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RESEARCH ARTICLE

IN VITRO EFFICACY OF *VITEX AGNUS-CASTUS* EXTRACTS AGAINST *TRICHOMONAS VAGINALIS*: A POTENTIAL THERAPEUTIC APPROACH

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Abstract

Aim and objectives: *Trichomonas vaginalis* is the most prevalent nonviral sexually transmitted infection globally. The current treatment, 5-nitroimidazole derivatives has raised concerns as a result of drug reliance, allergies, and drug resistance, driving efforts to identify alternative therapies. The *Vitex* species recognized for their pharmacological attributes including anti-inflammatory, antibacterial, antifungal, and antimicrobial properties are promising candidates for further investigation.
Methods: In this study, the potential of *Vitex agnus-castus* extracts against *T. vaginalis* was explored. The water, ethanol, and 60% aqueous ethanol extracts from both leaves and fruits were examined for their effects on metronidazole (MET)-resistant (TV 50143) and sensitive strains (TV-78). Cytotoxicity was evaluated on L929 mouse fibroblast cells to determine the minimum lethal dose for each extract under aerobic and anaerobic conditions.
Results: Antitrichomonial activity, cytotoxic activity and Selectivity Index (SI) values revealed distinct efficacy profiles. Leaf water extract displayed a balanced selectivity profile, while leaf 60% ethanol extract showed moderate to high selectivity. The fruit 60% ethanol extract exhibited significantly elevated selectivity with SI values of 2 and 1 under aerobic and anaerobic conditions, respectively, for the MET-sensitive reference strain, and 3 and 5, respectively, for the MET-resistant reference strain.
Conclusion: These findings underscore the potential of the fruit 60% ethanol extract as a promising candidate for future drug development. Further investigations into its mechanisms and optimization are warranted to enhance its efficacy against *T. vaginalis*.
Keywords: Drug discovery, parasitic diseases, sexually transmitted diseases, treatment failure, *Trichomonas vaginalis*.

INTRODUCTION

The global significance of sexually transmitted infections (STIs) is underscored by the daily incidence of over 1 million new infections, primarily attributed to the four most curable STIs caused by: *Treponema pallidum*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* (*T. vaginalis*), and *Chlamydia trachomatis*. This burden disproportionately impacts low- and middle-income countries[1]. *T. vaginalis* is considered the most prevalent nonviral STI globally and is not classified as a reportable disease. Global estimates suggest an annual incidence of 156 million new cases among women, surpassing the combined prevalence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and syphilis[2].

As an extracellular parasite, *T. vaginalis* predominantly targets the squamous epithelium of the genital tract exhibiting a common affinity for infecting the female lower genital tract (including the vagina, urethra, and endocervix) and the male urethra and prostate. Human transmission occurs primarily through sexual intercourse, its sole known mode of transmission, with infections in women demonstrating the potential for prolonged persistence, spanning months or even years, while men may experience shorter infection durations, often less than a month[3,4]. Approximately 85% of women and 77% of men infected with *T. vaginalis* exhibit no symptoms, while half of asymptomatic

women may develop symptoms within six months. Symptoms in women manifest as vaginal erythema, dyspareunia, dysuria, and diffuse, malodorous, yellow-green vaginal discharge, often accompanied by pruritus in the genital region[4,5]. Inadequate or untreated *T. vaginalis* infection is associated with a spectrum of clinical conditions, encompassing vaginitis, cervicitis, urethritis, and pelvic inflammatory disease, and is implicated in adverse birth outcomes like preterm delivery, premature rupture of membranes, and low-birth weight infants. Moreover, *T. vaginalis* can augment both the acquisition and transmission of human immunodeficiency virus (HIV)[6]. The link between trichomoniasis and increased HIV transmission is a critical concern, as the inflammatory response and disruption of the mucosal barrier may enhance the likelihood of HIV acquisition[7]. This interplay between trichomoniasis and other STIs underscores the importance of effective management and prevention strategies to mitigate associated morbidity and reduce the overall burden of the disease[8].

Currently, nitroimidazole derivatives, namely metronidazole and tinidazole are the only drugs indicated for the treatment of trichomoniasis. Concerns arise due to the limited availability of alternative treatments, particularly in the context of nitroimidazole- resistant trichomoniasis ("Trichomoniasis - STI Treatment Guidelines," n.d.) Reports indicate the emergence of drug resistance against metronidazole and tinidazole[9]. Although the incidence of 5-nitroimidazole-resistant *T. vaginalis* infections is relatively uncommon, the recognition of potential risks associated with relying solely on this drug class, coupled with the imperative to address allergies to these medications, has driven research efforts to identify alternative therapies for trichomoniasis. The ideal alternative therapy would be orally administered, well-tolerated, and exert its effectiveness against trichomonads through a distinct pathway from the 5-nitroimidazoles[10]. The *Vitex* species recognized in pharmacology for their medicinal attributes, including anti-inflammatory, antibacterial, antifungal, and antimicrobial properties represent a promising candidate for further exploration[11]. Previous literature has demonstrated that *V. agnus-castus* L. essential oil possesses significant antimicrobial properties against bacterial and fungal strains due to its rich composition of oxygenated monoterpenes[12]. In this study, we aimed to assess the *in vitro* efficacy of water, ethanol, and 60% aqueous ethanol extracts derived from the leaves and fruits of *V. agnus-castus* plant against MET-resistant and non-resistant strains of *T. vaginalis* in both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Preparation of plant extracts

The leaves and fruits of the *V. agnus-castus* tree were collected from the Manisa province, Turkey, located at latitude 38.630554 and longitude 27.422222, during the period between August and October 2022. A specimen voucher of plant material collected and authenticated by Dr. Husniye Kayalar in July 2022 was deposited under 1262/3 number in herbarium at Department of Pharmacognosy, Faculty of Pharmacy, Ege University. Extracts from the leaves and fruits of the *V. agnus-castus* plant were prepared using water, ethanol, and 60% aqueous ethanol. Percentage yields were calculated.

Water Extracts

Leaves and fruits were separately collected and dried at room temperature. The dried leaves were ground into a powder. To prepare a 2% water extract infusion, 2 g of powdered plant material was soaked in 100 mL boiling water for 10 minutes and then filtered using filter paper. The filtered portion was evaporated in a rotavap at a temperature not exceeding 50°C until dry. Subsequently, the lyophilized material was frozen at -80°C and stored at -20°C until activity studies were conducted.

Aqueous ethanol extract

Powdered leaves (2 g) and fruits (2 g) were extracted with 60% aqueous ethanol (100 mL) using the shaking maceration technique at room temperature. The solution was filtered, and the solvent was evaporated in a Rotavap at a temperature not exceeding 50°C. The extract was frozen at -80°C and lyophilized. The lyophilized material was stored at -20°C until activity studies were conducted.

Ethanol extract

Powdered leaves (2 g) and fruits (2 g) were extracted with ethanol (100 mL) using the shaking maceration technique at room temperature. The solution was filtered, and the solvent was evaporated in a rotavap at a temperature not exceeding 50°C. The extract was frozen at -80°C and lyophilized. The lyophilized material was stored at -20°C until activity studies were conducted. The percentage yields of all extracts were calculated.

Cytotoxic activities of plant extracts

Water, ethanol, and 60% aqueous ethanol extracts obtained from *V. agnus-castus* leaves and fruits were studied for cytotoxic activities. L929 mouse fibroblast cells were thawed from liquid nitrogen and dissolved in a 37°C water bath. The L929 mouse fibroblast cell line was chosen as it is the most widely used cell line for cytotoxicity studies. Then, 5 mL of RPMI 1640 medium was added to the transferred cell pellet in a 15 mL sterile falcon tube. The mixture was homogenized using a sterile pipette. The falcon tube was centrifuged at 1200 rpm for 5 minutes. After centrifugation, the supernatant was discarded, and 5 mL of RPMI-1640 medium was added to the cell pellet. The contents of the tube were gently mixed with a pipette until homogenized in the medium. Then, the content in the tube was transferred to T25 cell flasks. The cell flasks were placed in a 37°C incubator, and the cell line was left for incubation. Daily checks were performed, and cell growth was monitored.

For cytotoxic activity, $1x10^4$ cells were distributed into each well of sterile 96-well microplates. The microplates were incubated at 37°C for 24 hours. The next day, the media in the microplates were completely removed, and 100 µL of fresh medium was added to each well. Then, for each prepared extract, three rows

were separated, and 100 µL of the extract was added to the first wells, followed by serial dilution within the microplates. After all dilution processes, the microplates were incubated at 37°C. After 48 hours, the microplates were removed from the incubator, and the material inside the wells was emptied. Then, 100 µL of the solution from the CellTiter-Glo® Luminescent Cell Viability Assay kit was added to each well and left for 10 minutes of incubation. Afterward, the microplates were placed in the Luminoskan (Thermo Scientific™, Waltham, Massachusetts, United States) device for reading. Cytotoxic activities were determined at the end of the reading, and the concentrations that killed 50% of the cells (CC_{50}) were determined by GraphPad software (Boston, Massachusetts USA),

Preparation of TYM (Trypticase-Yeast Extract-Maltose) medium

In a sterile balloon, 1 g of L-cysteine, 0.2 g of L-ascorbic acid, 0.8 g of K_2HPO_4, 0.8 g of KH_2PO_4, 20 g of trypticase, 10 g of yeast extract, 5 g of maltose, and 0.5 g of agar were weighed and transferred. Distilled water (900 mL) was added to the balloon, and the added substances were completely dissolved. Then, it was adjusted to pH 6, and 100 mL of distilled water was added. The prepared suspension was autoclaved at 121°C for 20 minutes for sterilization. After autoclaving, it was distributed into sterile glass tubes to a volume of 4 mL. During the use of the medium, 1 mL of horse serum and 250 µL of the antibiotic mixture (Penicillin/Streptomycin, Gentamycin, Amphotericin B) were added.

Production of *T. vaginalis* parasites

Commercially obtained MET-sensitive *T. vaginalis* ATCC 30188 and MET-resistant *T. vaginalis* ATCC 50143 strains were thawed in a 37°C water bath. Then, they were inoculated into the prepared media. The inoculated media were placed in a 37°C incubator. The media were checked every other day, and the reproduction status of the parasites was monitored. To obtain a large number of parasites, media were added according to the reproduction status of the parasites (Figure 1). Figure 1 shows *T. vaginalis* trophozoites produced in TYM medium and which are morphologically healthy.

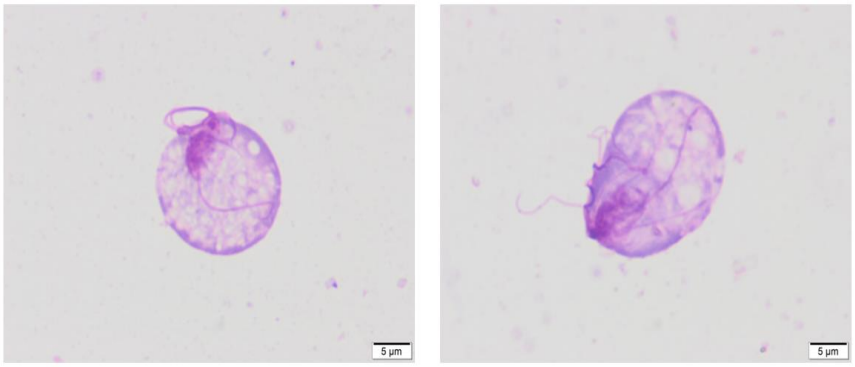

Figure 1: Giemsa stained *T. vaginalis* trophozoites (x100 augmentation).

Determination of antitrichomonal activity of prepared plant extracts

The antitrichomonal activity of the extracts was investigated by the broth microdilution method[13]. Efficacy is based on the lowest concentration of agent that immobilizes and completely kills parasites in the wells[9,14]. The determination of the concentration that inhibits half of the trophozoites (IC_{50}) values was carried out in aerobic and anaerobic conditions. Prepared microplates were placed directly in a 37°C incubator for aerobic conditions, and for anaerobic conditions, microplates placed in a jar with an added anaerobic environment kit were placed in a 37°C incubator. The study began when the reproduction density of parasites produced in the media was 10^5 parasites/mL. Sterile 96-well microplates were used for the determination of the *in vitro* activity of water, ethanol, and 60% aqueous ethanol extracts obtained from *V. agnus-castus* leaves and fruits in different ratios under aerobic and anaerobic conditions. Then, 100 µL of TYM medium was added to each well. Subsequently, three rows were created on the microplates for each extract, metronidazole control, and parasite control. After adding 100 µL of each extract and metronidazole to the first wells, serial dilution was performed within the microplates. No substance was added to parasite control wells. Then, 100 µL of a suspension of 10^5 parasites/mL density was added to each well of each extract, metronidazole control, and parasite control. Afterward, the microplates were incubated at 37°C under both aerobic and anaerobic conditions. At the end of the incubation period, samples were taken from each well of the microplates and stained with trypan blue dye. The percentage viability of parasites in each well corresponding to the concentration of each extract was calculated by staining with trypan blue and counting under a Thoma slide. The obtained percentage viability values were analyzed using the GraphPad software (Boston, Massachusetts USA), and the IC_{50} values were determined for both isolates. To validate the complete inactivation of parasites, the assessment of viability was conducted by transferring the pertinent wells into a fresh TYM medium. The same evaluation stages were repeated after 48 hours of incubation of the microplates. These processes were repeated three times.

Ethical statement

The ethical committee approval for the study was obtained from Manisa Celal Bayar University Local

Ethical Committee for Research Studies (Decision No: 319/Date: 22.08.2022)

RESULTS

Extraction results

The percentage of yield of different components of *V. agnus-castus* using various solvents during extraction are as follows. The fruit and leaves extracts provided a percentage yield of 39.23% and 42.77%, respectively, by extraction in water. For ethanol extraction, the fruits yielded 14.625%, while the leaf exhibited a higher yield at 27.51%. Notably, the fruit 60% ethanol extract displayed a yield of 17.625%, and the leaf 60% ethanol extract demonstrated the highest yield at 53.42%.

Cytotoxicity profiles of the *V. agnus-castus* leaves and fruits extracts

The cytotoxic effect of *V. agnus-castus* extracts, prepared using different solvents, were assessed on L929 mouse fibroblast cells. Toxicity in this context was defined as cytotoxic concentration (CC_{50}) values below 20 µg/mL, as established in previous studies[15,16]. Extracts with CC_{50} values <20 µg/mL were considered toxic, while those demonstrating CC_{50} values >20 µg/mL and a SI value >1.0 were presumed to exhibit potent bioactivity with acceptable toxicity[16,17]. The water extracts from fruit and leaves exhibited the lowest cellular toxicity, maintaining viability even at the highest concentrations (1200 and 2700 µg/mL, respectively). Conversely, ethyl alcohol (EA) extracts from fruit and leaves displayed more pronounced toxicity, with CC_{50} values of 96 and 74 µg/mL, respectively, while 60% EA extracts showed values of 700 and 725 µg/mL (Table 1).

Table 1: Cytotoxicity values of *V. agnus-castus* Extracts against L929 Fibroblast cells.

V. agnus-castus leaves and fruits extracts	CC_{50} (µg/mL)
Leaves water extract	>1200
Leaves ethyl alcohol extract	74
Leaves 60% ethyl alcohol extract	725
Fruits water extract	>2700
Fruits ethyl alcohol extract	96
Fruits 60% ethyl alcohol extract	700

Anti-trichomonal activity of the *V. agnus-castus* leaves and fruits extracts

The antitrichomonal activity of extracts obtained from *V. agnus-castus* using various solvents was investigated against both MET-sensitive clinical and MET-resistant reference isolates under both aerobic and anaerobic conditions. The extracts' efficacy was assessed using the broth microdilution method[16]. The most robust antitrichomonal activity was observed in ethyl alcohol (EA) and 60% EA extracts from the fruit under both atmospheric conditions. In aerobic conditions, IC_{50} values for these extracts in MET-sensitive strains were 138 and 290 µg/mL, respectively, while in anaerobic conditions, they were 98 and 464 µg/mL, respectively. In MET-resistant strains, the IC_{50} values were 112 and 227µg/mL in aerobic conditions and 59 and 141 µg/mL in anaerobic conditions. EA and 60% EA extracts from the leaves exhibited moderate antitrichomonal activity, while no activity was observed in water extracts. Upon examining the IC_{50} values for all extracts, the antitrichomonal activity did not exhibit significant variations based on metronidazole sensitivity or atmospheric conditions.

Table 2: Antitrichomonal efficacy values of *V. agnus-castus* extracts (µg/mL).

V. agnus-castus leaf and fruit extracts	ATCC 30188 Strain (IC_{50})				ATCC 50143 Strain (IC_{50})			
	Aerobe		Anaerobe		Aerobe		Anaerobe	
	24h	48h	24h	48h	24h	48h	24h	48h
Leaves (Water)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
Leaves (EA)	543	298	495	538	683	397	1115	416
Leaves (60% EA)	598	649	674	703	690	595	1025	567
Fruits (Water)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
Fruits (EA)	163	138	163	99	131	112	322	59
Fruits (60% EA)	610	290	560	463	629	227	718	141

EA: Ethyl alcohol extract, **IC_{50}**: Median inhibition concentration, **ATCC 30188**: Metronidazole sensitive *T. vaginalis* reference strain, **ATCC 50143**: Metronidazole resistant *T. vaginalis* reference strain

Table 3: Efficacy values of metronidazole against *T. vaginalis* reference strains (µM).

Metronidazole	ATCC 30188 Strain (IC_{50})		ATCC 50143 Strain (IC_{50})	
	Aerobe	Anaerobe	Aerobe	Anaerobe
24h	8	3	103	17
48h	7	2	42	3

IC_{50}: Median inhibition concentration, **ATCC 30188**: Metronidazole sensitive *T. vaginalis* reference strain, **ATCC 50143:** Metronidazole resistant *T. vaginalis* reference strain

Consistent results were obtained for both strains under both atmospheric conditions (Table 2). The IC_{50} values of ATCC 30188 reference strain against metronidazole were determined to be 8 µM and 3 µM after 24-hour incubation under aerobic and anaerobic conditions, respectively, while they were found to be 7 µM and 2 µM after 48-hour incubation. Similarly, the IC_{50} values of the ATCC 50143 reference strain against metronidazole were determined to be 103 µM and 17 µM after 24-hour incubation under aerobic and anaerobic conditions, respectively, while they were found to be 42 µM and 3 µM after 48-hour incubation (Table 3).

Table 4: Selectivity index values of *V. agnus-castus* extracts against *T. vaginalis* isolates.

V. agnus-castus leaves and fruits extracts	ATCC 30188 Strain (IC_{50})						ATCC 50143 Strain (IC_{50})					
	Aerobe			Anaerobe			Aerobe			Anaerobe		
	TV	L929	SI	TV	L929	SI	TV	L929	SI	TV	L929	SI
	IC_{50}	CC_{50}		IC_{50}	CC_{50}		IC_{50}	CC_{50}		IC_{50}	CC_{50}	
Leaves (Water)	>5000	>1200	<1	>5000	>1200	<1	>5000	>1200	<1	>5000	>1200	<1
Leaves (EA)	299	74	<1	538	74	<1	397	74	<1	416	74	<1
Leaves (60% EA)	650	725	1	704	725	1	595	725	1	567	725	1
Fruits (Water)	>5000	>2700	<1	>5000	>2700	<1	>5000	>2700	<1	>5000	>2700	<1
Fruits (EA)	138	96	<1	99	96	<1	111	96	<1	59	96	2
Fruits (60% EA)	290	700	2	464	700	1	227	700	3	141	700	5

EA: Ethyl alcohol extract, **IC_{50}**: Median inhibition concentration, **ATCC 30188**: Metronidazole sensitive *T. vaginalis* reference strain, **ATCC 50143**: Metronidazole resistant *T. vaginalis* reference strain, **SI**: Selectivity index

The selectivity index (SI) value was calculated for each extract. Except for the 60% EA extracts obtained from fruits and leaves, all other extracts were found to have SI values <1. For the 60% EA extracts obtained from fruits/leaves, the SI values under aerobic and anaerobic conditions were determined to be 2/1 and 1/1, respectively, for the MET-sensitive ATCC 30188 reference strain, and 3/1 and 5/1, respectively, for the MET-resistant ATCC 50143 reference strain (Table 4).

DISCUSSION

The primary objective of this study was to evaluate the *in vitro* efficacy of water, ethanol, and 60% aqueous ethanol extracts obtained from both the leaves and fruits of the *V. agnus-castus* plant. Specifically, the investigation aimed to assess the effectiveness of these extracts against both MET-resistant and non-resistant strains of *T. vaginalis* under aerobic and anaerobic conditions. The *in vitro* efficacy investigation of *V. agnus-castus* extracts against MET-resistant and non-resistant *T. vaginalis* isolates revealed distinct efficacy profiles. The selectivity index (SI), calculated as the ratio of cytotoxic concentration (CC_{50}) to inhibitory concentration (IC_{50}), served as the primary measure of efficacy. Most extracts displayed SI values of less than 1, indicating limited selectivity and potential cytotoxicity. However, the 60% EA extracts from fruits and leaves exhibited more promising results. For the 60% EA fruit extract, SI values were notably higher under both aerobic (SI=2 for ATCC 30188, SI=3 for ATCC 50143) and anaerobic conditions (SI=1 for ATCC 30188, SI=5 for ATCC 50143), suggesting a significant inhibitory effect on *T. vaginalis* with relatively low cytotoxicity. Similarly, the 60% EA leaf extract demonstrated an SI of 1 under all conditions, indicating moderate selectivity. These findings highlight the potential of 60% EA extracts, particularly from fruits, as effective agents against *T. vaginalis*, warranting further investigation into their mechanisms and optimization for enhanced efficacy.

Metronidazole resistance is a documented cause of treatment failure in *T. vaginalis* among women, with a Centers for Disease Control and Prevention (CDC) study performed between the years 2002-2008 revealing 66% metronidazole resistance in treatment-refractory cases[18]. However, the extent and optimal treatment for MET-resistant *T. vaginalis* infections in men remain unknown due to limited data and studies on drug resistance in this population[19]. Therefore, predicting the burden of untreatable *T. vaginalis* infections becomes challenging once benzimidazole derivatives, the sole available treatment option, lose efficacy.

In this regard, various synthetic drugs, including miltefosine, isatin and amine derivatives, proton pump inhibitors, and dual-function vaginal microbicides, have been explored for their anti-trichomonal effects. Nevertheless, none of these compounds have progressed to become approved pharmaceutical treatments for *T. vaginalis* infection[20]. Additionally, numerous natural products have been assessed for their antitrichomonal activity, both *in vitro* and *in vivo*, yielding varying success rates[20,21]. Despite numerous studies on natural products against *T. vaginalis*, none have advanced to clinical trials. On the other hand, the synthetic compounds under consideration, primarily derived from existing drugs, have undergone preliminary clinical testing, exposing a deficiency in substantial financial investments and an absence of identified alternative treatments for trichomoniasis[22].

Inadequate attention is directed toward *T. vaginalis*, the globally predominant non-viral sexually transmitted infection, likely attributed to its non-fatal nature, resulting in limited awareness among health professionals regarding its severe consequences. To bridge this gap, there is a crucial need to develop innovative treatment and prevention strategies aimed at effectively diminishing the burden of *T. vaginalis* globally[25,26]. Despite presenting challenges for drug discovery, natural products, and their analogs have historically played a significant role in pharmacotherapy, particularly for cancer and infectious diseases. Recent technological advancements, including improved analytical tools and microbial culturing strategies, are rekindling interest in natural products as potential drug leads, especially in tackling antimicrobial resistance[27]. Research on natural or plant-derived drugs offers a cost-effective approach to discovering new molecules and drugs, with biological macromolecules and plant-derived drugs constituting over 40% of newly approved therapeutic agents and half of the antiparasitic agents since 1981[22].

Despite the valuable insights garnered from current study on the *in vitro* efficacy of *V. agnus-castus* extracts against *T. vaginalis*, it is imperative to acknowledge certain constraints. A significant limitation lies in the absence of a comprehensive investigation into the active compounds present in the *V. agnus-castus* plant. The intricate chemical

composition of the plant and the identification of specific bioactive constituents remain unexplored in this research. Given the pivotal role that active compounds play in mediating therapeutic effects, current study serves as an initial exploration, establishing a baseline for future investigations dedicated to isolating and characterizing these compounds. This limitation underscores the necessity for a subsequent project aimed at unraveling the molecular intricacies of *V. agnus-castus*, providing a more nuanced understanding of its pharmacological potential against *T. vaginalis.*

Limitations of the study

The limitation of this study is that the main active ingredient in each extract was not determined and studied on the same parasite isolates.

CONCLUSIONS

In summary, the *in vitro* efficacy of *V. agnus-castus* extracts against *T. vaginalis* unveils promising prospects for alternative trichomoniasis treatments. The distinctive efficacy profiles observed in MET-resistant and non-resistant isolates, particularly the notable performance of fruit 60% ethanol extract under anaerobic conditions, underscore the potential of *V. agnus-castus* as a valuable resource in drug development. Despite inherent limitations, such as the absence of an active compound investigation, obtained findings emphasize the necessity for further research. The persistent challenges in trichomoniasis treatment, including drug resistance, highlight the exigency for novel therapeutic options. This study contributes to the escalating interest in natural products and their derivatives, specifically *V. agnus-castus*, as prospective leads for addressing *T. vaginalis* infections. The ramifications extend beyond trichomoniasis, addressing the broader landscape of emerging sexually transmitted infections and the imperative for developing targeted, efficacious treatments.

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AUTHOR'S CONTRIBUTION

Özbilgin A: writing original draft, methodology, investigation, conceptualization. **Çavuş İ:** writing original draft, methodology, investigation**. Tunalı V:** writing original draft, formal analysis, data curation**. Özel Y:** writing original draft, methodology, investigation**. Alagöz ÇY:** writing original draft, formal analysis, data curation**. Kayalar H:** formal analysis, data curation, conceptualization. Final manuscript was checked and approved by all authors.

DATA AVAILABILITY

Data will be made available on request

CONFLICT OF INTEREST

None to declare.

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