



Evaluating the Effect of Selected Medicinal Plant Extracts and their Synergistic Effect with Metronidazole against *Entamoeba histolytica*

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ABSTRACT

Introduction: Intestinal infection with *Entamoeba histolytica* is an important cause of diarrhoea world-wide especially where sanitation conditions are poor. Medicinal plants have played a significant role in various traditional systems of medications including intestinal infections caused by *E. histolytica*. **Objectives:** To investigate the antiameobic activity of pomegranate, castor and thyme aqueous and alcoholic extracts, in addition to thyme oil and pomegranate juice against *E. histolytica* and to determine the synergistic effect of these plant extracts with the antibiotic metronidazole. **Methods:** Three medicinal plants pomegranate (*Punica granatum*), castor (*Ricinus communis* L) and thyme (*Thymus vulgaris* L) were used in this study. Some of these plants were bought from fruit's market in Gaza and some were collected from some regions in Gaza; each plant was dried then extracted according to standard extraction method using Soxhlet apparatus. *E. histolytica* was cultured in a modified diphasic liver infusion agar medium *in vitro* conditions to study the effect of these plant extracts on *E. histolytica* and their synergistic effect with the antibiotic metronidazole. The type of *E. histolytica* was confirmed using PCR. **Results:** Successful cultures of *E. histolytica* were obtained. All the plant materials used in this study possessed antiameobic activity *in vitro* with different levels except the aqueous extract of *R. communis* leaves which also was the only one didn't show synergistic effect with metronidazole at tested concentrations. Alcoholic extract of *P. granatum* pulp had the strongest antiameobic activity with MIC of < 5 mg/ml while the aqueous extract of *T. vulgaris* leaves had the weakest antiameobic activity with a MIC of < 20 mg/ml. **Oils and Juice:** *P. granatum* juice possessed antiameobic activity and synergistic effect with a MIC < 12.5 % (v/v). *T. vulgaris* oil not only possessed antiameobic activity and synergistic effect but also had the strongest activity and a MIC of < 6.5% (v/v), and the best synergistic effect. Metronidazole is still effective against *E. histolytica* with a MIC < 20 µg/ml and exhibit greater amoebicidal activity as compared with the plant extracts. **It is recommended** that pomegranate and thyme should be advised by physicians to treat *E. histolytica* infection with or without metronidazole. The plant extracts which showed a good amoebicidal activity *in vitro* should be tested *in vivo* on experimental animals to evaluate their amoebicidal effect and their synergistic effect with metronidazole on *E. histolytica* and also recommended to raise awareness regarding the use of medicinal plants to treat parasitic infections.

KEYWORDS: Synergistic Effect, Pomegranate, Castor, Thyme, *Entamoeba histolytica*, Metronidazole.

INTRODUCTION

Entamoeba histolytica is a protozoan parasite which causes a parasitic disease known as Amoebiasis. It can pass through the stool of infected people or animals and spread everywhere such as soil, water, food or surfaces. The sever form of this disease is known as amoebic dysentery, while the symptoms include fever, stomach pain, bloody stools, and diarrhea. In rare cases *E. histolytica* can invades the liver causing hepatic amoebiasis and abscess. it may move to other parts of the body, such as the lungs or brain.^[1] The Published data about the prevalence rates of cyst passage varies from

place to place, increasing in the places with poor sanitation.^[2] Even though the Intestinal amoebiasis is considered a serious infection, the infected person may stay a carrier for many years and the trophozoites don't cause any damage or symptoms. But some who are infected will develop amoebic colitis or fulminant colitis and syphtomos will be exists.^[3] Metronidazole is a synthetic nitroimidazole, it is effective against some parasites such as *Trichomonas vaginalis* (trichomoniasis), *E. histolytica* (amoebiasis), and *G. lamblia* (giardiasis) and almost all obligate anaerobic bacteria including *Bacteroides fragilis*.^[4] In spite of

Metronidazole resistance in protozoa and helminthes is becoming a major public health problem, development of new drugs proceeding slowly, *E. histolytica* resistance to metronidazole is related to overexpression of iron superoxide dismutase, over expression of peroxiredoxin, decrease of the expression of ferredoxin and decrease in the expression of flavin reductase.^[5] This study was performed to investigate the antiamebic activity of pomegranate (*Punica granatum*), castor (*Ricinus communis L*) and thyme (*Thymus vulgaris L*) aqueous and alcoholic extracts in addition to thyme oil and pomegranate juice against *E. histolytica*, and to determine the synergistic effect of these plant extracts with the antibiotic Metronidazole.

MATERIALS AND METHODS

In the present study three plants were used as follows; *Punica granatum* (pomegranate), *Ricinus communis L* (Castore) and *Thymus vulgaris L* (thyme). These plants were collected from local markets in Gaza, then divided into small pieces. Those small pieces were shadow dried away from sunlight. Then the dried plant pieces were converted into fine powder using a mill. The air dried and powdered materials of *Punica granatum*, *Ricinus communis L* and *Thymus vulgaris L* using water and ethanol were extracted in 250 ml for 8 hours on Soxhlet apparatus. Then the solvents were removed by air drying using an oven over 3 days at 40°C to obtain a crude extract, this was then stored at 4°C in bottles.

Preparation of modified diphasic liver infusion agar medium used for *Entamoeba histolytica* cultivation.

A modified diphasic liver infusion agar medium was used for culturing *E. histolytica in vitro*. It is a very nutrient media and has the main components for parasite's growth with some supplements as rice flour used as a carbohydrate source, bovine serum as a source of lipids, antibiotics (Streptomycin 3 mg/ml and penicillin 1500-2000 Iu/ml) and antifungal (a drop of nystatin (originally named Fungicidin) were added to the media to prevent the growth of bacteria and fungi which sensitive to the used antibiotic and antifungal respectively. pH was adjusted to 7.2, which is the optimal pH for the growth of *E. histolytica*.

Sample collection: Thirty stool samples from patients with amoebic dysentery were obtained from UNRWA clinic in El-shatte' refugees camp and from some private medical laboratories in Gaza, and delivered to the Islamic University labs within 30 minutes. Direct smear examination using saline for each sample was first done, when viable trophozoites were seen, the sample was then cultivated.

Preparation of plant extracts and metronidazole stock solution

Stock solution of plant extract was prepared by weighing 1g of net extract in a known-weight tube using an electronic scale, with the volume brought up to 5 ml using D W as a solvent in the case of aqueous extracts or

10% (v/v) DMSO as a solvent in the case of alcoholic extracts and oils. This will lead to the preparation of 200 mg/ml concentration of plant extract. A stock solution for each plant extract was prepared. Then 2.5; 5; 10; 20; and 30 mg extract/ml media were prepared from the stock solution and 6.25% (v/v) and 12.5 % (v/v) for oils and pomegranate juice. Serial dilutions of metronidazole (5 mg/ml) using prepared media to obtain the concentrations of 10, 20, 40, and 80 µg/ml was prepared.

Plant extracts activity assay

From each tube containing well grown *E. histolytica* trophozoites, six subculture tubes were performed, according to the method of subculture explained previously. This was done after microscopic examination to make sure that the tube was containing many viable trophozoites in (H.P.F). These six tubes were used for: control; and for each plant extract concentrations of 2.5, 5, 10, 20, and 30 mg/ml. For oils and pomegranate juice, three subculture tubes were prepared: one for control; and two for the concentrations of 6.5% (v/v); and 12% (v/v). Triple trials for each concentration were conducted to ensure the accuracy of the results, each trial consisted of; control (culture medium and *E. histolytica*); and test (extract or metronidazole, culture medium and *E. histolytica*); and for the synergistic effect (extract, metronidazole, culture media and *E. histolytica*). After incubation for 24 hrs at 37°C the tubes were examined daily for 4 days.

Determination of minimum inhibitory concentrations (MIC) of plant extracts

The concentrations that inhibit the growth of parasite were determined by counting the total cells under microscope. According to Upcroft and Upcroft, (2001)⁶, (MIC) is "the lowest concentration at which > 90% of the trophozoites growth is inhibited". The tubes were chilled for 15 minutes to detach the trophozoites, and then the number of viable cells from every tube was counted twice. The results were calculated as: the growth inhibition with extracts compared with the controls grown without plant extracts.

Polymerase chain reaction for *Entamoeba histolytica*.

The protocol of this study was performed according to (Qiagen Inc., USA). DNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions.

Primers used in this study

The primers which used in this study as shown in table 1.

Table. 1: The primers used in the present study.

Primers	Nucleotide sequences	PCR product size	Reference
EH1 (P1) <i>E. histolytica</i>	P1:5'TCAAAATGGTCGTCTAGGC3'	125-bp	(Al-Hindi et al., 2005) ⁷
EH2 (P2)	P2:5'CAGTTAGAAATTATTGACTTTGTA3'	133-bp	
ED1 (P3) <i>E. dispar</i>	P3:5'GATCCTCCAAAAAATAAAGTTT3'		
ED2 (P4)	P4:5'ACAGAACGATATTGGATACCTAGTA3'		

RESULTS

Result of Polymerase chain reaction.

Using *E. histolytica* specific primer showed that the band size was 125 bp. This confirmed that *E. histolytica* was the type in the examined stool samples.

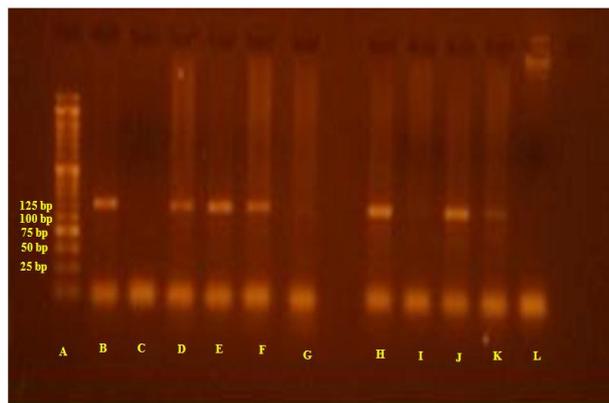


Fig. 1: A representative photograph of ethidium bromide stained 2% agarose gel showing, the PCR products for some stool samples. Lane A; ladder, lane B; Positive control, lane C; Negative control. Lanes D, E, F, H, J, and K for positive for *E. histolytica*, while Lanes G, I and L for negative.

The effect of plant extracts and their synergistic effect with metronidazole on *Entamoeba histolytica* trophozoite.

The cultivated samples were examined after 24 hrs of cultivation and for a week, if the trophozoites were seen during this week this was considered positive results. But when testing the effect of extracts the samples were tested for 96 hrs, if there wasn't any trophozoite seen during this period, this means the extract had antiamoebic effect.

The Minimum inhibitory concentrations (MIC) for plant extracts on *Entamoeba histolytica*.

The Minimum inhibitory concentration (MIC) for each plant extract was recorded as the lowest concentration which can inhibit the growth of *E. histolytica* trophozoites (table 2).

Table. 2: MIC values for each plant extracts used in this study.

Material	Type of extract	MIC
Pomegranate rind	Aqueous extract	<10 mg/ml
Pomegranate rind	Alcoholic extract	<10 mg/ml
Pomegranate pulp	Aqueous extract	< 10 mg/ml
Pomegranate pulp	Alcoholic extract	<5 mg/ml
Pomegranate juice	Juice	< 12.5 %
Castor leaves	Aqueous extract	No effect ***
Castor leaves	Alcoholic extract	<10 mg /ml
Thyme leaves	Aqueous extract	< 20 mg/ml
Thyme leaves	Alcoholic extract	<10 mg/ml
Thyme oil	Oil	< 6.5 %
Metronidazole	Aqueous solution	< 20 µg

*** means no effect at the tested concentrations

The relation between each plant extract separately and with metronidazole on *Entamoeba histolytica*.

To evaluate the synergistic effect between each extract and metronidazole, 10 µg/ml of metronidazole (half amount of MIC) and amounts less than MIC for extracts were used as shown in table 3.

Table. 3: The relation between each plant material and metronidazole on *E. histolytica*.

Material	Type of extract	The amount of Metronidazole	The effective amount of extract	Relation
Pomegranate rind	Aqueous extract	10 µg/ml	2.5 mg/ml	Synergism
Pomegranate rind	Alcoholic extract	10 µg/ml	2.5 mg/ml	Synergism
Pomegranate pulp	Aqueous extract	10 µg/ml	2.5 mg/ml	Synergism
Pomegranate pulp	Alcoholic extract	10 µg/ml	2.5 mg/ml	Synergism
Pomegranate juice	Juice	10 µg/ml	6.5 %v/v	Synergism
Castor leaves	Aqueous extract	10 µg/ml	10 mg /ml	No synergism ***
Castor leaves	Alcoholic extract	10 µg/ml	2.5 mg/ml	Synergism
Thyme leaves	Aqueous extract	10 µg/ml	10 mg/ml	Synergism
Thyme leaves	Alcoholic extract	10 µg/ml	2.5 mg/ml	Synergism
Thyme oil	Oil	10 µg/ml	3.25 % v/v	Synergism

*** No synergism at tested concentrations

DISCUSSION

E. histolytica was successfully cultured in this study. Because *E. dispar*, cysts and trophozoites and *E. histolytica* are morphological identical, laboratory reports should say "*E. histolytica* / *E. dispar*".^[8] Al-Hindi et al., (2005) reported that 30 % of suspected clinical amoebiasis in Gaza were negative for *E. histolytica* after analyzing them using (PCR) and they recommended the use of (PCR) for diagnosis *E. histolytica* in stool sample. In the present study, *E. histolytica* isolates were confirmed using (PCR).^[7]

T. vulgaris leaves extracts inhibited the growth of *E. histolytica*, both aqueous and alcoholic extracts. For alcoholic extract MIC was <10 mg/ml while in aqueous extract MIC was <20 mg/ml and in *T. vulgaris* oil MIC was < 6.5%. In particular, the essential oil of *T. vulgaris* exhibited stronger activity against *E. histolytica* trophozoites than the extracts. In fact, Lee et al. (2005)^[9] previously showed that the essential oil of *T. vulgaris* has greater antioxidant activity in comparison with its extracts. So in this study alcoholic extract, aqueous extract and *T. vulgaris* oil all of them showed antiamoebic activity. *T. vulgaris* essential oil and extracts seem to be good natural antiamoebic alternative for metronidazole.

This is agreed with a study carried out by Behnia et al., (2008)^[10] with effect of *T. vulgaris* on *E. histolytica* and differed with the effective concentrations. In their study the antiamoebic effects of hydroalcoholic and n-hexane extracts, as well as of the essential oil, of *T. vulgaris* against *E. histolytica* have been tested and (MIC) for this plant's hydroalcoholic extract, hexane extract, and essential oil after 24 hrs was 4.0, 4.0, and 0.7 mg/ml, respectively. In this study, the result of the effect of thyme leaves extracts and oil on *E. histolytica* is consistent with the findings observed by Behnia et al., (2008)^[10] but differed in MIC values, this is may be due to differences in the strain of the used *E. histolytica*, components of culture media or incubation period of *E. histolytica*, full maturity of the used thyme leaves, time of leaves collection, extraction methods and procedures, the time of extraction process, and differences in methodology details and techniques. According to Duke

et al., (2002)^[11] and kare, (2007)^[12] the thyme is used as antiseptic, antibacterial, antifungal, antiviral, antispasmodic, ascaricide, insecticide, vermifuge and for treatment many other diseases.^[13] *P. granatum*, Pulp; rind extracts and juice also were used. The (MIC) for aqueous extracts of rind and pulp were for both <10 mg/ml and for alcoholic extracts for rind and pulp were <10 mg/ml and < 5 mg/ml respectively, and for pomegranate juice was <12.5% (v/v). According to these results all of *P. granatum* extracts used in this study showed good results and exhibit amoebicidal activity. These results are consistent with the findings observed by Naqvi et al., (1992)^[14] who reported that, the aqueous extract of *P. granatum* rind has amoebicidal activity with (MIC) <10 mg/ml. Al –Tikrity et al., (2008) also studied the effect of aqueous and alcoholic extracts of *P. granatum* (pulp and rind) together on *E. histolytica*, according to their study the IC50 for aqueous extract was <0.75 mg/ml and for alcoholic extract was <1.25 mg/ml. So *P. granatum* (pulp and rind) aqueous and alcoholic extracts showed good results and have antiamoebic activity. The result of the effect of pomegranate extracts on *E. histolytica* is consistent with the findings observed by Al –Tikrity et al., (2008)^[15] in the antiamoebic effect but differed in MIC values due to of differences in the Strain of *E. Histolytica*, components of culture media or incubation period of *E. histolytica*, parts of pomegranate used, full maturity of pomegranate and time of pomegranate collection, extraction methods and procedures, the time of extraction process and differences in methodology details and techniques.

Rind of pomegranate is used for the treatment of diarrhoea and dysentery in addition to many other diseases.^[12] Aqueous and alcoholic extracts of *R. communis* leaves were tested for their antiamoebic activity; the aqueous extract didn't inhibit the growth of the trophozoites of *E. histolytica* completely at the tested concentrations, while the alcoholic leaves extract showed antiamoebic effect with MIC < 10 mg/ml. Metronidazole (Flagyl) is one of the few drugs used for the amoebic and anaerobic bacterial infections treatment in Gaza and has serious side effects. Metronidazole activity on *E. histolytica* was also tested in this study with different concentrations; MIC was < 20 µg/ml. Results of this

study consistent with those of Behnia et al (2008)^[10] and Nagvi., et al (1992)^[14] with the antiamebic effect of metronidazole, but differed in the MIC values. MIC for metronidazole in their studies were < 2 and < 5 µg/ml respectively. But in this study MIC was < 20 µg/ml. These differences in the MIC values may be due to the difference in the *E. histolytica* strains, the quality of the metronidazole component; the techniques used, and may be due to the indiscriminate use of metronidazole by patients in Gaza with or without medical advice. According to these results metronidazole still the drug of choice for treating *E. histolytica* infection but using it randomly and continuously without physician administrations may lead to emergence of parasite's resistant. In order to reduce the metronidazole side effects and also to avoid parasitic resistance to the drug, synergistic effect between each plant extract and metronidazole was tested. The concentrations less than the MIC for each plant material and metronidazole tested together for their antiamebic activity, each trial of the study was repeated three times. All the tested materials showed synergistic effect with metronidazole except the aqueous extract of *R. communis* leaves at tested concentrations.

Synergistic effect of plant materials and metronidazole has an important role to minimize the metronidazole dose which should be used by patients, for decreasing the drug side effects, parasite's resistance to drug and for increasing the drug effect on the parasite and also to avoid drug tolerance.

The alcoholic extracts in this study showed better result with strongest antiamebic activity and lowest MIC values than the aqueous extracts except pomegranate rind extract, where both showed the same effect with the same MIC values. In general, aqueous extracts showed less activity than ethanol extracts which may be explained in the light of what have been reported by De Boer et al., (2005),^[16] that the same active substances are present in water extracts, but in lower concentrations and/or that some active substances were more soluble in organic solvents and, therefore, not present in water extracts. Thyme extracts is an excellent example for the differences between the effect of alcoholic and aqueous extract, in this study alcoholic was better, this may be due to the presence of thymol. According to Beale and Block, (2010),^[13] Thymol is slightly soluble in water but extremely soluble in alcohols and other organic solvents.

CONCLUSIONS

It was found that modified diphasic liver infusion agar medium was useful for cultivation of *E. histolytica*. All the plant materials used in this study possessed antiamebic activity *in vitro* with different levels except the aqueous leaves of *R. communis* extract which also was the only one which didn't possess synergistic effect with metronidazole at tested concentrations.

It was concluded that metronidazole is still effective against *E. histolytica* with MIC < 20 µg/ml and exhibits greater amoebicidal activity as compared to plant materials.

Alcoholic extracts showed strongest amoebicidal activity and lowest MIC values compared with the aqueous extracts of plant materials used in the study except for the aqueous extract of *P. granatum* rind where the alcoholic and aqueous showed the same activity, MIC for both was < 10 mg/ml.

RECOMMENDATIONS

- 1- It is recommended to use plant extracts under the study with metronidazole for *E. histolytica* treatment.
- 2- The plant materials which showed good amoebicidal activity *in vitro* should be tested *in vivo* on experimental animals to evaluate amoebicidal affect and their synergistic affect with metronidazole on *E. histolytica*.
- 3- There are many other medicinal plants should be tested for their amoebicidal activity. These plants are cheap, available and safe. So this study recommends examining them in other studies.
- 4- Further studies should be done to know the active ingredients of *P. granatum* (pomegranates) pulp, rind and juice, *T. vulgaris* (Thyme) leaves and oil, and *R. communis* (castor) leaves, and then the active compounds which have amoebicidal activity can be isolated and can be used to invent new natural amoebicidal drugs.
- 5- Raising awareness regarding the use of medicinal plants to treat parasitic infection.
- 6- It is recommended to use two or more of the used medicinal plants together to investigate their synergistic effect against *E. histolytica*.
- 7- The effective medicinal plants should be *in vitro* tested on human lymphocyte cells to ensure that this plants safe and don't have any effect against human cells.

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