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Mycoplasma and *Neisseria* Prevalence in Asymptomatic Women and Investigation of their Susceptibility to *Tamarindus indica* and *Syzygium aromaticum* Aqueous Extracts

Hermann Joe Djeukoua Djinou [a,b], Didiane Mefokou Yemele [a,b], Steve Francky Sohanang Nodem [b,c], Benjamin Tangue Talom [a,b], Rosine Dicko Newe [a], Sodea Yangai [c] and Simeon-Pierre Chegaing Fodouop [a,b*]

[a] Department of Biomedical Sciences, Faculty of Sciences, University of Ngaoundere, P.O. Box 455, Ngaoundéré, Cameroon.
[b] Sunshine Diagnostics Research Laboratory of Ngaoundere, Cameroon.
[c] Department of Food Science and Nutrition, National Advanced School of Agro-Industrial Sciences, University of Ngaoundéré, P.O. Bo× 455, Ngaoundere, Cameroon.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: saafodouop@gmail.com, saafodouop@yahoo.fr;*

ABSTRACT

Neisseria and *Mycoplasma* are two prevalent bacteria in the female urogenital tract leading to gynecological infections and infertility. The aim of this study was to assess the antibacterial activity of *Tamarindus indica* and *Syzygium aromaticum* extracts against *Neisseria* and *Mycoplasma* isolates. It was a cross cectional study on 60 asymptomatic women at the Protestant and Regional Hospital of Ngaoundere. For this reason, a large consecutive sample of patients with several well-defined characteristics was assembled and urine and cervical-vaginal swab were collected using standard procedures. After being isolated on a specific medium, several strains of *Neisseria* and *Mycoplasma* were identified based on their morphological and biochemical characteristics. The antibacterial activity of the plant extracts was tested using the agar-well diffusion method. It was discovered that 70% of asymptomatic women had overall infection, with varying prevalence rates. The prevalence rates of *Neisseria* and *Mycoplasma* were 14.29% and 85.71% respectively. The aqueous extracts of *Syzygium aromaticum* against *Neisseria* produced inhibitory diameters of 43 mm, 40 mm and 32 mm at doses of 20 mg/mL, 10 mg/mL, and 5 mg/mL, respectively while, at the same doses, the aqueous extract of *Tamarindus indica* produced inhibitory diameters of 16 mm, 14 mm and 13 mm, respectively. The combined extract of *Syzygium aromaticum* and *Tamarindus indica* exhibits inhibitory diameters of 25, 23, and 30 mm at 20 mg/mL, 10 mg/mL, and 5 mg/mL, respectively. *Syzygium aromaticum* extract alone showed efficacy against *Mycoplasma*, with diameters of 16.5 mm, 13 mm and 10.5 mm at concentrations of 20 mg/mL, 10 mg/mL, and 5 mg/mL respectively. Inhibition diameters of 18 mm (for fosfomycin and ofloxacin), 22 mm (for chloramphenicol and ceftriaxone) and 26 mm (for levofloxacin) were found using *Neisseria* isolates. The only drugs that demonstrated efficacy against *Mycoplasma* were Minocycline and Josamycin. Given these findings, extracts of *Syzygium aromaticum* and *Tamarindus indica* can be investigated and used to treat infections caused by *Niesseria* and *Mycoplasma*.

Keywords: Asymptomatic woman; Neisseria; mycoplasma; extract; antimicrobial activity; infertility.

1. INTRODUCTION

Infections of the urinary and reproductive systems that affect both men and women are known as urogenital infections. The germs responsible for these infections are typically found in the vaginal tract and can be introduced through sexual contact or medical procedures [1]. These urogenital infections are the most prevalent transmissible disease in the world, and gonorrhea and *Mycoplasma* infections treat an increasing number of patients each year [2]. An estimated 250 million new cases of sexually transmitted illnesses occur globally. The frequency of gonorrhea in asymptomatic women varies from 0% to 1.4% in the international literature [3]. About 10% of women in France are estimated to carry *Mycoplasma hominis*, while 50% of women carry *Ureaplasma urealyticum* [4]. Africa is one of the regions with the highest incidence, with around 63 million cases [5]. *Neisseria gonorrhoeae* was found to be more common in 1.3% of Cameroonian women with secondary infertility cases than in controls (controls) in a case-control study. Furthermore, study conducted at the Garoua Pasteur Annex Center in 2018 revealed that 61.1% of women had *Mycoplasma* infection [6]. Sexually transmitted infections such as *Mycoplasma* and *Neisseria gonorrhoeae* can cause major complications that affect sexual and reproductive health, ultimately resulting in infertility [7]. The inability of a heterosexual couple to conceive a child is known as infertility. Thus, infertility is a disorder that occurs regardless of the efforts of the couple to become pregnant [8].

About eigthy million people worldwide struggle with infertility [9]. Around 40% of infertility in developing nations like Cameroon is caused by primary infertility, while 60% is caused by secondary infertility [10]. Even with all the progress made, infertility is still a problem that cannot be fully controlled, and the use of synthetic molecules can have unfavorable side effects [11]. For more than 40 years, treatments have been available for sexually transmitted diseases (STI) caused by gonorrhea and *Mycoplasma*. However, STIs continue to be a concern for public health in developed and, more importantly, emerging nations, and the majority of antibiotic compounds utilized are responsible for the recurring resistance that bacteria exhibit [12]. Today, more and more people are treating these infections with natural therapies [13]. The antibacterial activity of aqueous extracts and

methanolic extracts of the plants *Typha angustifolia* L and *Zingiber officinale* Roscoe at various doses (0.0031 and 0.05 mg/mL) in *Neisseria* strains was evaluated in studies conducted by Bashige et al. [14] using the dilution method. Thus, plant medicines in their many forms still have a place of preference even in the face of the emergence of synthetic drugs [15]. Among these, *Syzygium aromaticum* and *Tamarindus indica* are plants whose therapeutic qualities are used in traditional Indian medicine. Women in Cameroon, particularly in the Adamaoua region, are facing a growing number of infertility problems. Those who seek medical attention do so too late, as germs have developed some resistance and resulted in an upper tract genital infection (tubal infertility).

The objectives of the current study are to determine the incidence of *Neisseria and Mycoplasma* in asymptomatic women in the town of Ngaoundere and to demonstrate the antibacterial activity of *Tamarindus indica* and *Syzygium aromaticum extracts* against isolated microorganisms.

2. MATERIALS AND METHODS

2.1 Materials

Urine and vaginal swab samples were collected from 60 asymptomatic women treated at the Protestant and Regional Hospital of Ngaoundere. On the other hand, *Tamarindus indica* and *Syzygium aromaticum spices* were collected from the Ngaoundere city market (November 2022) and brought directly to the Sunshine Laboratory.

2.2 Methods

2.2.1 Sampling

Urines was collected using standardize method previously described [16]. The sampling was carried out using the morning urine collection method, also known as the "midstream" or "broadcast" method. This involves eliminating the first jet (20 mL) and then collecting the next 20 to 30 mL in a sterile bottle [16]. Concerning vaginal swab, samples were collected using standardize procedure previously described [17].

2.2.2 Cytobacteriological examination of urine

The enumeration process involved placing a precise amount of urine (2 to 5 µL) between a slide and a coverslip, then examining the entire sample under an objective microscope x40 while still fresh. The number of elements present per mL was reported.

Qualitative cytological examination: Gram staining was performed on the centrifugation pellet to observe any microorganisms present and to guide the choice of culture medium according to their morphology and affinity for dyes (pink staining for Gram negative bacteria and violet staining for Gram positive bacteria).

Bacteriological examination: Inoculation was carried out using the streak method from a drop of urine deposited with a platinum loop on Chocolate + VCN agar for *Neisseria* and on CLED agar for culture and isolation of other urinary tract germs. Readings were taken after 24 h of incubation at 37 °C.

2.2.3 Cervico-vaginal sampling (CVS)

Microscopic analysis: Fresh and stained states were performed using the method described by (Somita et al., 2003). A suspension of vaginal secretions was made using the swab and a few drops of physiological water. A drop of suspension was placed between the slide and the coverlip and examined under the microscope with an objective (x40). For staining, vaginal secretions were spread by carefully rolling the swab on a slide and pressing to obtain a homogeneous smear, then drying and stained. After Gram staining, the slides were examined with a microscope objective (x100) [17].

Culture, isolation, and purification : Pathogens were isolated using the method described by (Ngaba et al., 2014). Inoculation was carried out on chocolate + VCN agar (CHOC) for the isolation of *Neisseria gonorrhoeae*, Sabouraud agar (SAB) for the isolation of candida strains, EMB agar for the isolation of Gram-negative bacteria, and Chapman agar (CHAP) for the isolation of *Staphylococcus aureus*. All these mediums were incubated at 37°C for 24-48 h. Only chocolate medium was incubated in a 10% CO2 environment for *Neisseria gonorrhoeae* [18].

For purification, an individual colony was picked and streaked with a platinum loop, then incubated at 37 °C for 24-48 h.

***Mycoplasma* culture:** *Mycoplasma* culture was performed using the Freeze-Dried test kit. The

principle of the kit is based on the presence of specific substrates and an indicator (phenol red), which, in the event of a positive culture, visualizes a color change in the broth linked to an increase in pH. This gallery enables simultaneous culture, identification, counting, and sensitivity.

2.2.4 Identification and antibiogram

Neisseria colonies were identified on the basis of the control Gram, catalase, and oxidase tests.

Gram staining: The material was spread on a glass slide, which was then let to air dry before being stained with lugol and gentian violet for one minute each. Before applying the next dye, each was carefully washed with clean water. 96% alcohol was used to remove the stain and clean water was used to rinse the slides. After a minute, rinse the slide with clean water and cover it with diluted fuchsin (1 mL of fuchsin to 9 mL of water). After the slide was let to air dry, use an oil immersion microscope with a 100x objective to examine it under a microscope [19].

Catalase and oxidase tests: The test was carried out using the methodology described by Reiner (2013). A well-isolated colony from a pure culture (18 to 24 hours incubation) was selected and placed on the microscope slide using a sterile inoculation loop. Using a Pasteur pipette, a drop of 3% hydrogen peroxide was added to the colony, and then the Petri dish was immediately covered with a lid [19]. The test was conducted using the procedure described previously [19]. An oxidase disk was placed on an object slide with forceps and a stick was used to collect a well-isolated colony representative of the fresh culture to be tested. The colony was gently rubbed on the disc until a violet coloration appeared in 30 seconds [19].

Antibiotic resistance of characterized germs: Antibiotic susceptibility of *Mycoplasma* was performed directly using freeze dried test kit and following the procedure described by manufacturer. Twelve different antibiotics were included such as Spectinomycin, Levofloxacin, Minocycline, Ofloxacin, Roxithromycin, Azithromycin, Clarythromycin, Josamycin, Spectinomycin, and Gatifloxacin. Concerning *N. gonorrhoeae,* antibiotic susceptibility was carried out using the technique described by Otto et al. [20]. After young colonies were isolated, a bacterial solution was prepared and then inoculate on CHOC medium. Using sterile forceps, antibiotic discs were placed on dried agar plates. The entire set was then incubated for 24 h at 37 °C with 10% CO_2 [20].

2.2.5 Préparation of *T. indica* and *S. aromaticum* extract

Two grams (2g) of dry ground plants (*T. indica and S. aromaticum*) were weighed and extracted with 25 mL of distilled water. The mixture was left for for 48 h at room temperature (25 ± 2°C). After 48 h, the mixture was filtered and resulting solution was dryed at 40 °C for 24 h. The dry extract obtained is weighed and stored in the refrigerator until use.

2.2.6 Evaluation of the antimicrobial activity of extracts

The antibacterial activity of the extracts was performed using the agar well diffusion method as described previously [21]. After incubation, inhibition zone was measured and the antimicrobial activity as been classified as sensitive, very sensitive, extremely sensitive, or resistant based on the inhibition diameter [21].

Preparation of culture medium and bacterial inoculum: From young precultures of each *Neisseria gonorrhoeae* isolate, microbial suspension was prepared with a concentration equal to 0.5 Mc Farland [21].

Concerning antibacterial tests with *Mycoplasma* strains, when the medium showed a positive result, 100 µL of the culture on the negative control (Kit) collected and inoculated on MH agar medium. After, 200 µL of extract was applied to 6 mm diameter agar wells and Petri disches were incubated at 37 °C for 48 h. The diameters of inhibition were measured.

Inoculation: Petri dishes filled with chocolate medium were aseptically inoculated with the different bacteria using a sterile swab that had been dipped in the microbial suspension over the whole surface of the medium. The bacteria were released and distributed on the entire agar surface in tight streaks [21]. A sterile punch was used to cut the wells once the plates dried. The resulting 10 mm cavities were filled with extracts (approximately 200 µL each well) after their bottoms were sealed with agar. After the plates were closed and allowed to pre-diffusion for 15 minutes at room temperature [21], they were incubated for 48 hours at 37 °C with 10% CO_2. The diameters of inhibition were measured.

2.3 Ethical Considerations

The several hospitals in the city of Ngaoundere, the regional health delegate, and the dean of the Faculty of Science of the University of Ngaoundéré had all granted permission for the research. For the proper conduct of our study, we obtained written informed consent from the participants.

2.4 Statistical Analysis

Data were analyzed using Sphinx and EXCEL 2016 software.

3. RESULTS

3.1 Etiology of Urogenital Infections in the Study Population

The prevalence of genital infections in asymptomatic women has been calculated and data are illustrated in Fig. 1. Forty-two of the 60 women who received the treatment had *Mycoplasma* and *Neisseria* infections, representing a frequency of 70%. Similarly, the prevalence of *Neisseria* and *Mycoplasma* in the target population is mentioned in Fig. 2. Of the 42 women who had infections, six had gonorrhoeae, which encountered a prevalence rate of 14.29%, and 36 had *Mycoplasma*, which represented a prevalence rate of 85.71%. There are a few species that could be involved with *Mycoplasma* and two of them were studied. Fig. 3 shows the prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum*. With a prevalence rate of 22.22%, *Mycoplasma hominis* was the most prevalent species. On the other hand, the coinfection of *Ureaplasma urealyticum* and *Mycoplasma hominis* showed a higher prevalence rate of 58.33%.

Several antibiotics were tested against the *Neisseria* bacterial strain; Table 1 shows the results. The isolated germ is resistant to five drugs, including ciprofloxacin, amoxyclav, azithromycin, tobramycin, and erythromycin, as this table demonstrates. Table 1 shows that only two antibiotics, josamycin and minocycline, were shown to be responsive to *Mycoplasma*.

3.2 Antimicrobial Activity of *Tamarindus indica* (TI) and *Syzygium aromaticum* (SA) Extracts

Table 2 shows the inhibition diameters of *Tamarindus indica* and *Syzygium aromaticum* on the isolates of interest. Depending on the concentration, each of the three extracts exhibited different levels of activity against *Neisseria gonorrhoeae* isolates. With inhibitory diameters of 43, 40 and 32 mm, respectively, at 20 10 and 5 mg/l, the aqueous extract of *Syzygium aromaticum* exhibited the highest activity. This was followed by a 1/1 mixing of the two extracts. Regarding the activity of the extracts in *Mycoplasma*, the same table demonstrates that the only extract that inhibits these isolates is the aqueous extract of *Syzygium aromaticum* at varying doses.

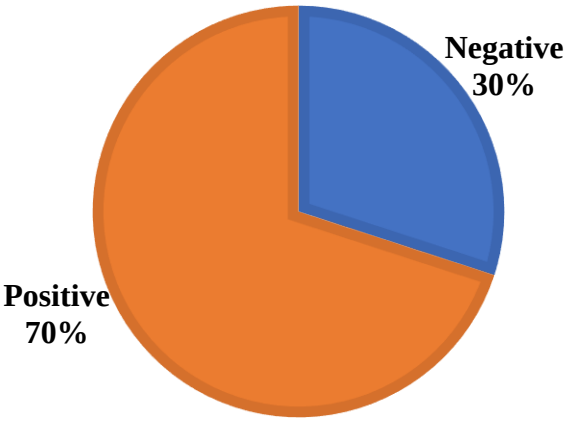

Fig. 1. Prevalence of genital infections in the sudied population

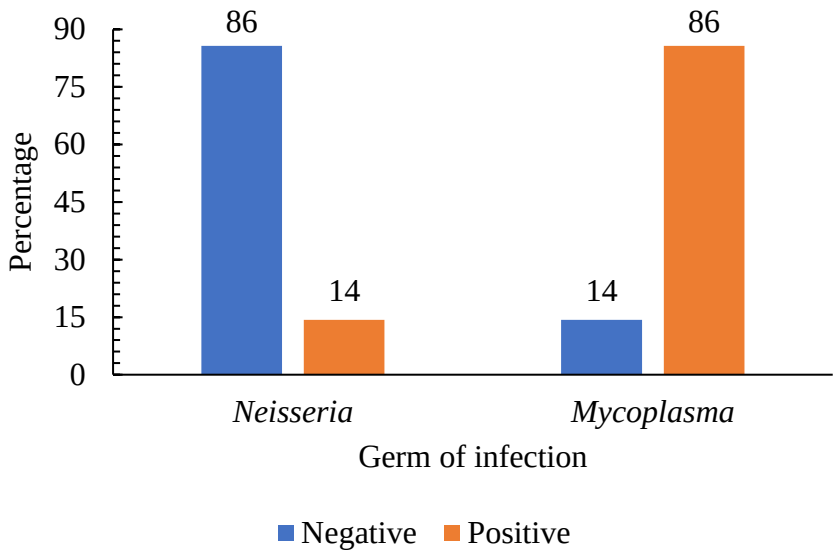

Fig. 2. *Neisseria gonorrhoeae* and *Mycoplasma* infections in the population

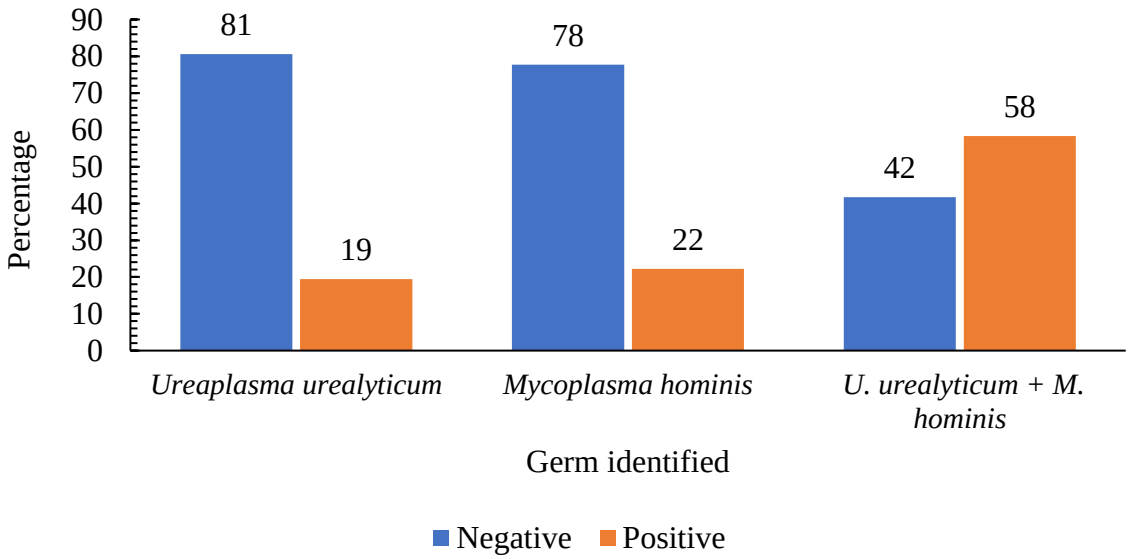

Fig. 3. Prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum*

Table 1. Diameter of inhibition for various antibiotics with respect to isolated microorganisms

	ATB	Diameter of inhibition (mm)
Neisseria	Fosfomycin	18
	Chloramphenicol	22
	Ofloxacin	18
	Ciprofloxacin	-
	Amoxyclav	-
	Levofloxacin	26
	Ceftriaxon	22
	Azithromycin	-
	Tobramycin	-
	Erythromycin	-

	ATB	Diameter of inhibition (mm)
Mycoplasma	Josamycin	a
	Minocyclin	a
	Doxycyclin	-
	Ciprofloxacin	-
	Ofloxacin	-
	Sparfloxacin	-
	Roxithromycin	-
	Azithromycin	-
	Clarithromycin	-
	Spectinomycin	-
	Levofloxacin	-
	Gatifloxacin	-

(-) no bacterial inhibition; (a) microbial inhibition

Table 2. Inhibition diameters of extracts against *Neisseria* and *Mycoplasma* strains

	Extracts	Concentrations (mg/mL)	Inhibition diameter (mm)
Neisseria	ESA	20	43
		10	40
		5	32
	ETI	20	16
		10	14
		5	13
	EST	0.2	30
		0.1	25
		0.05	23
	Cefixim	0.2	50
		0.1	36
		0.05	27
Mycoplasma	ESA	20	16.5
		10	13
		5	10.5
	Ofloxacin	0.2	45
		0.1	38
		0.05	30

ESA = Syzygium aromaticum extract ; ETI = Tamarindus indica extract ; EST = Syzygium and Tamarindus extract.

4. DISCUSSION

4.1 Etiology of Urogenital Infections in the Study Population

To carry out this study, we conducted a campaign at the end of which we received 60 women. The overall prevalence of *N. gonorrhoeae* and *Mycoplasma* infections in asymptomatic women showed that 70% of the study population were ill. This shows that women are highly exposed to these infections. This could be explained by inadequate lifestyles and sexual activity of women. Of these 70% infections, 14.29% of the study population suffered from *N. gonorrhoeae*. This could be explained by a lack of awareness and a low rate of systematic screening. This result differs from that obtained by the WHO on the general prevalence in the asymptomatic female population, which ranged from 0% to 1.4%, and is close to that obtained by Karim Safae in Morocco [22], which was 14.1% obtained in women received at the Anatomopathology Laboratory of the Hassan II University Hospital in Fez. Our results also differ from those of Tissier et al. [3] who detected five cases of *Neisseria* in symptom-free patients out of 320 patients screened with a prevalence of 1.56%.

Secondly, we also observed a high prevalence of 85.71% due to infection caused by *Mycoplasma*. This is explained by the fact that *Mycoplasma*s are commensal germs of the genital tract, which explains their presence in a greater number. Concerning *Mycoplasma* species two were found

in this study *Ureaplasma urealyticum* and *Mycoplasma hominis*. Of the 85.71% of *Mycoplasma*s obtained, 19.44% represented the prevalence of *Ureaplasma urealyticum*. *Mycoplasma hominis*, with a prevalence of 22.22%, is the more common of the two species.

The prevalence of coinfection of the two species is the highest, at 58.33%. This would mean that women are more affected by the two germs than by a single one, which just goes to show the seriousness of this infection in that it is much more asymptomatic; in fact, the germs have time to proliferate depending on the various factors, leading to miscarriage and subsequent infertility. These results differ from those of Djigma & Ouermi [23] who in a study carried out in Burkina Faso found that among 120 HIV-positive women, 10% were carriers of *U. urealyticum* and 0.8% were carriers of *M. hominis*, and the prevalence of coinfection of the two species was 7.5%. Furthermore, our results are superior to those obtained by Ezeanya-Bakpa et al. [24] who, in a study carried out in South Africa among asymptomatic women, obtained a prevalence of 8% and 2%, respectively, for *M. hominis* and *U. urealyticum*, and a prevalence of 28.6% among those harboring both species.

4.2 Antimicrobial Activity of *Tamarindus indica* and *Syzygium aromaticum* Extracts

The phenomenon of antibiotic resistance observed in microbial strains is due to the frequent use of antibiotics, which generates several mutants from naturally sensitive strains. With this in mind, we set out to investigate the antimicrobial activity of tamarind and clove. Several extracts were obtained: aqueous extract, ethanolic extract, and hydroalcoholic extract.

According to the results obtained, only three extracts were active in the *Neisseria* strains tested. The highest activity was observed with the aqueous extract of *Syzygium aromaticum* (43 mm), followed by the hydroalcoholic extract of *S. aromaticum* and *T. indica* (30 mm), and the lowest was observed with the aqueous extract of *Tamarindus indica* (16 mm). Our study differs from that carried out by Bashige et al. [17], which shows an antimicrobial effect of aqueous and alcoholic extracts on *Neisseria* strains.

For *Mycoplasma*, only the aqueous extract of Syzygium aromaticum had a significant effect, the inhibition diameters for its different concentrations being 16.5, 10 and 10.5 mm. Therefore, we can conclude from this result that *S. aromaticum* has antimicrobial activity against *Mycoplasma* and not *T. indica*.

5. CONCLUSION

From the isolation and identification of strains, it emerged that the highest prevalence was that of *Mycoplasma*, with a rate of 85.71%. *Neisseria gonorrhoeae* was 14.29%. Two *Mycoplasma* germs are responsible for *Mycoplasma* infections, and the species most frequently encountered is *Mycoplasma hominis* (22.22%). In terms of antimicrobial activity, *Syzygium aromaticum* extract was found to have an antimicrobial effect on *Neisseria gonorrhoeae* and *Mycoplasma* strains, while *Tamarindus indica* had a considerable effect on *Neisseria gonorrhoeae* but not on *Mycoplasma* strains. The combination of concentrations of aqueous clove extract and antibiotics showed a synergistic effect on *Neisseria gonorrhoeae* strains. Based on the results obtained, we can say that *Syzygium aromaticum* and *Tamarindus indica* extracts are effective in the microorganisms tested, so they can be used as alternative molecules for the treatment of gonorrhoeae and *Mycoplasma* infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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