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Effect of Citrus Limon and Menthapiperita oil on the Balantidium coli in Vitro

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ABSTRACT

Mint and lemon are medicinal plants that contain many effective compounds, such as oils against many pathogens, including the *Balantidium coli* parasite.In the current study, a crude hexane extract was prepared from lemon peels and mint leaves for the purpose of isolating and purifying lemon and mint oils from them by using thin layer chromatography, and the flow coefficient was determined for each oil separately. The effectiveness of purified oils was examined onto a water agar medium against the parasite *Balantidium coli*.Page solution was added to the medium for the purpose of activating the effectiveness of oils. The results showed a high effectiveness of lemon oil within four days, compared with the antibiotic (Albendazole) and peppermint oil against the parasite.Due to health and economic risks resulting from infection with the parasite, in addition to the concentration of medicines used in treating this parasite depending on chemical drugs, while natural materials drugs have not been taken to the same extent in the research and attention. Therefore, this study was conducted to test the anti-parasitic efficacy of lemon and mint extracts in vivo to understand the extent of their effectiveness against this parasite.
Keywords: Extract; herbal medicines; parasite; pathogen; protozoan; trophozoite.

Introduction

Balantidiosis is a zoonotic disease that can be acquired by humans via fecal-oral route from the normal host. Infected humans may remain asymptomatic, or may develop dysentery similar to that caused by *Entamoeba histolytica*. *Balantidium coli* can become an opportunistic parasite in immunosuppressed hosts living in urban environments[1]), where pigs are not a factor in infection. As opportunistic organisms, the trophozoites of *B. coli* tend to become invasive in penetrating the linings of mucosa and sub-mucosa of the damaged intestine and within the lymphoid tissue of affected hosts, from which they transfer throughout the rest of the body[2].Microscopy is still the first choice for the identification of these ciliates in stool samples. Establishment of all these organisms in culture is far from a routine procedure and is usually less sensitive than microscopy as a detection mechanism. In contrast to bacteria, these organisms are difficult, expensive, and labor-intensive to maintain in the diagnostic laboratory. Due to the few previous researches on the *Balantidium*, is an often-neglected pathogen[3].The xenic culture was reported for the first time for culturing *B. coli* in 1921, demonstrating the use of a simple culture medium comprising saline and human serum. Over the years, several authors have isolated and maintained this protozoan in different culture media using different protocols. The culture media typically used for the isolation and maintenance of *B. coli* are those standardized for amebae, particularly *Entamoeba histolytica*, such as Boeck and Drbohlav medium and Pavlovaxenic medium modified by Jones[4].Herbal medicine (also known as traditional, folk and alternative medicine) comprises medical knowledge systems that developed over generations within various societies before the era of modern medicine[5].Herbal drugs have been in use since ancient times for the treatment of parasitic diseases in human and could be of value in preventing the development of resistance. *Citrus limon* belongs to family rutaceae is (also known as Assam lemon) one of the most important crops of Assam and other parts of north eastern region. Fruits are widely used for culinary, beverages, industrial and medicinal uses[6]. Preliminary qualitative phytochemical screening of *C. limon* peel revealed the presence of phenolic compounds, terpenoids, flavonoids and steroids. Also, Extracts of *Menthapiperita* species have compounds, such as terperno´ides, flavonoids and phenolics that have activity against bacteria and protozoa. These phytochemicals are responsible for the biological activities in plants, and their use have been increased during the recent years due to the search for new bioactive compounds with anti-parasitic potential for several animals, parasites and protozoa that were considered from common contaminants. Such contaminants may come from water used in moistening vegetables includes *Ascarislumbricoides*, *Cryptosporidium* spp., *Entamoebahistolytica*/dispar, *Enterobiusvermicularis*, *Giardiaintestinalis*, *hookworm*, *Hymenolepis* spp., and *Trichuristrichiura*[7]. Consequently, this study was conducted to examine the anti-parasitic efficacy of lemon and mint extracts in vivo to understand the extent of their effectiveness against this parasite.

Methodology

Sample Collection and parasite diagnosis

Samples were collected from pork droppings and the parasite was morphologically and genetically diagnosed by the same

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authors in a previous research (Unpublished).

Preparing the water agar medium

5 g of the agar were added into 250 ml of distilled water and 0.25 g of chloramphenicol to prevent the growth of bacteria, then sterilized with an autoclave. The medium was poured into Petri dishes (9 cm diameter) for the purpose of parasite culturing[8].

Preparing the Page solution

The Page solution was prepared according to (Page, 1988) from the following materials dissolved in a liter of distilled water: NaCl (0.12) g, $MgSO_4.7H_2O$ (0.004) g, $CaC1_2$ $2H_2O$ (0.004) g, Na_2HPO_4 (0.142) g and KH_2PO_4 (0.136) g, with the addition of 200 units / ml of each of Streptomycin and Penicillin[9]

Sample Collection of the study plants:

Samples (lemon and mint) were collected from the local market in Basra province, brought to the laboratory and washed with running water. Then, the lemon peels and mint leaves were cut, dried and milled well to obtain powders for the plants for the purpose of the extraction process.

Extraction:

5 g of the powder from each plant (lemon peel and mint leaves) were weighed and placed separately in a 100 ml conical flask, then 50 ml of hexane solvent was added to each beaker and placed on a magnetic stirrer for 24 hours. Then the samples were dried onto sterile petri dishes. After sample drying, the oils were obtained and placed in sterile tubes until use[10].

The oils of Lemon and mint were purified by using thin layer chromatography. Lemon and mint oils were isolated and purified separately from the special hexane extract for each of them according to the method[11, 12].

UV-Visible absorption spectroscopy

The UV-Visible absorption spectra are measured from 190–900 nm using a T90+ UV-visible spectrophotometer (PG Instruments Ltd., Leicester, UK) with a conventional quartz cell having a 1 cm optical path in the Department of Chemistry, University of Thi-Qar, Iraq. The concentrations of limonene and menthol solutions are 20% (v/v) in hexane.

Fourier-Transform infrared (FTIR) spectroscopy

FT-IR spectra were recorded in the 500-4000 cm^{-1} range on a SHIMADZU/FT-IR Affinity-1 spectrophotometer in the department of Chemistry, College of Science, University of Thi-Qar[13].

Preparation of the antibiotic metronidazole

This antibiotic was prepared at a concentration of 250 mg / ml, where used as a control group.

The efficacy test of the purified oils and antibiotic against the parasite

Samples were cultured onto the water agar that provided with 1 ml of the parasite suspension (contains 500 trophzoite), and 2 ml of phosphate buffer saline (pH 7.4), and 6 ml of page solution. Then, the extracts (oils) and antibiotic were added in a volume of 1 ml each separately with 3 replicates for each group. The dishes were incubated at 36 ˚C for 48 hours and the parasite count was calculated daily, using the counting slide (Neubauer Chamber) after making sure the parasite was appeared under a light microscope[14].

Results

Effects of extracts were significant on the parasite

The results proved the direct effect of the extracts and the antibiotic on the growth of *Balantidium coli* parasite, and the effects of extracts were significant on the parasite, as their addition led to decreasing the numbers of parasite. The effect of lemon extract was higher than the effect of mint extract. While, the antibiotic effects were positive as it led to an increase in preparing the parasite, which was considered as a control group**Table1**.

UV absorption spectroscopy of limonene and menthol oils

UV-Visible absorption spectroscopy

The UV absorption spectrum of limonene in hexane in Fig. (1, 2) show a peak at 315 nm, which is attributed to π-π*

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transition of C=C bonds. Both peaks in menthol spectrum occurred at 262 nm and 399 nm, which are due to π-π* transition of OH group. The very weak peak at 648 nm was belonging to trace impurities.

FT-IR of limonene and menthol oils

The structures of limonene and menthol were characterized by FT-IR spectroscopy. The FT-IR spectra and their assignments are represent in Fig. 1, 2 and Tables 2, 3 respectively. The FT-IR spectrum of menthol shows broad peak within the range of ca 3313-3165 cm^{-1} which is belong to O-H stretching. Significant peaks at 2918.69, 2855.08 cm^{-1} are attributed to alkene C-H stretching. The peaks at 1525.45, 1458.66, 1378.10 cm^{-1} could correspond to aliphatic C-H bending and C-O stretching. The spectral band at 3006.03 cm^{-1} is due to alkene C-H stretching. Significant peaks are found at 2923.06, 2855.59 cm^{-1} corresponding to aliphatic C-H stretching. The band at 1743.72 cm^{-1} is attributed to the C=O stretching vibration. The peak at 1525.41 cm^{-1} is ascribed to C=C stretching vibration. Both peaks corresponding to alkene C-H stretching and C=C stretching support the presence of non-saturated C=C bonds in structure of limonene. Aliphatic and aromatic C-H bending peaks occurred at 1460.09 and 1156.73 cm^{-1} respectively.

Table 1. Maintenance periods of *B. coli* trophozoite on variable culture media

Extracts	**Maintenance period (days)after incubation for 48h.**			
	1st day	2nd day	3rd day	4thday
Citrus limon	3500	3250	2750	1750
Menthapiperita	4250	5000	3000	2250
Metronidazole(control group)	6000	8000	7750	10250

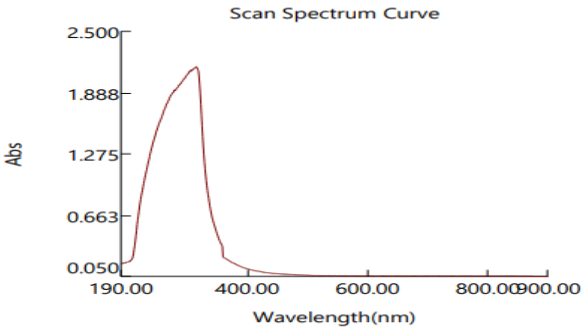

Figure 1. UV absorption spectrum of limonene oil

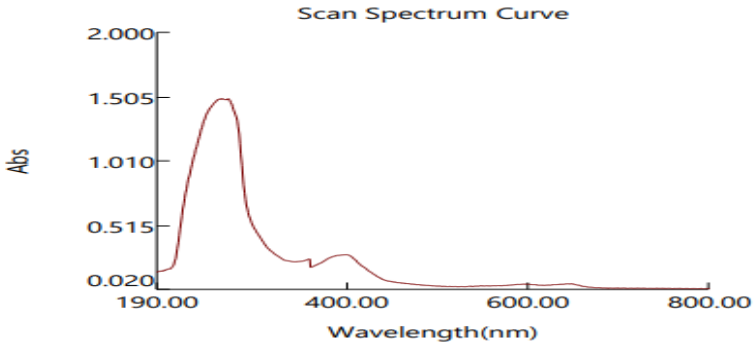

Figure 2. UV absorption spectrum of menthol oil

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Table 2. FT-IR frequencies (cm^{-1}) and their assignments of **limonene**.

Wave number (cm^{-1})	**Assignment**
3006.03	Alkene C-H stretching
2923.06, 2855.59	Aliphatic C-H stretching
1743.72	C=O stretching
1525.41	C=C stretching
1460.09	Aliphatic C-H bending
1156.73	Alkene C-H bending

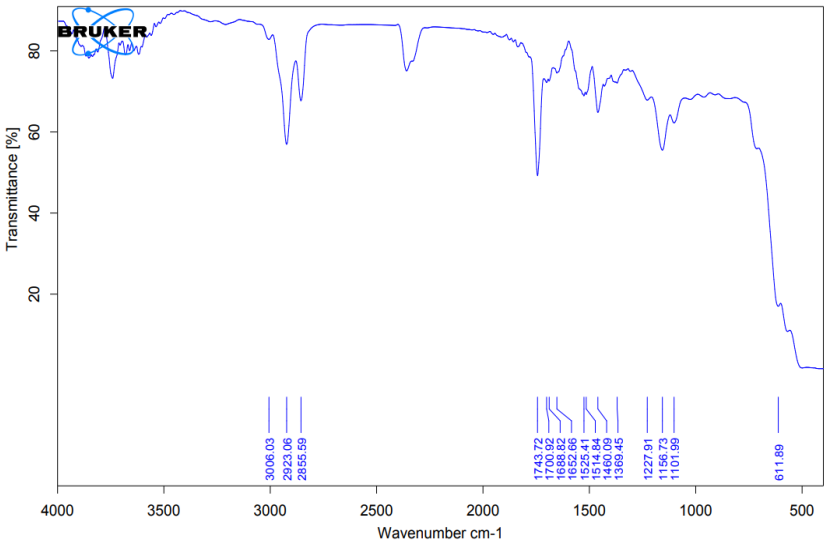

Figure 3. FT-IR for limonene oil

Table 3. FT-IR frequencies (cm^{-1}) and their assignments of **menthol**.

Wave number (cm^{-1}	**Assignment**
3313-3165	O-H stretching
2918.69, 2855.08	Aliphatic C-H stretching
1688.23	O-H bending
1525.45, 1458.66, 1378.10	Aliphatic C-H bending and C-O stretching

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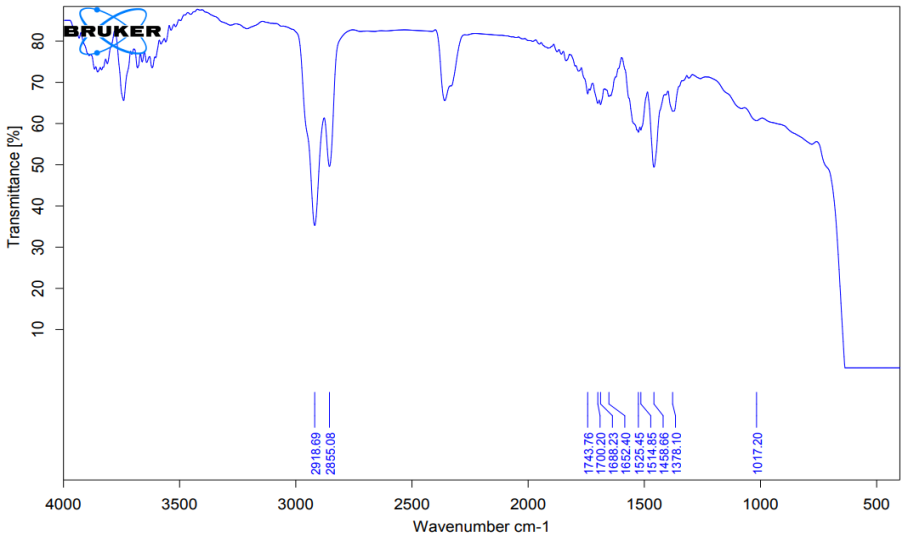

Figure 4. FT-IR for menthol oil

Discussion

Parasitic infections are one of the most common infections in humans, and as a result of the emergence of many parasitic isolates that are resistant to antibiotics, especially those with multiple resistance of antibiotics at the same time. So, interest has increased in recent years about the possibility of introducing medicinal compounds of plant origin in pharmaceutical drugs[15].In the late nineties, it was found that 80% of the world's population relied on traditional folk medicine to treat many clinical infections, as many plant extracts are used as anti-parasites[16]. Therefore, the researchers' attentions were directed towards the plant kingdom, which is an abundant and inexhaustible treasure, and among these plants known in various countries of the world for their biological effectiveness are lemon and mint. Xavier-Junior[17] have mentioned that lemon peel contains lemon oil, which contains many essential oils that are known in their anti-parasite effectiveness. These oils are characterized by their ability to combine with the cell protein and its precipitation. Also, they are good solvents for fatty substances due to their ability to decompose the membranes of live cells of the parasite and as a result, the internal components come out of the cell, so the parasite cell dies. This is in accordance with a study of Maaroufi et al.[18] who proved the effectiveness of lemon peel against parasites.

Peppermint oil (menthol oil) also had a parasite fatal effect because it contained fatty acids such as menthol, isomenthol and neomenthol that have a parasitic effect. This finding is consistent with Morgan [19]who stated that the parasite cell is surrounded by a protoplasmic membrane consisting of several materials, including fatty substances.The results of this study evidently supported the use of these plants in the treatment of *Balantidiasis*. Similar findings have been reported by Burt [20].

Conclusion

The phytochemicals of some herbal medicines, such as lemon and mint have been reported to be responsible for the biological activities in plants, and their use have been increased in last decades due to the need of promising bioactive compounds with depressionactivity against several parasites, especially for those consideredas common contaminants. In the current study, the isolated compound (menthol and limonene oils)illustrated promising anti-parasite accomplishments against *B. coli* as the most predominant parasite in intestine.

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