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Anti-*Trichomonas vaginalis* Activity of Marine Ascidians (Tunicates; Ascidiacea) from the Bushehr Province, Iran

İran'ın Buşehr Eyaletindeki Deniz Ascidianlarının (Tunikatlar; Ascidiacea) Anti-Trichmonas vajinalis Aktivitesi

Hamideh Malekhayati[1], Afshar Bargahi[2,3], Soleiman Khorami[1], Mohsen Khataminejad[3], Moradali Fouladvand[4,5]

[1]Bushehr University of Medical Sciences Faculty of Paramedical Sciences, Department of Laboratory Sciences, Bushehr, Iran

[2]Bushehr University of Medical Sciences Faculty of Medicine, Department of Biochemistry, Bushehr, Iran

[3]The Persian Gulf Marine Biotechnology Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran

[4]Bushehr University of Medical Sciences Faculty of Medicine, Department of Microbiology and Parasitology, Bushehr, Iran

[5]The Persian Gulf Tropical and Infectious Diseases Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran

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ABSTRACT

Objective: The aim of the current research is to evaluate the antiparasite effects of compounds isolated from marine ascidian tunicates on *Trichomonas vaginalis*.

Methods: Ascidian tunicates after collection were cut into small pieces, freeze-dried, and powdered. The resulting material was subjected to extraction in double-distilled water, ethanol, n-hexane, and dichloromethane. To fractionate the extracts and identify the most bioactive compound, silica gel column chromatography and GC-M/S analysis were used.

Results: Fraction 18 of silica gel column chromatography of ethanol extract was the most effective against *T. vaginalis*. The respective IC_{50}, CC_{50}, and SI values for fraction 18 were 28.62 µg/mL, >800 µg/mL, and >27.95. GC-M/S analysis of this fraction identified a major phenolic compound (2, 4-bis (1, 1-dimethyl ethyl), whose toxicity against *vero* cells was only 10.15%.

Conclusion: The ethanolic fraction containing phenol-2,4-bis (1,1-dimethylethyl), which has a potent lethality effect on *T. vaginalis*, may be considered as an antiparasite drug candidate.

Keywords: *Trichomonas vaginalis*, tonicate, ascidians, chromatography, GC-MS, metronidazole

ÖZ

Amaç: Mevcut araştırmanın amacı, deniz ascidian tunikatlarından izole edilen bileşiklerin *Trichomonas vajinalis* üzerindeki antiparazit etkilerini değerlendirmektir.

Yöntemler: Ascidian tunikatlar toplandıktan sonra küçük parçalar halinde kesildi, dondurularak kurutuldu ve toz haline getirildi. Nihai malzeme, çift damıtılmış su, etanol, n-heksan ve diklorometan içerisinde ekstraksiyona tabi tutuldu. Ekstraktları parçalara ayırmak ve en biyoaktif bileşiği belirlemek için silika jel kolon kromatografisi ve GC-M/S analizi kullanıldı.

Bulgular: Etanol ekstraktının silika jel kolon kromatografisinin 18. fraksiyonu *T. vajinalis*'e karşı en etkili olanıydı. Fraksiyon 18 için ilgili IC_{50}, CC_{50} ve SI değerleri 28,62 µg/mL, >800 µg/mL ve >27,95 idi. Bu fraksiyonun GC-M/S analizi, *vero* hücrelerine karşı toksisitesi yalnızca %10,15 olan önemli bir fenolik bileşiği (2,4-bis (1,1-dimetil etil) tanımladı.

Sonuç: *T. vajinalis* üzerinde güçlü öldürücü etkiye sahip olan fenol-2,4-bis (1,1-dimetiletil) içeren etanolik fraksiyon antiparazit ilaç adayı olarak değerlendirilebilir.

Anahtar Kelimeler: *Trichomonas vajinalis*, tunikat, ascidianlar, kromatografi, GC-MS, metronidazol

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Address for Correspondence/Yazar Adresi: Moradali Fouladvand, Bushehr University of Medical Sciences Faculty of Medicine, Department of Microbiology and Parasitology; The Persian Gulf Tropical and Infectious Diseases Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran
Phone/Tel: +00989177722394 **E-mail/E-Posta:** mfouladvand35@gmail.com **ORCID ID:** orcid.org/0000-0002-3406-1624

Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Trichomonas vaginalis (*T. vaginalis*) as a parasitic protozoan is pathogenic agent of trichomoniasis. It is the most common sexually transmitted non-viral disease with worldwide spread. This infection is asymptomatic in some women and most men, but it can cause various side effects in women, such as unhealthy pregnancy, infertility, pelvic inflammatory disease and cervical malignancy. Men with trichomoniasis are mostly asymptomatic but symptomatic infected women have a wide range of clinical symptoms from mild to severe (1-3). The global incidence of Trichomoniasis is 276 million cases per year and is more common than gonorrhea, syphilis and chlamydia infections (4). Trichomoniasis causes at least a two-fold increase in the risk of HIV transmission as well as a four-fold increase in the spread of the virus (5,6). Studies in Iran have reported the prevalence of trichomoniasis as follows: 9.2% in Tabriz, 3.3% in Zanjan, 3.2% in Tehran, 2% in Yazd and Kashan, and 1.2% in Hamadan city (7-12).

Metronidazole and tinidazole are the only FDA-approved drugs for the treatment of trichomoniasis, but there have been reports of drug resistance and treatment failure against these medications (13-15).

Although research on the effects of the natural substances against *T. vaginalis* is scarce in scientific databases but some studies have published reports on the effects of marine organisms such as seaweeds, microorganisms and marine fungi against *T. vaginalis* (16-20). Tunicates are a diverse group of marine invertebrate animals and members of the phylum chordata and subphylum tunicata (21).

To date, 3051 species of tunicates have been identified. These marine organisms were classified into four major classes: Ascidiacea, thaliacea, apendicularins and sorberacea, consisting of 2800 species in ascidiacea, 72 species in thaliacea and 69 species in appendicular (21,22). Ascidians have various chemical defenses to combat predators in the marine ecosystem, most of which are synthesized by symbiotic bacteria. These marine organisms are the deposits of various chemical compounds such as didemnin, appelidin and trabectedin which are effective against many types of cancers. In addition, various studies indicate the presence of antimicrobial compounds in isolated compounds from Ascidians (23,24).

Most of the compounds extracted from Tunicates have antibiotic, antitumor, antiviral and immunosuppressive activities (25). The aim of the current study was to evaluate the effect of the isolated compounds from the ascidian tunicates on *T. vaginalis* viability.

METHODS

Sample Collection

Ascidian tunicates were collected on ice from the Haleh shure of, Bushehr province, Iran. The samples were washed with tap water and distilled water to remove the contaminants and stored at -70 °C until used.

Extraction

To prepare the aqueous, ethanol, n-hexane and dichloromethane ascidians extracts, the samples were cut into small pieces, chopped and were freeze-dried. The dried samples were powdered by a home grinder. The samples were extracted in double distilled water and in ethanol 70% (V/V) at 40 °C by overnight maceration with interval shaking. Also, the n-hexane and dichloromethane extractions were performed using Soxhlet extractor. All extracts were filtered with filter paper (Watman, no.1, Merk, Germany) and the filtrate was concentrated under vacuum to eliminate the solvent using a rotary evaporator apparatus (Laborota 4000, Heidolph, Germany), dried, powdered and reserved at -20 °C as the crude ascidian extracts.

TLC and Silica Gel Column Chromatography

Thin-layer chromatography (TLC Silica gel 60 F_{254}) was used to choice the appropriate solvent and pre-evaluation of sample solubility for next silica gel G-60 column chromatography. After each column chromatography the TLC were performed for identity and composition analysis of the obtained fractions.

Column Chromatography

The silica gel G-60 column chromatography was performed using (5×70 cm) glass column and ethanol as the mobile phase. Then, the ethanolic crude extract which having more anti parasitic activity was loaded on the column and every 15 minutes the 2.5 mL fractions were recovered. The resulting fractions were concentrated, dried and refrigerated until used.

GC-MS Analysis

The ascidian F18 fraction with the most cytotoxic effect on *T. vaginalis* was subjected to the Gas chromatography-mass spectroscopy (GC-M/S) analysis equipped with Chemstation software. For this purpose, 1 µL of samples was injected to the GC-MS instrument equipped with HP-5MS and mass detector, placed at 50 °C for 1 min with a heating rate of 1 °C/min. Then, used 300 °C with a heating rate of 15 °C/min, hold time of 20 min, run time of 66/37 min, injector temperature of 120 °C, MS detector temperature of 230 °C, ion source and quadrupole temperature of 230 and 150 °C, respectively. Helium gas with flow of 21.7 mL/min and pressure of 4 psi at a ratio of 30:1 ratio were used as the carrier. In this experiment, a quadrupole mass spectrometer with ionization energy of 70eV was used. The corresponding chemical structure of each peak was identified by comparing the mass spectra obtained from the chromatogram with the National Institute of Standards and Technology (NIST) library and by searching the PubChem database. After detecting the name, molecular formula and chemical structure of each compound, the biological and industrial applications of each compound were determined using the aforementioned database.

Evaluation of Ascidian Effects on *T. vaginalis*

The *T. vaginalis* was obtained from Pasteur Institute of Iran. It was cultured in diamond medium (Merk, Germany) at 37 °C. After the parasite reached the logarithmic phase, 125 µL of the culture medium containing 1×10^6 trophozoites were transferred to the 96 well flat bottom microplate and 125 µL of 20, 40, 80, 100, 200, 400 and 800 µg/mL doses from aqueous and organic extracts, silica gel G-60 column chromatography fractions, (as tests) were added to the wells containing *T. vaginalis* and incubated at 37 °C (two test sets, 24 and 48 h) The metronidazole as positive control was used.

For cell viability evaluations, the trypan blue dye exclusion and MTT assay tests were used. All experiments were performed in triplicate.

Cytotoxicity Assay

For cytotoxicity evaluations on mammalian cells, the *vero* cells were affected by the obtained ascidian fractions. Ten thousand of *vero* cells were cultured (DMEM medium, Gibco, USA) at 37 °C and 5% CO_2 for 48 h in pre- trypsinized, PBS washed micoplate in the presence of 20, 40, 80, 100, 200, 400, 800 µg/mL of ascidian fractions. Also, the wells containing only *vero* cells (as negative control) and cell-free wells (as blank) were considered. The cell viability was performed by the trypan blue exclusion and MTT assay test.

Statistical Analysis

The experiments were replicated three times. Statistical analysis was performed using SPSS Software version: 16.0 (SPSS Inc., Chicago, IL) for data analysis, Kruskal-Wallis test and Mann-Whitney U test were used and p-value <0.05 was considered significant.

RESULTS

The results showed that the ascidian extracted in water, ethanol, n-hexane and dichloromethane solvents have increasing lethal effect on *T. vaginalis* in dose and time dependent approach. The lowest percentage of lethality was recorded for the water extract at the concentration of 20 µg/mL after 24 h incubation, which almost had no lethal effect. But, the highest lethal effect was for the ethanolic extract, which at dose of 800 µg/mL caused 60% of *T. vaginalis* trophozoites to be killed after 48 hours (Table 1).

Separation and identification of ethanolic extract by silica gel G-60 column chromatography resulted in the fractions which subjected to subsequent bioassays. The evaluation of cytotoxic effects of obtained fractions (tests) and the metronidazole (positive control) at 20, 40, 80, 100, 200, 400 and 800 µg/mL doses on the *T. vaginalis* viability by trypan blue dye exclusion and MTT cytotoxicity assay revealed that fraction 18 was more active against target cells (Table 2).

Fraction 18 at a dose of 80 µg/mL showed 100% toxicity (Table 2) which the IC_{50} was 28.62 µg/mL (Table 3).

The GC-M/S analysis was used to identify the chemical composition of the bio-active fractions. The analysis of GC-M/S peaks and searching in the NIST library and PubChem database led to the identification of the phenol-2, 4-bis (1,1-dimethylethyl) compound in Fraction 18 (Table 4).

Table 1. Time and dose dependent screening of Ascidian crude extracts on *T. vaginalis*

Solvent		**Water (%)**		**Ethanol (%)**		**n-Hexane (%)**		**Dichloromethane (%)**	
Incubation time		**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
Concentrations (µg/mL)	**20 µg/mL**	0	0	3	6.7	0	2	0	0
	40 µg/mL	2	3.2	5	10	1	2.40	0	2
	80 µg/mL	3.4	5.5	7.5	18.50	2.24	3	1	3.2
	100 µg/mL	4	6.2	11.45	24.50	3.33	5	3	6.50
	200 µg/mL	5.75	7.62	26	33.56	6.67	8.60	6	0
	400 µg/mL	6.55	8	31	51	9.57	12.34	7.20	13.25
	800 µg/mL	8	10	49	60	14.50	18.50	10	15

Table 2. Lethality effects of silica gel G-60 column chromatography fractions on *T. vaginalis*

Fraction		**F14**		**F15**		**F16**		**F17**		**F18**		**Metronidazole**	
Incubation time		**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
Concentration (µg/mL)	**20**	15	17.3	18	20	20	21	25.11	47.19	22.16	41.33	61.18	82
	40	27	30	33	35.20	34.5	36.50	49.1	69.74	44.73	78.14	88.29	100
	80	38.3	41	41.36	43.74	46	48.63	86.33	100	89.07	100	100	100
	100	47	50	56.23	56.50	62.38	65	93.42	100	94.66	100	100	100
	200	59.28	64	70.5	71	76	79.30	100	100	100	100	100	100
	400	80	81.28	80	83.40	83.04	87.64	100	100	100	100	100	100
	800	90	95	95	99	97	99	100	100	100	100	100	100

Table 3. Cytotoxicity effects of silica gel G-60 column chromatography fractions on *T. vaginalis*

Fraction	**F14**		**F15**		**F16**		**F17**		**F18**	
Incubation time	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
CC_{50} against *vero* cells	800>	800>	800>	800>	800>	800>	800>	800>	>800	>800
IC_{50} against *T. vaginalis*	122.50	100	93.34	81.70	76.24	82.46	42.09	28.83	44.08	28.62
Selectivity index	6.53>	8.00>	8.57>	9.79>	10.49>	>9.70	>19.00	27.75>	>18.15	>27.65

CC_{50}: Cytotoxic concentration, IC_{50}: Inhibitory concantration

Table 4. GC-MS compositional analysis of Ascidian F18 fraction

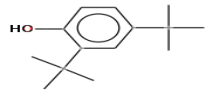
Fraction	Compound	Mulecular formula	Stracture
F18	**phenol-2, 4-bis (1,1-dimethylethyl)**	**C14H22O**	

Table 5. Toxicity effects of silica gel G-60 column chromatography fractions on *vero* cells

Fraction		F14		F15		F16		F17		F18	
Incubation time		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Concentration (µg/mL)	20	2.67	1.87	0.11	1.00	0.00	0.49	1.00	1.87	0.21	0.77
	40	2.79	2.89	0.70	1.58	0.33	1.00	0.67	1.00	1.00	1.41
	80	3.00	3.65	1.00	1.63	1.89	4.66	1.00	1.91	1.91	2.91
	100	3.51	4.00	1.74	2.00	7.67	6.00	1.49	5.00	2.00	3.69
	200	5.00	6.00	2.37	2.81	9.00	11.00	4.00	10.80	4.63	6.29
	400	6.00	6.51	14.00	15.00	9.30	13.89	4.06	11.00	5.89	9.67
	800	10.50	13.89	14.50	17.93	10.00	16.88	7.00	16.89	10.00	10.15

Also, the results showed that the metronidazole (as positive control) at dose of 80 µg/mL caused 100% toxicity after 24-h incubation and at 40 µg/mL after 48 h incubation (Table 2).

The *vero* cell line was used to evaluate the toxicity of ascidian compounds on mammalian cells. Incubation of *vero* cells in the presence of fraction 18 at doses of 20-800 µg/mL showed, that this fraction has the most inhibition effect on *T. vaginalis* growth with very low toxicity against *vero* cells although at high doses (Table 5).

DISCUSSION

Trichomoniasis is the most common non-viral sexually transmitted infection. There have been many reports of a relatively high incidence of this infection especially, in developing countries (4,7-11). On the other hand, there are frequent and reliable reports of the underlying cause of this infection for cervical and prostate malignancies in men, as well as facilitating HIV transmission (1-6).

Given that no approved vaccine has been available to prevent this infection so far, we need to think about a new drug to treat this infection effectively. The current treatments of choice for trichomoniasis are metronidazole and tinidazole. Studies have shown that metronidazole not only has significant side effects, but also there are reports of many cases of treatment failure and drug resistance (13-15). So, finding an effective and low-cost drug to treat this infection is one of the priorities.

In the past few decades, marine organisms have been one of the promising sources for finding medicinal and nutritional compounds. Among the wide range of marine organisms, Tunicates, especially the Ascidians, have received special attention, and promising reports have been published on the efficacy of secondary metabolites extracted from these marine organisms against bacteria, viruses, fungi, and malignancies, but there are few studies to evaluate the effect of these metabolites against *T. vaginalis* (26-35). Our study showed that among the solvents used to achieve the extracts from ascidians, ethanol as a suitable solvent was able to extract more effective compounds (60% parasite death at 800 µg/mL dose). The silica gel G-60 column chromatography resulted in the separation of 18 different fractions and, the bioassay against *T. vaginalis* revealed that fraction 18 showed significant dose and time dependent lethal effects. The IC_{50}, CC_{50} and SI values were 28.62 µg/mL, >800 µg/mL and, 27.95 respectively. Cytotoxic analysis of F18 against *vero* cells showed that this fraction at the concentration of 800 µg/mL had the lethality rate of 10.15% and the maximum concentration of this fraction (800 µg/mL) could not have a 50% inhibitory effect against *vero* cells, so, it was not possible to calculate the CC_{50} definitive values for this fraction. Therefore, the CC_{50} values for all fractions were estimated as >800 µg/mL. Due to the very low toxicity of all fractions against *vero* cells, especially the fraction18 that have shown high toxicity against *T. vaginalis*, the calculated SI value for this fraction was >27.95.

The GC-M/S analysis of fraction 18 showed that, phenol-2,4-bis (1,1-dimethylethyl) with the chemical formula of C14H22O; is a phenolic compound. Some studies confirmed the antioxidant (36) and antifungal activity of phenol-2,4-bis (1,1-dimethylethyl) against *Aspergillus niger, Fusarium oxysporum* and *Penicillium chrysogenum* (37). Also, in a study which investigated the phenol-rich extracts from plants against *Giardia lamblia*, different concentrations of these extracts inhibited the growth of the protozoa in the culture medium. In that study, the most inhibitory effect was 275 µg/mL (38). In another study, the effects of extracts containing phenolic compounds at the concentrations of 125 and 250 mg/mL were investigated on *L. infantum, Leishmania braziliensis* and *Trypanosoma cruzi* in the culture medium and the results as lethality percentages were 54.27%, 80.39% for *Leishmania infantum*, and 68.61%. 80.159% for *Leishmania braziliensis* and 54.45%, 22.26% for *Trypanosoma cruzi* respectively (39). Another study evaluated the ethanol extract of three plant species containing phenolic compounds against *Leishmania* spp. and *Trypanosoma* spp., and found that the lethality percentages were 63.8% for *Leishmania braziliensis*, 95% for *Leishmania infantum* and 63% for *Trypanosoma cruzi* (40). As in

our results, Ezz Eldin and Badawy (41) investigated the effects of two plant extracts containing phenolic compounds on *T. vaginalis* and showed that these compounds have an increasing lethality in time and dose dependent manner.

CONCLUSION

The biological assay of F18, containing the phenol-2,4-bis (1,1-dimethylethyl, showed that this fraction at the concentration of 80 µg/mL caused 100% parasite death and its IC_{50}, CC_{50}, and SI values were 28.62 µg/mL, >800 µg/mL and >27.95 respectively. Then, based on the bioassay results from the evaluation of fraction F18 effect on the *T. vaginalis*, it showed potent anti-parasite effects. Other studies on the phenolic potential compounds such as phenol-2,4-bis (1,1-dimethylethyl) and other alkaloid derivatives indicated a wide range of biological effects and caused death or inhibition of cancer cells, bacteria, viruses, fungi and the parasites. Therefore, our study indicates that ascidians, which are a source of biologically active compounds especially the phenolic, with anti *T. vaginalis* activity, can be considered as a therapeutic drugs after further more details investigations.

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*** Ethics**

Ethics Committee Approval: This study was approved in Research Committee of Bushehr University of Medical Sciences with ethics code IR.BPUMS.REC.1396.160.

Informed Consent: This study was conducted *in vitro*, so, it doesn't involve any patients or human samples.

***Authorship Contributions**

Design: M.F., Data Collection or Processing: H.M., A.B., S.K., M.K., M.F., Analysis or Interpretation: H.M., A.B., S.K., M.K., M.F., Writing: H.M., A.B., S.K., M.K., M.F.

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