

# Evaluation of the Susceptibility of *Trichomonas vaginalis* Isolates to *Melaleuca cajuputi*

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**Abstract— Introduction:** Trichomonosis is the most common non-viral sexually transmitted disease (STD) in the world. Due to the development of metronidazole-resistant isolates, therapeutic alternatives to 5-nitroimidazole are being investigated. *T. vaginalis* can be naturally infected with *Mycoplasma hominis*, *Mycoplasma girerdii* and *Trichomonas vaginalis virus (TVV)*. **Objective:** In this research, the essential oil from plant *Melaleuca cajuputi* used to test the anti-trichomonas activity. Meanwhile, symbiosis was checked to determine whether any correlation between sensitive and non-sensitive *T. vaginalis* isolates with essential oil. **Materials and methods:** A total of 32 *T. vaginalis* isolates from Hue province and Sassari University subjected to susceptibility testing against essential oils by broth microdilution method. Polymerase chain reaction (PCR) was used to detect the presence of *M. hominis* and *M. girerdii*. TVV harboring protozoan was identified by total RNA extraction. **Result:** The *M. cajuputi* essential oil showed antitrichomonal activity at the mean of MIC at  $0.08 \pm 0.05\%$  after 24 hours and  $0.06 \pm 0.05\%$  after 48 hours. There was no significant differences in MIC of EO to *T. vaginalis*-microorganism-infected and *T. vaginalis*-free. **Conclusion:** *M. cajuputi* essential oil can be used as potential therapeutic natural resource for development of antitrichomonal drugs, to not only *T. vaginalis*-free isolates but also *T. vaginalis*-infected isolates.

**Keywords—** *Melaleuca cajuputi*, *Mycoplasma girerdii*, *Mycoplasma hominis*, TVV, *Trichomonas vaginalis*.

## I. INTRODUCTION

*Trichomonas vaginalis* is the causative agent of human trichomoniasis, the most common non-viral transmitted disease with 156 million new cases each year (according to WHO, 2016). The clinical manifestations of trichomoniasis in women can range from asymptomatic to severe vaginitis, while in men this infection typically has no symptoms. This disease is associated with unfavorable outcomes of pregnancy[1] and increased risk of HIV infection[2] as well as invasive cervical cancer[3] and prostate cancer[4]. The treatment options for trichomoniasis are limited to nitroimidazole compounds (metronidazole and tinidazole), it has been unchanged since 1959. However, the *T. vaginalis* strains which are resistant to metronidazole has been noted for years and may be increasing in prevalence. *T. vaginalis* drug resistance is relative and can lead to increased doses of metronidazole[5]. In addition, many side effects of these drugs such as headache, dry mouth, metallic taste, glossitis, and urticaria caused by lengthy treatment or high doses[6]–[8] or reinfection[9]. Occasionally, patients who are allergic to nitroimidazoles may manifest an immediate-type

hypersensitivity reaction[5]. So it is necessary to improve new chemotherapy against *T. vaginalis* infection, and natural products with high effectiveness and low toxicity have shown promising therapeutic sources.

Essential oils (EOs) are very important natural plant products with various biological properties. It is estimated that out of 15 anti-parasitic medications accepted by health authorities from 1981 to 2010, 50% are natural products or derivatives[10]. The anti-trichomonas activity of natural plant products has been investigated and increased in the past decade[10]. *Melaleuca cajuputi* which is cultivated and extracted in Hue - Vietnam with strong antimicrobial activity was used in this research to test whether having the antitrichomonas activity or not.

*T. vaginalis* is an extracellular pathogen; in order to establish an infection, it must mediate adherence to host epithelial cells, evade the host immune system and compete with the vaginal microbiota[11]. In addition, surface and secreted proteins and exosome, especially the *T. vaginalis*-microbes interactions include *T. vaginalis* virus, a double-stranded RNA totivirus, and two different mycoplasma species, likely contribute to *T. vaginalis* pathogenesis[11], [12]. TVV infection can lead the host immune towards an antiviral inflammatory response that is incapable of antiparasitic activities[13]. *Mycoplasma hominis*, another common symbiont, has been shown not only increasing *T. vaginalis* growth rate but also contributing to *T. vaginalis* inflammation[11]. And the correlation between metronidazole resistance with the presence of *M. hominis* inside *T. vaginalis* is still debated. Therefore, this study will also detect the presence of symbiotic agents including *M. hominis*, *M. girerdii* and dsRNA inside *T. vaginalis* and use a statistical test to determine whether any connection between essential oil susceptibility and symbiosis.

The main Objectives of this study:

1. To determine the susceptibility of *T. vaginalis* isolates to *Melaleuca cajuputi* essential oil.
2. To identify the presence of *Mycoplasma hominis*, *Mycoplasma girerdii* and dsRNA virus inside *T. vaginalis* strains and illustrate the correlation between symbiosis to essential oil susceptibility.

## II. MATERIALS AND METHODS

### A. Study Design

This was a cross-sectional study and in vitro study.

All experiments were carried out in Parasitology Department - Hue Medicine and Pharmacy University and in the Immunology lab of Biomedical Science Department – Sassari University from 3/2019 to 1/2020.

The ethics committee of Hue University of Medicine and Pharmacy approved the study.

**B. Sample Collection**

**1. Trichomonas vaginalis isolates**

A total of 32 *T. vaginalis* isolates were analyzed, 16 isolates from Hue Medicine and Pharmacy University (HMPU) and 16 isolates from Sassari University. Since 2016, *Trichomonas vaginalis* isolates were stored in the department of Parasitology – HMPU. 16 samples had been collected till the end of August 2019 from the Hospital of HMPU and health care center in Hue Central. Italian isolates were from the *T. vaginalis* bank of the laboratory of Protozoology, Department of Biomedical Sciences – University of Sassari, and were tested from September to December 2019.

**2. Essential oil**

*Melaleuca cajuputi* was purchased from commercial company with the quality assurance by Vietnam National institute for food control.

**3. Laboratory procedure**

*T.vaginalis* was detected directly by light microscopy (x40). Positive samples were then inoculated in Diamond's Trypticase-yeast medium (TYM), modified by adding 10% fetal bovine serum, antibacterial, antifungal drugs. The medium was changed daily with observation to remove miscellaneous matter. The same procedure was used for the -80°C freezer samples. After 6-7 days cultivation, the total medium was separated into 3 tubes: One for susceptibility assays, one to extract RNA to detect dsRNA virus and the last one to extract DNA to detect *M. hominis* and *M. girerdii*.

**3.1. Determination of susceptibility of T.vaginalis to essential oil**

A standard inoculum  $5 \times 10^4$  trophozoites/ml was prepared in Diamond medium, called A solution (solA).

DMSO was used to dissolve essential oil in Diamond medium. The stock solution (solB) at 2x concentration of each oil had 2% of essential oil and 8% of DMSO in Diamond medium; and it was mixed thoroughly with a vortex mixer until obtaining a homogenous suspension. A 8 % DMSO solution was prepared in Diamond medium without essential oils to be used as a control.

Susceptibility assays were performed by broth double - dilution method in a 96 well flat bottom, tissue culture plate (Corning-Costar®, USA). The wells containing diluted concentrations  $2^n$  (n= 1,2,3,4,5,6,7,8) of each essential oil were obtained in duplicate and ranged from 1% to 0.008%.

Then the plates were placed in the incubator at 37°C in a 5% CO<sub>2</sub>. Plates were examined using inverted microscope to determine the MIC after 24 and 48 hours of incubation.

**3.2. Detecting the presence of M. hominis and M. girerdii, TVV.**

Total DNA from *T. vaginalis* previously isolate and cultivate will be extracted by using Gen Elute™ Mammalian Genomic DNA Miniprep Kit - G1N305 - Sigma Aldrich.

PCR was designed for the detection of *M. hominis* with RNH1-RNH2 primers and *M. girerdii* with OUT-M1 primers. RNA was extracted from *T. vaginalis* trophozoites using Trizol reagent instruction. Nucleic acid obtained was run electrophoresis in agarose gel 0.8% and visualized under UV light.

**4. Data analysis**

Statistical analysis was performed using Rstudio software. Mann-Whitney U test was used to compare data and considered significant at p-value < 0.05.

**III. RESULT**

**A. Susceptibility of T. Vaginalis to M. Cajuputi Essential Oil**

Antitrichomonad activity was assessed for essential oil from *Melaleuca cajuputi* on 16 isolates from Italia and 16 isolates from Vietnam. All 32 *T. vaginalis* strains were incubated with serial dilutions and viability was checked after 24 and 48 hours in duplicate. The result showed the cytotoxic to *T. vaginalis* with MIC range from 0.03 to 0.25% at 24 hours, from 0.02 to 0.25% at 48 hours; the mean of MIC at 24 hours was  $0.08 \pm 0.05\%$  and at 48 hours was  $0.06 \pm 0.05\%$ .

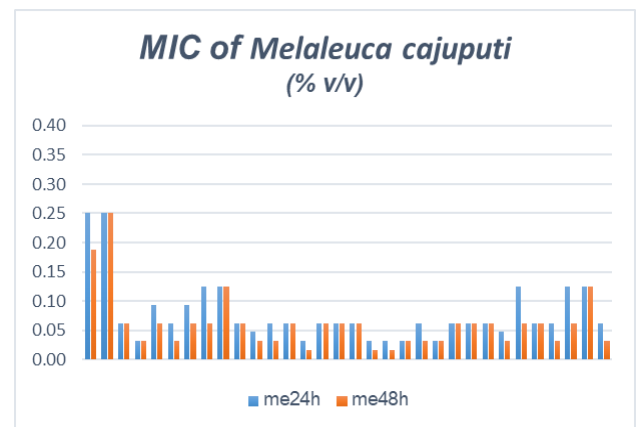


Figure: The MIC distribution of *Melaleuca cajuputi* EO to 32 *T. vaginalis* isolates tested after 24 and 48 hours

**Time dependency of the effect of M. cajuputi EO on T. vaginalis**

Median values of MIC of the oil against the 32 trichomonad strains, after 24 and 48 hours, were calculated and compared. As shown in table 1, no differences were observed in the MIC values after 24 and 48 hours (p>0.05).

TABLE 1: The comparison of MIC of *M. cajuputi* to *T. vaginalis* isolates after 24 hours and 48 hours of incubation time

	MIC %	
	24 hours	48 hours
Range	0.03-0.25	0.02-0.25
Median	0.06	0.06
p-value	p>0.05	

**Dependence of the Susceptibility of T. vaginalis strains on geographic origin**

According to table 2, after 48 hours of incubation, *M. cajuputi* had the same effect with Italian and Vietnamese strain (p>0.05)

TABLE 2: The comparison of MIC of *M. cajuputi* to *T. vaginalis* isolates between Hue province and Sassari

MIC %	<i>T. vaginalis</i> (number)	
	Huê (16)	Sassari (16)
Range	0.02-0.25	0.02-0.13
Median	0.06	0.05
p-value	p>0.05	

**B. Effect of the Presence of Symbionts on the Susceptibility of *T. Vaginalis* to Essential Oils**

All 32 trichomonad strains were tested by PCR to verify the presence of the symbionts *M. hominis*, *M. girerdii* and TVV. To investigate on the possible influence of the different symbionts on the sensitivity of trichomonad strains to each essential oil, they were divided in groups positive and negative for each symbiont.

TABLE 3: The percentage of *T. vaginalis* with and without *M. hominis*, *M. girerdii* and TVV after 48 hours incubation

<i>T.vaginalis</i>		MIC của <i>M. cajuputi</i> (%v/v)	p-value
with <i>M.hominis</i>	+	0.06	>0.05
	-	0.06	
with <i>M.girerdii</i>	+	0.06	>0.05
	-	0.06	
with Trichomonavirus	+	0.06	>0.05
	-	0.06	

When we compared the median MIC of *M. cajuputi*, the MIC to *T. vaginalis* – free isolates were equal to *T. vaginalis* – infected isolates (p-value > 0.05). It was true for all *T. vaginalis* symbionts: *M. hominis*, *M. girerdii*, and TVV.

**IV. DISCUSSION**

In this work, the effectiveness of essential oil extracts of *Melaleuca cajuputi* plant against 32 *T. vaginalis* isolates, 16 isolates from Hue and 16 isolates from Sassari University, was investigated. We set up micro-dilution assays to detect the minimal inhibitory concentration of EO to *T. vaginalis* under aerobic condition. This research showed that *M. cajuputi* essential oil had antitrichomonal activity at MIC range from 0.03 to 0.25% at 24 hours, from 0.02 to 0.25% at 48 hours. There was no differences observed in the MIC values after 24 and 48 hours with p>0.05; it means that the effect of EO to *T. vaginalis* was time-independent (table 1). In addition, *M. cajuputi* showed not significant differences in MIC between 16 *T. vaginalis* isolates from Hue and 16 isolates from Sassari (p-value >0.05). It means that the antiparasitic effects of the EO was the same in two far apart countries such as Vietnam and Italy, suggesting that the effectivity of the EO is independent from the geographical origin of *T. vaginalis* strains.

To the best of our knowledge, in vitro antitrichomonal activity of *M. cajuputi* has never been studied and there are not data for comparison with our results. However, in recent years, significant numbers of studies have reported the antitrichomonal effects of some medicinal plants. Most plant species with antitrichomonal activity belong to the three families of Asteraceae, Lamiaceae and Myrtaceae[14]. Avocado, with scientific name *Persea americana* has been reported as the most effective plant on *T. vaginalis* till now. In

this study, the chlorofomic and ethanolic extracts of *P.americana* seeds showed significant activity against *T. vaginalis* at IC<sub>50</sub> of 0.424 and 0.533 µg/ml, respectively, in comparison with IC<sub>50</sub> at 0.037 µg/ml for metronidazole [15]. Among essential oils, the one showing the most potent trichomonacidal effect is *Ocimum basilicum*, which inhibited growth of *T. vaginalis* (100%) at concentration of 30, 20 and 10 µg/ml after 24, 48 and 96 hours, respectively[16]. Other notable antitrichomonal essential oils extracted from plant that have been reported are *Amomum tsao-ko* (MLC = 44.97µg/ml for Tv1 and 89.93µg/ml for Tv2, after 48hrs)[17], *Nectandra megapotamica* (IC<sub>50</sub> = 98.7µg/ml, after 24hrs)[18], Brazilian red propolis (IC<sub>50</sub> = 100µg/ml, 24 hrs)[19], *Foeniculum vulgare* (MLC = 1600µg/ml, 48hrs)[20], *Aframomum sceptrum* (MLC = 1.72µl/ml, 48hrs)[21], *Lavandula angustifolia* and *Lavandula intermedia* (MLC<1%, 96hrs)[22]. *M. cajuputi*, the essential in our work, has MIC of 0.08±0.05% and 0.06±0.05% after 24 hours and 48 hours of incubation time, respectively. These concentration corresponded to 727±454µg/ml and 545±454µg/ml with the specific gravity of oil d = 0.909 g/ml (at 20°C – according to production company). ). The *M. cajuputi* essential oil have a good effect against *T. vaginalis*, although its efficacy of this oil is weaker than metronidazole and some EOs such as *Amomum tsao-ko*, *Nectandra megapotamica*, Brazilian red propolis (MLC < 350±175µg/ml, 48 hrs). These results indicated that a rich variety of *M. cajuputi* essential oil can be used as potential therapeutic natural resource for development of antitrichomonal drugs. As far as we know, this is the first demonstration of the potential anti-*T. vaginalis* activity of *M. cajuputi* essential oil.

One of the most intriguing aspects of *T. vaginalis* pathobiology is the complex relationship with intracellular microbial symbionts: a group of dsRNA viruses belonging to family of Totiviridae, named TVV (*T. vaginalis* virus), and eubacteria belonging to the *Mycoplasma* genus: *Mycoplasma hominis* and *Mycoplasma girerdii*. These microorganisms seem to strongly influence the lifestyle of *T. vaginalis*, suggesting a role of the symbiosis in the high variability of clinical presentation and sequelae during trichomoniasis. [11], [12]. To answer the question: is there any correlation between the presence of any symbiont and the susceptibility to essential oils cytopathic effect? In this case, our work and statistical analysis showed that with p-value > 0.05 there was no significant difference in MIC of EOs to *T. vaginalis* – infected and *T. vaginalis* – free. Results were the same with all five EOs and all *T.vaginalis* symbionts: *M. hominis*, *M. girerdii* and TVV, suggesting that the presence of the symbionts does not affect the activity of EOs. These results are consistent with the fact that EOs are complex mixtures with a great number of constituents, that seem to have no specific cellular targets. The antimicrobial activity of EOs results mainly from their lipophilic properties and is associated with damage of cell wall and cytoplasmic membrane structures, and their increased permeabilization[23], [24]. A study of M.Dai suggested that essential oil from *A.tsao-ko* exerts its anti-*T. vaginalis* activity by damaging the cell membrane and organelles[25]. An other study of H.Eldin from Iran demonstrated the possibility of

using *P.lentiscus* mastic and *O.basilicum* oil as anti-*T. vaginalis* agents, and they showed considerable damage of the membrane system of the trophozoites, abnormal vacuolization and extensive destruction of the cytoplasm[16]. However, to better comprehend the mechanisms of anti-*T. vaginalis* activities of essential oils further studies should be done in future.

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