement was performed in duplicate for each sample with solvent delay for 2 min. [40]. For a difficulty of extracting a concentrated of each compound in *C. limon* fruits extract because its needed to a high cost and time, and since a limonene compound, it's a most concentrated in *C. limon* fruits extract according to the researchers [51; 32;52 and 50] , they were analyzed essential oils by GC-MS technical where they found a main bioactive compounds with high content in *C. limon* fruits was limonene compound, So it was chosen to test its effectiveness on *E. vermicularis* worm *In Vitro*.

#### **Collection of live *E. vermicularis* worm sample**

The live adult worms were collected directly from an anus of children at midnight from Hilla center districts, Babil Province, Iraq. Then a worms washed with distal water and the parasites were kept in Petridishes with phosphate buffer saline (PBS) in 37°C in incubator until the *in vitro* evaluation was started [53].

#### **Microscope assay**

The samples of helminths were collected from patients and diagnosed by light microscope using wet mount preparation for adult pinworms or eggs detection at Parasite Lab. of biology department , science college, University of Babylon.

#### **The Effects of plant extracts on *E. vermicularis* worm *In Vitro***

Preparation of stock solutions of both extracts of the *Citrus limon* plant fruits which brings the melt (2) g of dry extract in (5) ml of distal water, therefore, stock solution become (400) mg/ml. Stock solutions are used for concentrations prepared (100,200 and 300) mg /ml, control is prepared from phosphate buffer saline(PBS) only .The efficacy of both water extracts were compared with albendazole drug by the same concentrations. Four groups of worms, each group with five worms, one of them represented a control group, each group have been treated with an desired concentration of drug and extracts, the time for complete paralysis and death was recorded. External stimuli were applied as ascertain the paralysis time. The time taken for worm become motionless was considered as paralysis time and lethal time was ascertained by death of motionless worm followed by fading of their body color [54].

#### **Administration of extracts**

The worms were assembled in petridishes which contain one ml of (PBS)then put in incubator, after one hour, different concentrations of cold, hot and secondary compound extracts for *c. limon* plant fruits have been added to a petridishes that contain worm each on its own by adding one ml of each concentrations to a petridishes that contain parasite each on its own.In another petridishes that contain worm a same concentrations of albendazole drug was added [55].

### **The effects of D-limonene on adult *E. vermicularis* worm *In Vitro***

D-limonene was purchased from FedEx company (Weifang CHENGMAO, International Tradeco 402, China). D-limonene compound is liquid with 0.86g/ml concentration. The stock solution was prepared by adding 1ml from D-limonene compound with adding 0.1% of Tween 80 (polysorbate 80) that used as a solvent and volume complemented to 1L of distal water therefor a stock solution become 860 $\mu$  and from it a concentrations (25, 50, 100, 200 and 400  $\mu$ g/ml) were prepared, then taken one ml of each concentrations and have been added to a petridishes that contain parasite each on its own [56].

### **The effects of albendazole drug with microgram concentration on adult *E. vermicularis* worm *In Vitro***

The stock solution of albendazole drug was prepared by adding 200mg of this drug with 20ml of distal water to become 20mg/ml or 20000 $\mu$ /ml and a concentrations (25, 50, 100, 200 and 400  $\mu$ g/ml) were prepared in addition to control group, the time of paralysis and death was record with three replicates then taken one ml of each concentrations and added to a petridishes that included worm each on its own for albendazole drug [56].

### **Estimation of pinworms worm Viability *In Vitro***

The movement and death of worms has been monitored by looking, no movement or death of worm was identified [55].

### **Statistical analysis**

Analyze of data take place by using factorial experiments with completely randomized (C.R.D) and using least significance differences at level ( $P<0.05$ ) by using the statistics system (SPSS).

### **The results**



Figure 4. Adult females of *E. vermicularis*(40X)



Figure 5. Ova with larvae of *E.vermicularis*(400X)

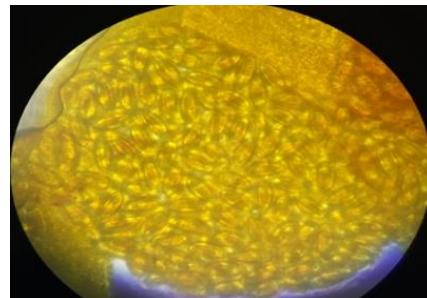


Figure 6. eggs of *E.vermicularis* (40X)



Figure 7. adult of *E.vermicularis*(10X)



Figure 8. Front portion of *E.vermicularis* body(200X)

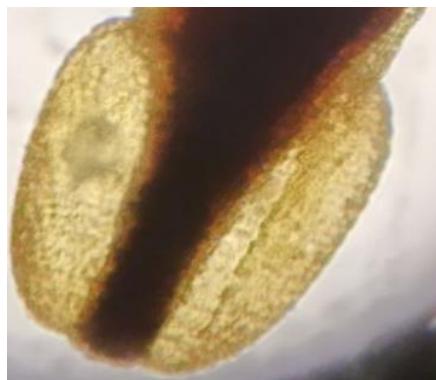
Figure 9. Head of *E.vermicularis* (400X)Figure 10. End portion of *E.vermicularis* (20X)Figure 11. Med portion of *E.vermicularis* body(20X)

Table1

The effect of plant type factor on worm paralysis and die for cold and hot aqueous extract *In Vitro*

Plant type	Time per minute for worm to die	Time per minute for worm to paralysis
	Mean	Mean
Limon plant	477.00	405.87

Veingar date	609.58	504.58
Total	543.29	455.23
LSD at probability level 0.05	12.61	10.24

Table 2

The effect of extract type factor on worm paralysis and die for cold and hot aqueous extract for both plant *In Vitro*

Extract type	Time per minute for worm to die Mean	Time per minute for worm to paralysis Mean
Cold extract	514.33	441.88
Hot extract	572.25	468.58
Total	543.29	455.23
LSD at probability level 0.05	12.61	10.24

Table 3

The effect of concentration type factor on worm paralysis and die for cold and hot aqueous extract for both plant *In Vitro*

concentration mg/ml	Time per minute for worm to die Mean	Time per minute for worm to paralysis Mean
300	150.08	86.33
200	197.50	121.17
100	283.08	200.83
control	1542.50	1412.58
Total	543.29	455.23
LSD at probability level 0.05	17.83	14.48

Table 4

The effect of extract concentrations overlapping for cold and boiling aqueous extracts for *Citrus limon* plant and date Veingar on worm paralysis and die *In Vitro*

Citrus limon plants at e Ve in	Extract type	concentration mg/ml	Time per minute for worm to die	Time per minute for worm to paralysis
			Mean	Mean
cold extract	cold extract	300	57.33	29.00
		200	62.33	43.00
		100	73.67	46.00
		Control	1542.00	1400.67
Hot extract	Hot extract	300	104.00	67.33
		200	171.33	111.67
		100	245.33	146.00
		Control	1560.00	1403.33
		300	193.00	110.00

cold extract	200	255.00	141.00
	100	389.00	342.33
	Control	1542.33	1423.00
Hot extract	300	246.00	139.00
	200	301.33	189.00
	100	424.33	269.00
	Control	1525.67	1423.33
	Total	543.29	455.23
LSD at probability level 0.05		35.65	28.95

Table 5  
The effect of albendazole drug on worm paralysis and die *In Vitro*

Concentration mg/ml	Time per minute for worm to die	Time per minute for worm to paralysis
	Mean	Mean
300	70.00	48.00
200	116.00	61.00
100	134.00	80.00
control	1544.00	1424.33
Total	466.00	403.33
LSD at probability level 0.05	34.25	34.55

Table 6  
The effect of Secondary compound's type factor for *Citrus limon* plants on worm paralysis and die *In Vitro*

Secondary compound type	Time per minute for worm to die	Time per minute for worm to paralysis
	Mean	Mean
Terpenoids compound	426.17	367.33
Phenolics compound	443.67	391.33
Alkaloids compound	454.17	396.00
Total	441.33	384.89
LSD at probability level 0.05	13.47	12.45

Table 7  
The effect of concentration type factor of secondary compounds for *Citrus limon* plants on worm paralysis and die *In Vitro*

concentration mg/ml	Time per minute for worm to die	Time per minute for worm to paralysis
	Mean	Mean
300	49.67	30.11
200	68.56	42.56
100	96.56	61.00
control	1550.56	1405.89

Total	441.33	384.89
LSD at probability level 0.05	15.56	14.38

Table 8  
The effect of extract's concentration overlapping for Secondary compound's extract for *Citrus limon* plant on worm paralysis and die *In Vitro*

Secondary compound type	concentration mg/ml	Time per minute for worm to die Mean	Time per minute for worm to paralysis Mean
Terpenoids compounds	300	33.00	18.00
	200	46.67	27.00
	100	65.00	38.00
	Control	1560.00	1386.33
Phenolics compounds	300	53.00	32.33
	200	64.00	38.67
	100	106.00	70.00
	Control	1551.67	1424.33
Alkaloids compounds	300	63.00	40.00
	200	95.00	62.00
	100	118.67	75.00
	Control	1540.00	1407.00
Total		441.33	384.89
LSD at probability level 0.05		26.95	24.91

Table 9  
GC-MS analysis of bioactive compounds in essentials oil of Iraqi *Citrus limon* plant fruits

Peak Number	compound Name	Retention Time	% Peak Area
1	2-furan-carboxaldehyde	9.260	0.71
2	2,5-Furandione, 3-methyl-	13.500	4.93
3	2-Furancarboxaldehyde, 5-methyl	14.386	1.20
4	D-Limonene	17.651	0.71
5	4H-Pyran-4one,2,3-dihydro-3,5-dihydroxy-6-methyl-	23.251	0.83
6	2-Furaldehyde, 5-(hydroxymethyl)-	27.194	6.15
7	5-(2-Thienyl)pentanoic acid	46.798	1.74
8	Nonadecane	53.936	0.30
9	Hexadecanoic acid, methyl ester	54.742	0.50
10	n-Hexadecanoic acid	55.999	11.66
11	9-Octadecenal, (Z)-	56.559	1.75
12	9,12-Octadecadienoic acid	60.006	2.91
13	9-Octadecenoic acid	60.320	1.70
14	Cyclopropaneoctanal, 2-octyl-	60.480	4.40
15	9,12-Octadecadienoic acid	61.406	13.27

16	9-Octadecenoic acid, (E)-	61.618	40.43
17	1-Heptadecanecarboxylic acid	62.263	6.80

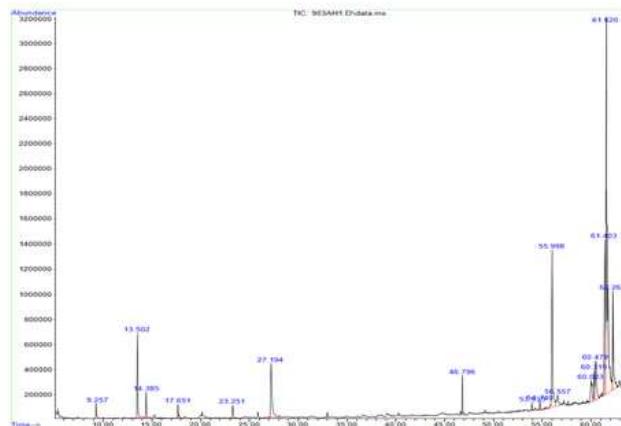


Figure 12. Chromatogram of the essentials oil of Iraqi *Citrus limon* plant fruits.

Table 10  
Limonene compound concentrations effects on worm paralysis and die *In Vitro*

Concentration of limonene ( $\mu$ /ml)	Time per minute for worm to paralysis	Time per minute for worm to die
	Mean	Mean
400	10.00	30.00
200	13.00	36.00
100	15.00	41.00
50	20.00	52.00
25	34.00	66.00
control	1554.6	1551.0
Total	274.4	296.0
LSD at probability level 0.05	2.84	1.77

Table 11  
Albendazole drug effects with  $\mu$ /ml concentrations on worm paralysis and die *In  
Vitro*

Concentration( $\mu$ /ml)	Time per minute for worm to paralysis	Time per minute for worm to die
	Mean	Mean
400	145.00	243.00
200	177.00	301.00
100	192.00	382.00
50	235.00	465.00
25	322.00	583.00
control	1551.00	1553.33

Total	437.00	587.89
LSD at probability level 0.05	1.78	1.97

## Discussion

*Enterobius vermicularis* (Nematoda), a pinworm, is a global helminthic parasite of humans that is especially common in temperate climates [3]. Medicinal plants represent the most ancient form of medication, used for thousands of years in traditional medicine in many countries around the world [57], it's treating and preventing various diseases [58].

### **Effect of cold and boiling water extract concentrations in a paralysis and die of *E. vermicularis* worm in different times *In vitro*.**

The results of this study demonstrate a high affectivity for a cold water comparative with a boiling water of *citrus limon* plant fruit extract on a paralysis and die of *E. vermicularis* helminths, this finding appears that a cold water of *citrus limon* plant fruit extract was very active and have ability to destroy or killed a worm and this is effect may be that a cold water extract contain active substances that effect on neuromuscular system and led to die this a ability may be because of an extract contain many active components in the fruit includes flavonoids and also other compounds such as a phenolic acids, coumarins, carboxylic acids, aminoacids and vitamins [59], or may be because it's contain or rich with nutrients such as vitamin C (ascorbic acid), carbohydrates and minerals [35] , or may be because it's a rich with phenolic (acids ferulic acid, synapic acid) that alters a PH media of worm and leads to kills it, or a phenolic compound adhere with tegument cells of helminths and stopped it's activity , where a phenolic compounds are dissolve in water [60] , or an activity of *citrus limon* plant fruit a cold water extract may be due to that active components or substances high decomposes in cold water that lead to concentration of this substance that effect on parasite more than in boiling water because a hot may be broke or damage an effective substances in plant that lead to reduces its activity on worms .

The statically analysis appears that a high effect on a paralysis and die of *E. vermicularis* helminths which was by a highest concentration(300mg/ml) for cold limon extract with significance differences , this result shows the great effectiveness for *Citrus lemon* plant extract fruits with a high concentration , this results fits with a finding of [61] , they were get on a great anthelmintic activity of aqueous extract for *Citrus Limon* plant on adult earthworm *Eisenia Foetida* in(20mg/ml) concentrations , this effect of *Citrus lemon* plant fruits extract may be attributed to it's a high amounts of components of phenolic compound as phenolic acid, hydroquinone, resorcinol, thymol, phenolic acids, tannins, stilbenes and lignans [42;60and62] , the valuable biological activity of *C. limon* is determined by its high content of phenolic compounds [63; 34;64 and 65] . Phenolic compounds have been reported to have multiple biological effects mainly flavonoids as diosmin, hesperidin, limocitrin and phenolic acids such as ferulic, synapic, p-hydroxybenzoic acids and other biologically active compounds which are rare in other compounds [34; 64 and 65] , The wider pharmacological actions of *C.limon* fruit extract, juice and essential oil made it used as

antibacterial, antifungal, anticancer, anti-inflammatory, hepatoregenerating and cardioprotective activities [66;67], where the pharmacological potential of *C. limon* is determined by its rich chemical composition that are the reason for the important of *C. limon* in the food and cosmetics industries [68] . Citrus lemon fruits have a high beneficial impacts on the human health because it's have nutritional , Anthelmintic activity and antioxidant properties [69;34] .

The extract of Date Veingar have effective roles in a paralysis and die of *E. vermicularis* helminths with increase of concentration and period time, where was the water cold extract in 300 mg/ml concentration has greater impact than hot water extract , this result is fits with result of [70] ,they were get on a high effective of cold acetic acid on giardiacidal activities that was 40.6% after 3 hours was at 24°C, also agree with finding of[71] , they were founds that cold veingar have an effective roles in kills all an eggs of *Ascaris lumbricoides* at 5% concentration after 30 minutes ,this activity of cold Veingar may be due to a presence of phenolic substance promotes antioxidant action [72] ,or may be attributed to that Vinegars contain bioactive compounds which are expecting have synergistic actions to modify the entire body system as sinapic acid reacted with protocatechuic acid, ferulic acid reacted with dihydro ferulic acid, p-coumaric acid or salicylic acid with protocatechuic acid or dihydro ferulic acid exhibited antagonistic effect [31] , or may be due to a presence of organic acids and melanoidins ,tetramethylpyrazine and some bioactive compounds such as lovastatin those have antioxidant action, results to reduction risk of diseases [28;31] , or may be due to a presence to interactions among organic acids, amino acids and phenolic compounds suggest that amino acids have a synergistic effect on the antioxidant activity of phenolic compounds, while organic acids exhibit an antagonistic effect [73] .

Therapeutic effects of vinegar arising from consuming bioactive components including providence energy, regulation of cell metabolism, immunoregulation, antioxidation, anticoagulation [30;29 and 31],differential vinegar varieties having different impact on human health as well as body metabolism [72] .vinegar had greater activity than other simple disinfectant material [70] ,vinegar act as medicinal and therapeutic potential advantages as anti-carcinogenic, anti-glycemic, anti-cardiovascular , antioxidant , antimicrobial [74; 28]. From this study we conclude that a best water extract that lead to a paralysis and die of *E. vermicularis* helminths in short period time was *C. lemon* plant aqueous extract that shows an efficiency of active substances found in *C. lemon* plant more than active substances found in veingar extract.

#### **Effect of plant type factor on a paralysis and die of *E. vermicularis* helminths *in vitro***

From a results of this study appearance that *C. lemon* plant is a best used plant in a paralysis and die of *E. vermicularis* helminths, this findings confirms an effectiveness of *C. lemon* plant, this finding is fits with a finding of [54;75;61 and76] , they were get on a high effective of *C. lemon* plant that have an effective roles against parasites, this finding may be due to *C. lemon* plant contains a high amounts of limonene that possess antimicrobial activity, [43] or may be attributed to vitamin C Which strengthens the immune system, acts as an antioxidant and

protects cells from radical damage [54] , or to that *Citrus limon* extracts contain many effective minerals that may be synergic with other compound found in *Citrus limon* extracts as phenols , terpiens and flavenoids that effect on parasites. *Citrus limon* extracts are found to do superior potential anthelmintic or antiparasitic activity [54;76] .

**Effect of water extract concentration type factor on a paralysis and die of *E. vermicularis* helminths in vitro**

The statics analysis demonstrates extract concentration factor important where was a high concentration (300mg/ml)for water extract has great and effective roles on a paralysis and die of *E. vermicularis* helminths comparative with others concentrations ,this findings is fits with a results of [70;71;54;75;61 and 76] they proved and confirms that a great concentrations possess a high effects on parasites ,this effects may be attributed to that *Citrus limon* extracts contains valuable source of phenolic and other biologically active compounds [75] ,where a high concentration makes an amount of active substance is more and therefore its effect is greater.

**The interference effect of Secondary compounds extracts concentrations for *Citrus limon* plants In vitro**

This part of the study aims to find out the impact of an effectiveness of secondary compounds in paralysis and die of worm where a study proven that terpenoids compound of *Citrus limon* plants are more influential than other secondary compounds it led to worm paralysis and death in a short period of time, this finding appears that terpenoid compounds may be effect by it's a high amount of limonene that have ability to dissolve a cholesterol of cells [77] , that led to die of cell or may be that terpenoid interesting activity could be related to the lipophilic properties of it that were shown to contribute in the disruption of parasite intracellular metabolic pathways [78]. Also this effect of terpenoids compounds may be due to a presence of pipecolic acid, scopoletin, *pulegone*, terpinene, *carvone*, *menthone*, *myrcene*, isopiperitenol, geranial and *linalool* in *Citrus limon* plant as active components that ascribed to observed effects [79], or to efficacy some major terpenoids such as  $\alpha$ -pinene,  $\beta$ -pinene,  $\gamma$ -terpinene, menthone, linalool, limonene which are known to be present in lemon [79;24].

The effects of terpenoids Secondary compounds may be attributed to interactions among components are assumed to be mediated by the emission of terpenoids [80] , terpenoids have been widely considered as therapeutic agent [24] , as well as recent studies showed that terpenoids could also individually or in mixture contribute to the whole antioxidant ability [26] ,also terpenoids as important bioactive constituents of essential oil [24;26]. This study appears that phenolic compounds has clear activity on paralysis and die of *E. vermicularis* helminths where was a time for a worm to paralysis was 32.33 minute and for worm to die was 53.00 minute, this effect of phenolic compounds probably attributed to its rich with tannins [60] ,tannin affects protein composition in worms exposed to high concentrations and thus affecting the neural receptors which leads to the worm's paralysis and death [55], tannins polyphenols binding to protein through hydrogen bonds and forming a tannin–protein complex [81] ,or tannins destroys

an organism's cell membrane through its effect on the fats and proteins in it and then the organism loses its ability to grow or penetrates a cell membranes and obscures the active sites of some enzymes inside the cell which is necessary for growth [82;83]. Phenolic compounds are recognized as being responsible for an antioxidant and antihelminthic ability [60;26].

The results of this study appear that alkaloids compounds have antihelminthic activity on paralysis and die of *E. vermicularis* helminths, this activity of alkaloids compounds can be attributed to that alkaloids may be disrupting the action of the enzyme, receptors and proteins by forming hydrogen bonds with this compound where they have functional groups, a proton accepting nitrogen atom, and one or even more proton donating amine hydrogen atoms [84], such as pergularinine and tylophorinidine alkaloids both of them inhibit the activity of dihydrofolate reductase that are responsible for nucleic acid synthesis [85], or an alkaloids may be binds to protein that important in cell division with high affinity causing inhibition of proteins and its enzyme activity which led to inhibition of cell division [86], or an alkaloid may be acts through a detergent-like mechanism that led to disruption of outer membrane of microorganisms [87], also an activity of alkaloids may be attributed to inhibition of virulence factors of microorganisms [88]. Alkaloids have many pharmacological properties, such as central nervous system stimulants, anticholinergic agents oxytocic and vasoconstrictor activity, anti-inflammatory and antimalarial activity [84;89], the anti-inflammatory activity of alkaloids, involving inhibition or regulation of important inflammation mediators [89]. This is results proven that terpenoid compounds it's the most influential of other secondary compounds and have a significant clear impact on a paralysis and die of *E. vermicularis* helminths therefore terpenoid compounds were chosen as the best secondary compounds, including the essential oil for GC-MS analysis (Gas Chromatography Mass Spectroscopy) and extract the most effective substance in essential oil to try it on *E. vermicularis* helminths *in vitro*.

#### **Concentration type factor effects for Secondary compound for *Citrus limon* plants on paralysis and die for worm *In Vitro***

Results or findings of this study appears that a well concentration of secondary compound extracts was a high concentration (300mg/ml) that possess an effective roles on a paralysis and die of *E. vermicularis* helminths comparative with others concentrations, this results was agree with findings of [90;91;55;92 and 93], they were proved that concentration of secondary compound extracts have clear and a great effect on parasites, this finding may be attributed to that a high concentration offers a great adhesive for active components in secondary compound with a cells of parasites and kills it or a high concentration limonene is one of the major constituents of citrus fruits, and its presence in the citrus fruits contributes to their smell [45].

#### **The interference effects of Albendazole drug in gram /ml concentrations on paralysis and die for worm *In vitro***

The static analysis for findings of interference effects of albendazole drug concentrations demonstrated that albendazole drug possess a high activity on a paralysis and die of *E. vermicularis* helminths especially with 300 mg/ml

concentration where its led to a paralysis and die in very short period of time, this result are fits with [94;95;96;55;97;98;99;53 and 100] , these researchers founding that a high activity of albendazole drug against various helminths ,these effective of albendazole drug may be attributed to a its vermicidal activity mainly depends on inhibiting the absorption of molecules that are critical for parasite growth [101] , or by action of mechanism of the drug's through binding intracellular microtubules and preventing their elongation [101;102]. Albendazole characterized by higher dissolution properties in fats medium [103] and very dynamic distribution and high rate of metabolism of albendazole drug [99] .Albendazole belongs to the benzimidazole class of drugs and is used to treat a wide range of parasitic diseases [104],it is generally effective and safe drugs [102] ,also its possess a broad spectrum anthelmintic for treatment of various helminthiasis , it is now used for chemotherapy of toxocariasis, gnathostomiasis, echinococcosis(cystic hydatid disease), taeniasis, and cysticercosis [105].

**Isolation and diagnosis of limonene compound from terpenoid compounds extracts of *Citrus limon* plants by GC-MS and show its impact on a paralysis and die of *E. vermicularis* helminths *in vitro***

The results of gas chromatography mass spectroscopy GC-MS technical showed that terpenoid compounds of Iraqi *Citrus limon* plants fruit contain limonene compound where it was a retention time of this compound very close to a retention time of stander limonene compound in a library search report of GC-MS apparatus , this findings was similar to findings of [40] in Taiwan ,they are used gas chromatography equipped with mass spectrometry detector to investigate the constituents of commercial *citrus limon* plant essential oils ,their results indicated that limonene was found in *citrus limon* plant and represents as the most abundant compound in *citrus limon* plant essential oils that accounting 57.71 area. Also this findings was similar to findings of [51] in Iraq ,they are analysis essential oil of *Citrus* sp. plant fruits peel (orange, lemon, mandarin) and its contents of limonene is (83.2189, 65.2867and 83.0271)respectively ,they showed that limonene compounds is the most abundant component in essential oil of *Citrus limon* plant fruits peel where that analysis by GC-MS technical is mainly composed of limonene, linalool, citronellal, nerol, geranial as major compound ,among many other components , also this findings was fits to findings of [32] they were analyzed essential oils by GC-MS technical where they found a main bioactive compounds with high content in Omani sweet lemon essential oil was limonene compound(84.73%) and Omani sour lemon essential oil was composed of limonene (53.57%).

As well as [50] they were extracted peel essential oil of *C. limon* and analyzed by GC/MS technical to identified a compounds that was limonene (55.40 %) and neral (10.39 %) were found as major compound followed by *trans*-verbenol (6.43 %) and decanal (3.25 %), also a study was conducted on *Citrus limon* grown in Iraq by [52] to appear chemical composition and biological properties of the essential oil that extracted by hydro-distillation method and analyzed by GC-MS, a results demonstrated that major constituents of essential oil of *C. limon* peels were limonene (29.52 %),  $\beta$ -pinene (23.89 %), citronellal (11.53 %) and thymol (9.79 %). Gas Chromatography Mass Spectroscopy, quick and reliable platform for essential oil analysis in both qualification and quantification [40] ,it is a

hyphenated system is a very compatible technique and the most commonly used technique for the identification and quantification purpose [106] ,It is a tool with good selectivity and high sensitivity is necessary for natural essential oil because they are usually composed of many different ingredients [40] .

Its applications include development of new pharmaceuticals and analysis of their purity, detection of chemical warfare agents and explosives, screening of athletes' urine for banned performance-enhancing substances, and analyzing soil samples on Mars [41] ,as GC-MS is an essential technology in modern analytical chemistry [106;41] , many GC columns separate compounds by boiling point where low-boiling substances move faster and have lower retention times than higher-boiling substances. Natural plant essential oils were prepared simply by dilution with methanol and a short separation gradient of GC coupled with autosampler made this platform more efficient [40]. In this study, we conducted that GC-MS is very precise technical in separation and detection of components of secondary compounds and limonene compound the most predominant compound of Iraqi *Citrus limon* plants fruit.

The limonene compound led to paralysis and die of *E. vermicularis* helminths especially with 400,200and100  $\mu$ /ml concentration where its led to a paralysis and die of worm with high efficient indicates that limonene compound has therapeutic capacity against this parasite , an activity of limonene compound may be attributed to that limonene effect on tegument then muscular and nervous system of worm by dissolve a cholesterol of cells and led to paralysis where its limonene that an excellent solvent for cholesterol, limonene has been used clinically to dissolve cholesterol-containing gallstones [77] ,or to that limonene raised an acidity that effect on metabolism enzyme and receptors of worm cells. Limonene was the major chemical compound in the *C. limon* peel essential oil which possess many medicinal properties could be used as a pharmaceutical industries so it can be used in the treatment of different diseases [50] ,

The high availability of limonene in nature, its safety and its wide mechanism of action make this monoterpenes a promising preventive agent, and could be also used in a complementary approach to conventional therapeutic drugs using anti-inflammatory and anti-infectious drugs [45] .The principal emerging applications of limonene in the food industry as antimicrobial, herbicidal and antioxidant agent [43] , the broad use of limonene in soft drinks, cosmetics and many other flavoring products has raised interest in the antimicrobial, anticancer, toxicity, antiparasitic and many other properties of limonene [107;43] . *C. limon* peel essential oil limonene could also be implemented for the herbal formulation of cost effective, easily available antimicrobial and antioxidant drugs. [50] . it is generally recognized as safe for human consumption as a synthetic flavoring substance [45;43]. The tolerable daily intake (TDI) of limonene was estimated at 0.27 mg / kg body weight, lethal dose (LD50s) of oral d-limonene in mice and rats are generally exceeding 5g/kg body weight. The dermal LD50 in the rabbit is also greater than 5g/kg body weight. Intraperitoneal LD50s range from 1.3g/kg and 4g/kg in mice and rats, respectively [45] .we conducted in this study that limonene compound has very effective activity on *E. vermicularis* adult helminths *In Vitro*.

### **Effect of Albendazole with Microgram unit on a paralysis and die of *E. vermicularis* helminths *in vitro***

Statically, a results of the albendazole drug's activity analysis showed a great efficiency on a paralysis and die of *E. vermicularis* helminths with 400and 200 $\mu$ /ml *in vitro*, this result are fits with [55;97;98;99;53 and 100] , these researchers confirmed that a great activity of albendazole drug against various helminths ,this activity of albendazole drug may be due to that albendazole causes degenerative alterations in the tegument and intestinal cells of the worm by diminishing its energy production, ultimately leading to immobilization and death of the parasite, It works by binding to the colchicine-sensitive site of tubulin, thus inhibiting its assembly into microtubules , as cytoplasmic microtubules are critical in promoting glucose uptake in larval and adult stages of the susceptible parasites, the glycogen stores of the parasites are depleted, degenerative changes in the endoplasmic reticulum, the mitochondria of the germinal layer, and the subsequent release of lysosomes result in decreased production of adenosine triphosphate , which is the energy required for the survival of the helminth [108;102 and 103].

Albendazole drug has a broad-spectrum anthelmintic agent with good efficacy in the treatment of echinococcosis, hydatid cysts, and neurocysticercosis caused by nematodes and cestodes [102;103] .Eating fatty meals enhances absorption significantly, which is important for tissue parasites, absorption is fast in humans and animals; maximum blood levels are achieved within 2–3 hours [109;102] .A fraction of ADZ is metabolized in the intestinal mucosa during absorption, and when it reaches the plasma is rapidly metabolized in the liver to ADZ sulfoxide and finally undergo biotransformation by cytochrome P-450 enzymes into the inactive metabolite albendazole sulfone [102;103 and 110].

### **Conclusion**

- The cold water extract of *Citrus limon* plant has a high effect on paralysis and die of *E. vermicularis* worm more than boiling water extract , then cold water extract and followed boiling water extract of veingar , respectively *In Vitro*.
- The terpenoid compounds of *Citrus limon* plant with 300mg/ml dose has a high efficient on paralysis and die of *E. vermicularis* worm, then phenolic compound followed by alkaloid compounds
- We conducted in this study that limonene compound has very effective activity on *E. vermicularis* adult helminths *In Vitro*.

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