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Evaluation the effects of Urtica dioica and Zingiber officinale extracts on Cryptosporidium hominis cultured on free-cell media by using RAPD technique

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ABSTRACT

The aim of this study was to explore the potential activities of the alcoholic and aqueous extracts of *Urtica dioica* leaves and *Zingiber officinale* rhizome on *Cryptosporidium hominis* that were grown *in vivo*,and evaluate the anticryptosporidial effect(s) at the molecular level by measuring the DNA concentration and using random amplification of polymorphic DNA (RAPD) analysis of the genomic DNA extracted from the control (untreated) group and plant extracts-treated groups. The results demonstrate that the alcoholic extract of *U. dioica* has an effect on the DNA concentration of tested parasite more than of *Z. officinale* .

1.Introduction

All species of the genus *Cryptosporidium* are eukaryotic organisms, including obligate and intracellular parasites which are a significant enteropathogen of immune-competent and immune-compromised vertebrate hosts with worldwide distribution [1]. *Cryptosporidium* has a complex life cycle which takes place in the epithelium of intestine and including both sexual and asexual reproduction [2]. The transmission form is a sturdy, environmentally resistant oocyst, excreted in the stool, which can be remaining for long periods of time in the environment [3]. The most important clinical sign in cryptosporidiosis is gastroenteritis that is may be self-limiting, but in persons who are immunocompromised, it may cause severe or chronic diarrhea [3]. *Cryptosporidium* is naturally resistant to many drugs with known anti-protozoal activities. Even though an extensive broadcast experiment

indicating some activity for 40 out of 101 tested drugs [4; 5]. Only a few were able to hold the parasite development back completely at low concentrations *in vitro*[6]. Plants show a rich source of bioactive compounds and have a long history of use for prevention and treatment of various human [7; 8] .

Urtica dioica has an important areas in drug progress with many pharmacological activities *U. dioica* has recently been shown to have antibacterial, antioxidant, analgesic, anti-inflammatory, antiviral, anti-colitis, anticancer and anti-Alzheimer activities [9]. *Zingiber officinale* is one of the most important plant with numerous medicinal and nutritional values[10; 11] .Frequent pre-clinical studies have supported their importance in the treatment of diabetes, obesity, diarrhoea, allergies, pain, fever, rheumatoid arthritis, inflammation and various forms of cancer and used extensively worldwide to treat a variety of diseases like, nausea, vomiting, asthma, cough, palpitaion, inflammation, dyspepsia, loss of appetite, constipation, indigestion and pain [12]. The present study was aimed to test aqueous and alcoholic effects of *U. dioica and Z. officinale* on *Cryptosporidium hominis* samples cultivated on free cell media and by using random amplification of polymorphic DNA RAPD technique.

2.Materials and methods

2.1 Cryptosporidium genotyping and purification

Cryptosporidium hominis oocysts, isolated from stool samples of infected human patients from Al-askari Hospital at Tikrit city and firstly diagnosed by modified Zeihl Nelson staining method , then genotyped by using Nested PCR-RFLP technique according to Pedraza-Díaz and *et al*, [13]. Samples were purified by using ether extraction, as earlier described [14]. The oocysts were stored in phosphate buffered saline (PBS) with antibiotics (100 IU/mL penicillin and 0.1mg/mL streptomycin) at 4C until use.

2.2 Excystation of oocysts and purification of sporozoites

To stimulate excystation, purified oocysts were resuspended in acidic water (pH 2.5–3) containing 0.5% trypsin/EDTA and incubated at 37C for 30 min. Subsequently, the suspension was centrifuged at 2000 g / 10 min. and resuspended in sterile water. Then, the suspension was re-eluted in 5 ml of preservation media (2% potassium dichromate) and incubated at 37 C in a water bath for at least 3 hr. and by this way the sporozoites will full released of from the oocysts. This suspension was then filtered to remove all oocyst shells [15].

2.3 Preparing of plant extracts

Method used by Prabhakar and *et al*, [16] was followed to obtain the plant extraction.*U. dioica* leaves and of *Z. officinale* rhizomes were collected; isolated; washed and left to dry at room temperature. fifty grams of each plant were separately crushed to a powder form by using sterilized mortar and pestle. These grind materials were extracted sequentially into 200ml of ethanol and sterilized water. The mixture was continuously stirred at room temperature for 2 hr and filtered through filter paper. Resulting extracts in two solvents were evaporated and concentrated to dryness using the rotary evaporator at 45 C° and stored at 4 C°. Serial double fold dilution was done to each stock aqueous and alcoholic extracts solutions in sterile labeled tubes and the concentration tested were (25, 50 and 75) mg/ml.

2.4 Cell-free cultivation

Following purification of sporozoites 20 ul (1×10^3) of suspension was taken and added to 13 micro tubes (2ml) containing cell-free media (RPMI-1640) containing 0.03 gL-glutamine, 0.2 g sodium bicarbonate, 0.88 mg/ml ascorbic acid, , 10% fetal calf serum,

0.25 g HEPES buffer, 10,000 U penicillin G and 5 mg/ml streptomycin, adjusted to pH 7.4 as described by Hijjawi and *et al.* [15]. Cultures were incubated at 37 C and 5% CO_2 until the next step. After 24 hr of cultivation the sporozoites in the media, the tested concentrations of extracts were added to 12 media tube and one tube remain as a control group. The growth and development of parasite was followed up at 24,72,120 and 180 hr by microscopic examination for media samples staining with giemsa stain . At seventh day the parasite centrifuged and the sediments suspended with PBS and stored at – 20C at deep freeze for the next step, [15].

2.5 DNA extraction and RAPD PCR

A randomly amplified polymorphic DNA (RAPD) PCR was used to demonstrate the effect of the plant extractions on *C.hominis*. Oocysts were purified from media materials by sequential sucrose and CsCl gradients and then washed as described earlier by Morgan and *et al.* [17]. DNA of the parasite was extracted manually and concentrated by Nano drop as explained by [18]. PCRs were carried out in a buffer containing 1.5 mM $MgCl_2$, and solution contains dNTPs (dATP,dGTP,dCTp and dTTP)(Bio NEER), and each reaction mixture also contained 20 ul/IU of *Taq* polymerase , the template and eight random primers (OPM-01, OPH-14, OPO-11, OPP-04, OPV-20, OPR-10, OPG-05 and OPZ-08) were used and for RAPD according to the following program: 40 cycles of denaturation at 94°C for 5 min, annealing 36°C for 1 min, and extension at 72°C for 1 min followed by a final cycle at 72°C for 10 min. All reactions were carried out in a model 110S Tempcycler II thermocycler (Coy). Agarose gel electrophoresis, photography, Southern transfers, and hybridizations were performed under standard conditions [18].

3.Results

Table 1 shows a decrease in the concentrations of parasite DNA of treated groups with alcoholic extract of *Urtica dioica* at (25 , 50 mg/ml) and the aqueous extract for the same plant at (50 mg/ml) after 7 days of plant extract exposure. It is clear that there were decrease in concentration of parasite DNA in groups that treated with alcoholic and aqueous extracts of *Zingiber officinale* at (75 mg/ml) and (50 ,75 mg/ml) subsequently compared with control group .

Table 1: DNA Concentration of *C. hominis* treated with plant extracts

plants	Type of extracts	Extracts concentration mg/ml	DNA concentration ng/ µl	X value
Urtica dioica	Alcoholic	25	63	82 a
		50	72	
		75	112	
	Aqueous	25	115	108.6 b
		50	67	
		75	144	
Zingiber officinale	Alcoholic	25	237	160.6 c
		50	188	
		75	57	
	Aqueous	25	211	123.3 d
		50	83	
		75	76	
Control (untreated) group	-	-	432	432 e

Different letters refer to significant differences at ($p \leq 0.05$)

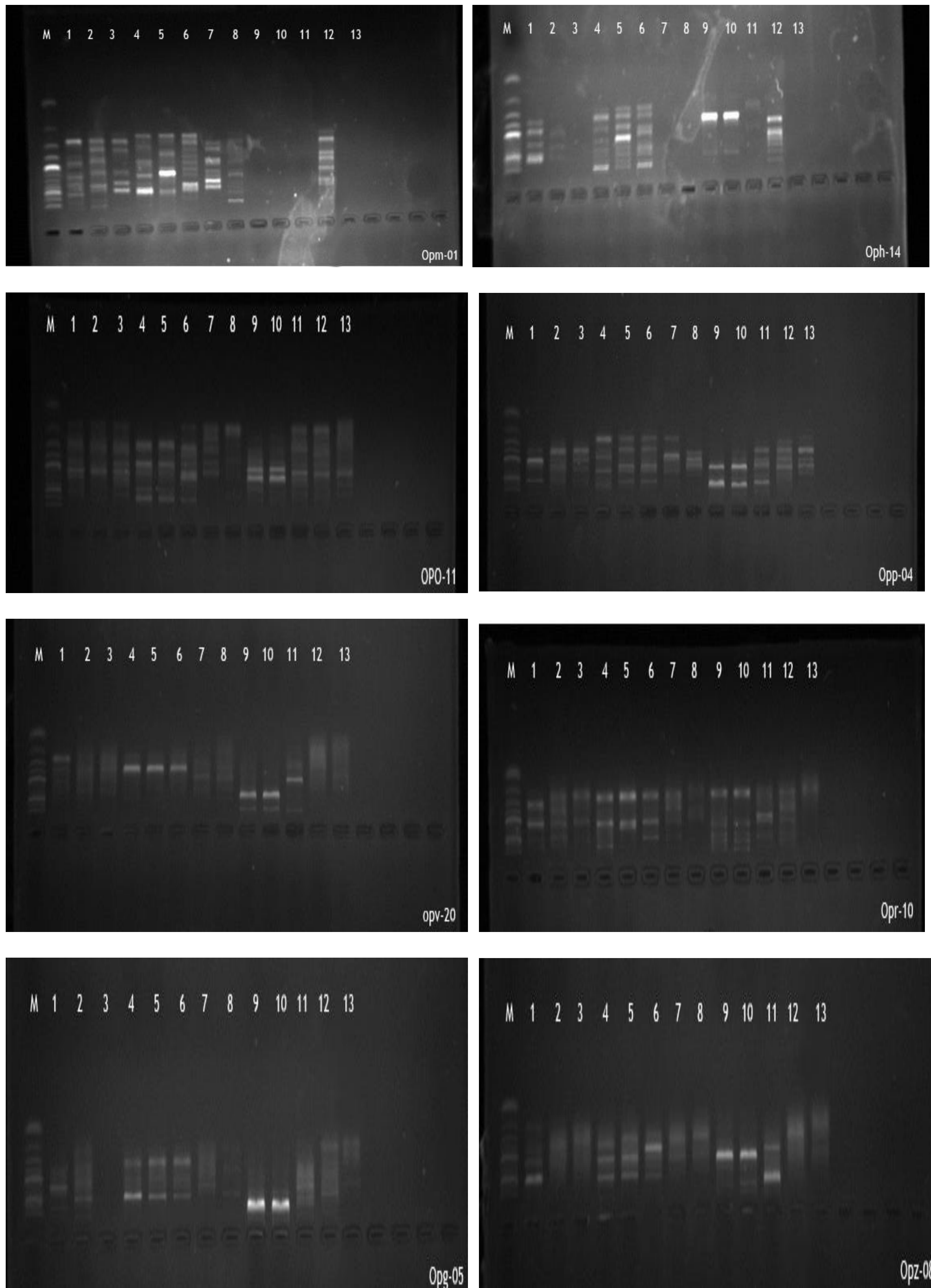

Fig:1 (RAPD profile of *C.hominis* after the treatment with different concentrations of alcoholic extract (CE) and aqueous extract (QE) of *Urtica dioica*; **1**, treated with 25 CE; **2**, treated with 50 CE; **3**, treated with 75 CE; **4**, treated 25 QE; **5**, treated with 50 QE and **6**, treated with 75 QE. And alcoholic extract (CE) and aqueous extract (QE) of *Zingiber officinale* ; **7**, treated with 25 CE; **8**, treated with 50 CE; **9**, treated with 75 CE; **10**, treated 25 QE; **11**, treated with QE and **12**, treated with 75 QE. ; whereas; **13**, untreated (control))

The constancy of *C.hominis* genomic DNA after the treatment with the plants extracts was evaluated using RAPD analysis. The RAPD results illustrated in Figure 1 shows polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for treated parasite compared with untreated group.The results illustrate that the highest number of polymorphic bands among treated *C.hominis* was generated in reactions with the primers OPZ-08 which was 43 genetic bands and their molecular weights ranged between (600 – 1600 bp) .

4.Discussion

According to the current study, it is evident that *U. dioica* has an effect on *C. hominis* activity and growth. Many researches showed the pharmacological functions of this plant as anti-inflammatory,antiviral, [19,20], analgesic, antiandrogenic, antihyperglycemia, anti-hyperlipidemia, antioxidant [21] and anticancer activities, among others[9]. As the current information shows, it is also possible that Scopoletin, polysaccharides, isolectins and sterols might be useful in the development of new drugs to treat various diseases. *U. dioica* has frequently known phytochemical compounds included: flavonoids, tanins, volatile compounds and sterols [22]. Formic acid, histamine and serotonin are also identified as the pain-inducing agents in the stinging hairs of *U. dioica* [23]. Carvacrol (38.2%), carvone (9.0%), naphthalene (8.9%), (E)-anethol (4.7%), hexa-hydrofarnesyl acetone (3.0%), (E)-geranyl acetone (2.9%), (E)-β-ionone (2.8%) and phytol (2.7%) are characterized as the main components of *U. dioica* essential oil [22;24]. Leaf extracts from *U. dioica* acts by switching Th1 derived responses to Th2; therefore it may inhibit inflammatory events of rheumatoid arthritis [25].

Aside from culinary uses, ginger (*Z. officinale*) and its major components, are known to have useful medicinal properties. Ginger and its metabolites have been recognised as potent anti-oxidants due to their ability to inhibit the oxidation of various free radicals and the production of nitric oxide. Ginger has strong antibacterial and to some extent antifungal properties [11;26].

Development in molecular techniques created a great effect on the taxonomy, biology, pathogenesis, epidemiology and on valuation of cryptosporidiosis treatment [17]. Thus, among the molecular methods RAPD is the mostly used for both clinical and environmental samples [13].

The result of this study illustrated that the RAPD technique confirm the genetic effect of the extract. The results analyze revealed a polymorphic banding pattern when comparing between the untreated group and those treated with different concentrations of the alcoholic and aqueous extract .This results suggesting the ability of extracts to make point mutation as a result of deletion compromising at least one nucleotide as showed by the disappearance of many genetic bands and change in limiting endonucleases sites comparing with untreated group and may made a deletion in one or more loci which affect gene expression and interruption in biochemical pathways of DNA and protein synthesis consequently [27;28].

Further research should identify the active ingredients in *U. dioica* and *Zingiber officinale* to improve understanding of the mode of action and to increase the therapeutic range and usefulness by applying isolated and well-defined active compounds **.**

5.Conclusion

Alcoholic and aqueous extracts of *Urtica dioica* leaves and *Zingiber officinale* rhizome exhibited the DNA concentration of *Cryptosporidium hominis* .

6.Acknowledgment

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تقييم تأثير مستخلصات أوراق نباتي القريص والزنجبيل على طفيلي البويغيات الخبيئة المستزرعة في وسط الخلية الحرة باستخدام تقنية RAPD

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الخلاصة

كان الهدف من هذه الدراسة هو الكشف عن التاثيرات المحتملة للمستخلصات الكحولية والمائية لأوراق نباتي القريص *Urtica dioica* والزنجبيل *Zingiber officinale rizome* على طفيلي البويغيات الخبيئة *Cryptosporidium hominis* الذي تمت تنميته في الزجاج ، وتقييم هذه التأثير على المستوى الجزيئي عن طريق قياس تركيز الحمض النووي واستخدام تقنية التضاعف العشوائي المتعدد الاشكال لسلسلة الدنا (RAPD) للحمض النووي الجيني الذي تم استخلاصه من مجموعة السيطرة (غير المعالجة) والمجموعات المعالجة بالمستخلصات النباتية. أظهرت النتائج أن المستخلص الكحولي لنبات القريص له تأثير اعلى على تركيز الحمض النووي للطفيلي مقارنة مع نبات الزنجبيل