Mycoplasma and Neisseria prevalence in asymptomatic women and investigation of their susceptibility to T*amarindus indica* and *Syzygium aromaticum* aqueous extracts

Abstract:

Two prevalent bacteria in the female urogenital tract, Neisseria and Mycoplasma, can lead to gynecological infections and infertility. By evaluating the antibacterial activity of *Tamarindus indica* and *Syzygium aromaticum* extracts, the current study aims to prevent Neisseria and Mycoplasma infection. For this reason, a large consecutive sample of patients with certain, well-defined characteristics was assembled. The appropriate procedures were followed to gather urine and cervical-vaginal samples. After being isolated on a specific medium, several strains of Neisseria and Mycoplasma were identified based on their morphological and biochemical characteristics. Plant extracts were tested for their antibacterial activity using the agar-well diffusion method. It was discovered that 70% of asymptomatic women had infection overall, with varying prevalence rates.Asymptomatic women had an overall 70% infection prevalence; separate prevalence rates for Neisseria and Mycoplasma were reported to be 14.29% and 85.71%, respectively. 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 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* displays inhibitory diameters of 25, 23, and 30 mm at 20 mg/mL, 10 mg/mL, and 5 mg/mL, respectively. The *Syzygium aromaticum* extract alone shown 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 in the investigation of Neisseria sencibility to antibiotics. The only drugs that demonstrated efficacy against Mycoplasma were Minocycline and Josamycin. Given these findings, extracts from *Syzygium aromaticum* and *Tamarindus indica* may be investigated and utilized to treat infections brought on by Niesseria and Mycoplams.

Key words: 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 diseases in the world, and both gonorrhea and mycoplasma infections are treating an increasing number of patients year [2]. An estimated 250 million new instances of sexually transmitted illnesses occur globally. The frequency of gonorrhea in asymptomatic women varies from 0% to 1.4% in international literature [3]. It is estimated that 10% of women in France carry *Mycoplasma hominis*, while 50% of women carry *Ureaplasma urealyticum* [4]. Africa is among 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 research [6]. In addition, a 2018 study conducted at the Garoua Pasteur Annex Center by Alexandre et al. revealed that 61.1% of women had Mycoplasma infection. Sexually transmitted infections such as Mycoplasma and Gonorrhoeae can cause major complications that impact 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 couple's efforts to become pregnant [8].

80 million individuals globally 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 that has been made, infertility is still a problem that cannot be fully controlled, and using synthetic molecules can have unfavorable side effects [11].

For more than 40 years, treatments have been available for sexually transmitted diseases (S.T.I.) brought on by gonorrhea and mycoplasma. Even so, S.T.I.s continue to be a concern for public health in developed and, more importantly, emerging nations, and the majority of the antibiotic compounds utilized are to blame for the recurring resistance that the bacteria exhibit [12]. Nowadays, more and more people are treating these

infections with natural therapies [13]. The antibacterial activity of aqueous (EAQ) and methanolic (EME) extracts of the plants *Typha angustifolia* L and *Zingiber officinale* Roscoe at several doses (0.0031 and 0.05 mg/ml) on Neisseria strains was evaluated in studies conducted by Bashige et al., [14] using the dilution method. Thus, plant medicines in their many forms still hold a place of preference even in the face of the emergence of synthetic pharmaceuticals [15]. Among these, *Syzygium aromaticum* and *Tamarindus indica* are plants whose therapeutic qualities are employed in traditional Indian medicine. Women in Cameroon, particularly in the Adamaoua region, are facing a growing number of infertility issues. Those who do seek medical attention do so too late, as the germs have developed some resistance and resulted in an upper tract genital infection (tubal infertility).

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

2. MATERIALS AND METHODS

2.1.Living organisms

ECBU and PCV biological samples from 60 asymptomatic women treated at the Hôpital Protestant et Régional de Ngaoundéré were the source of the strains used in our experiment. In November 2022, T. indica and S. aromaticum were acquired from the Ngaoundéré city market and brought straight to the Sunshine Microbiology Laboratory.

2.2.Methods

2.2.1. Cytobacteriological Examination of Urines (ECBU)

Cytobacteriological examination of the Urine was carried out according to the method described by REMIC in 1998. Sampling was carried out using the morning urine collection method, also known as the "midstream" or "broadcast" method. This involves eliminating the first jet (around 20 ml) and then collecting the next 20 to 30 ml in a sterile bottle [16].

2.2.2. Cervico-vaginal sampling (CVS)

The Cervico-Vaginal Sampling (PCV) It was carried out following the method described by Somita et al., in 2003. After installing the patient in the gynecological position, the speculum is introduced into the vagina atromatically in a vertical position then we carry out a rotation of 90°C on the horizontal. Arriving in contact with the cervix, the speculum is opened, the cervix must be clearly visible, then using a first sterile swab, we take from the level of the ectocervix, the second and third sterile swabs, allow us to take at the level of the endocervix [17].

Cervico-vaginal sampling (CVS) is performed according to the method described by Somita et al. in 2003. After the patient has been placed in the gynaecological position, the speculum is introduced into the vagina atromatically in a vertical position, then rotated 90°C horizontally. Once in contact with the cervix, the speculum is opened, the cervix must be clearly visible, and the first sterile swab is used to sample the ectocervix. The second and third sterile swabs are used to sample the endocervix [17].

2.2.3. Cytobacteriological examination of urine (ECBU)

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

2.2.3.2. Qualitative cytological examination

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

2.2.3.3. 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 24h incubation at 37°C.

2.2.4. Cervico-vaginal sampling (CVS)

2.2.4.1. Microscopic analysis

Fresh state and stained state 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 slide and coverslip 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 dried and stained. After Gram staining, the slides were examined under a microscope objective (x100) [17].

2.2.4.2. 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, Chapman agar (CHAP) for the isolation of *Staphylococcus aureus*. All these media were incubated at 37°C for 24-48 h. Only the Chocolate medium was incubated in a 10% CO2 environment for *Neisseria gonorrhoeae* [18].

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

2.2.4.3. 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.

2.2.5. Identification and Antibiogram

Neisseria colonies were identified on the basis of the control gram, catalase test and oxidase test.

2.2.5.1. Gram staining

The material was spread out 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 fuchsin to 9 ml water). After letting the slide air dry, use an oil immersion microscope with a 100x objective to examine it under a microscope [19].

2.2.5.2. Catalase test

The test was conducted using the methodology outlined by Reiner (2013). A well-isolated colony from a pure culture (18 to 24 hours incubation) was selected and put 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 the Petri dish was then promptly covered with a lid [19].

2.2.5.3. Oxidase test

The test was conducted utilizing the procedure outlined in (Reiner, 2013). An oxidase disk was placed on an object slide with forceps, and a stick was used to pick up 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 within 30 seconds [19].

Using the Freeze-Dried test kit, antibiotic susceptibility testing was done directly for mycoplasma, showing sensitivity to 12 different antibiotics: Spectinomycin, Levofloxacin, Minocycline, Ofloxacin, Roxithromycin, Azithromycin, Clarythromycin, Josamycin, Spectinomycin, and Gatifloxacin.

The antibiogram for gonorrhoeae was carried out using the technique outlined by Otto et al.,) [20]. After isolating young colonies and preparing a bacterial solution, flooding was used to inoculate CHOC media. Using sterile forceps, the antibiotic discs were placed on the dried agar plates. The entire set was then incubated for 24 hours at 37°C with 10% CO2 [20].

Preparation of plant extracts at different concentrations

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

Two grams (2g) of ground dry plants (*T. indica and S. aromaticum*) were weighed and extracted with 25 ml distilled water.The mixture was left to stand for 48h to achieve ideal extraction. After 48h, the mixture was filtered from the filter paper, then the resulting solution was left to stand in the oven for 24H at 40°c until completely dry. The dry extract obtained is weighed and stored in the refrigerator until use.

2.2.7. Evaluation of the antimicrobial activity of extracts

To ascertain the extracts' antibacterial activity, the well method was selected. The method's basic idea is that, following a specific amount of time for the product and the target microbe to come into contact, the antimicrobial chemical will diffuse across a solid medium in a Petri dish, resulting in the formation of a concentration gradient. By evaluating a zone of inhibition, the antimicrobial product's impact on the target is evaluated. The product under test can be categorized as sensitive, very sensitive, extremely sensitive, or resistant based on the inhibition diameter [21].

2.2.7.1. Preparation of culture medium and bacterial inoculum

Mycoplasma and Neisseria were isolated and their sensitivity to plant extracts was investigated using chocolate agar.

The preserved microbial strains were allowed to defrost and then be kept at room temperature. From juvenile pre-cultures of each *Neisseria gonorrhoeae* strain, a few well-isolated, identical colonies were selected using a sterile platinum loop. Then, to create an inoculum with a concentration equal to 0.5 Mc Farland [21].

When the medium showed a positive result for Mycoplasma, indicated by a pink tint.In order to determine the inhibitory zone diameters of the extracts against mycoplasma, we inoculated 100 µl of the negative control (Kit) onto chocolate agar. Following the medium's solidification, 200

µl of extract were applied to 6 mm diameter agar wells. Using a graduated ruler, the diameters of the zones of inhibition were measured following a 48-hour incubation period at 37°C.

2.2.7.2. Inoculation

Petri dishes filled with chocolate or MH 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 over the whole agar surface in tight streaks [21]. A sterile punch was used to cut out wells once the plates had dried. The resulting 10 mm cavities were filled with extracts (about 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% CO2. By measuring the diameter of the zone of inhibition for each petri dish, the results can be assessed.

2.2.7.3. Synergistic activity of *Syzygium aromaticum* and antibiotics on *Neisseria gonorrhoeae* strains

The synergistic activity of the extract with the largest diameter was assessed according to the method described by Otto et al., 2014 [20]. Each antibiotic disc was coated with 5 µl of extract at different concentrations (0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml), then placed on the surface of Chocolate agar plates previously inoculated with a bacterial suspension. After a 24-hour incubation period at 37°C with 10% CO2, the diameter of the inhibition zones surrounding the discs was measured.

2.3.Statistical analysis

Data were analyzed using Sphinx and EXCEL 2016 software.

3. RESULTS

3.1.Etiology of urogenital infections in the study population

Figure 1 shows the total frequency of Mycoplasma and Gonorrhoeae infections in asymptomatic women. 42 of the 60 women who were given the treatment had both Mycoplasma and Neisseria infections, representing a 70% frequency.

The target population's prevalence of Neisseria and Mycoplasma is depicted in Figure 2. Of the 42 women who had infections, 6 had gonorrhoeae, which encountered a prevalence rate of 14.29%, and 36 had mycoplasma, which represented a 85.71% prevalence rate.

There are a few species that could be involved with Mycoplasma, and two of them were studied. Figure 3 displays 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%.

A number of antibiotics were tested against the Neisseria bacterial strain; Table 1 displays the results. The isolated germ is resistant to five medicines, including ciprofloxacin, amoxyclav, azithromycin, tobramycin, and erythromycin, as this table demonstrates.

Table 1 demonstrates that just 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 mm, 40 mm, and 32 mm, respectively, at 20 10 and 5 mg/l, the Syzygium aromaticum (SA) aqueous extract exhibited the highest activity. This was followed by a 1/1 mixing of the two extracts. Regarding the extracts' activity on mycoplasma, the same table demonstrates that the only extract that inhibits these isolates is the aqueous extract of Syzygium aromaticum at varying doses.

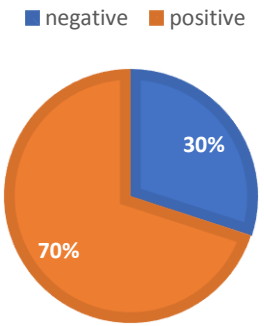

Figure 1 : prevalence of gonorrhoeae and Mycoplasma infections in the study population

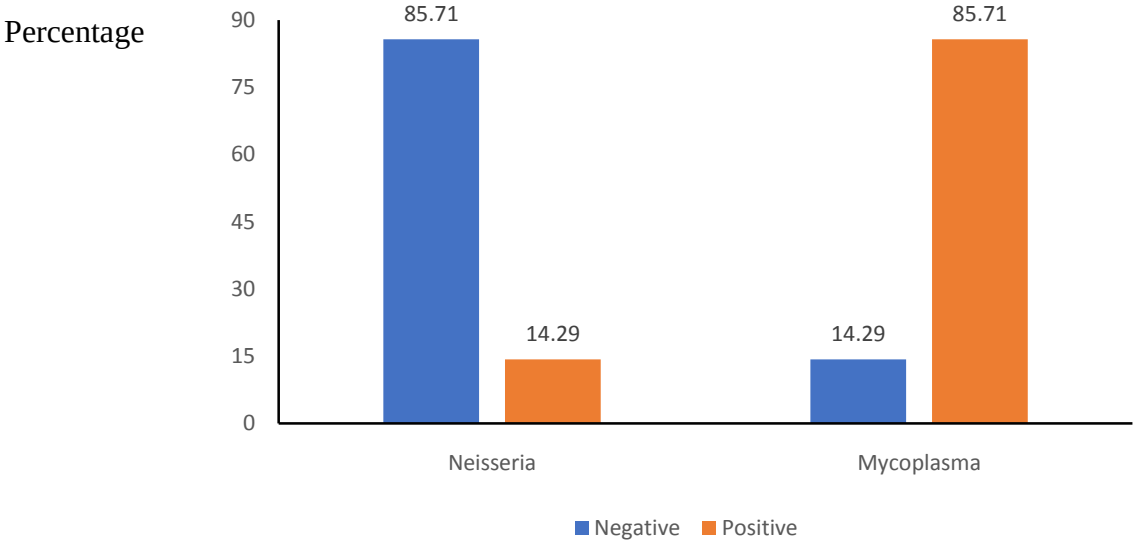

Figure 2 : prevalence of gonorrhoeae and Mycoplasma in the target population

Percentag

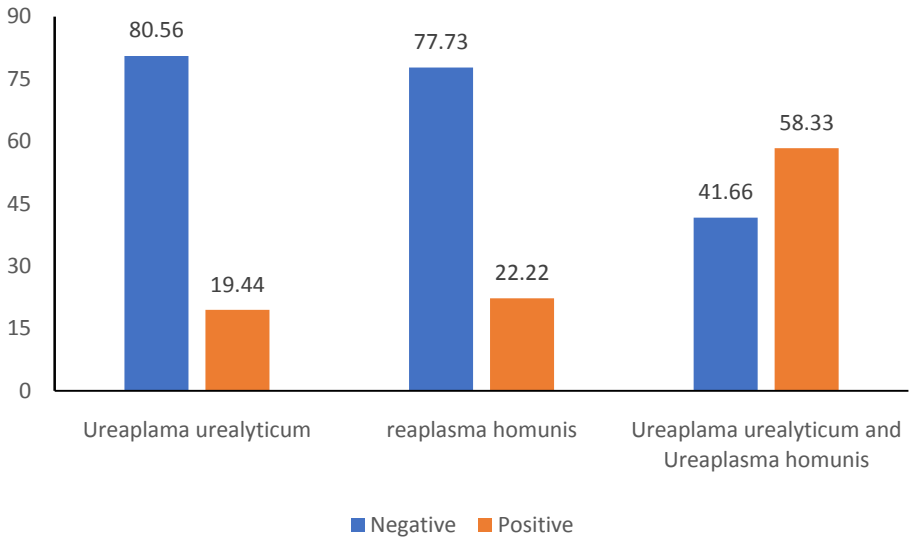

Figure 3 : Prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum*

Table 1 : diameter of the inhibitory zones for various antibiotics in respect to isolated microorganisms

	ATB	Diameterof inhibition (mm)
Neisseria	Fosfomycin	18

	Chloramphenicol	22
	Ofloxacin	18
	Ciprofloxacin	-
	Amoxyclav	-
	Lévofloxacin	26
	Ceftriaxon	22
	Azithromycin	-
	Tobramycin	-
	Erythromycin	-
Mycoplasme	Josamycin	a
	Minocyclin	a
	Doxycyclin	pa
	Ciprofloxacin	pa
	Ofloxacin	pa
	Sparfloxacin	pa
	Roxithromycin	pa
	Azithromycin	pa
	Clarithromycin	pa
	Spectinomycin	pa
	Levofloxacin	pa
	Gatifloxacin	pa

(-)Resistant (a) activity, (pa) no activity

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 our study we conducted a campaign at the end of which we received 60 women. The overall prevalence of 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 women's inadequate lifestyles and uncontrolled sexual activity. Of these 70% infections, 14.29% of the study population suffered from 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 2018 in Morocco, which was 14.1% obtained in women received at the Anatomo-pathology Laboratory of the Hassan II University Hospital in Fez. Our results also differ from those of (Tissier et al., 2015) who detected 5 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. Our results show that mycoplasmas are indeed widespread in our society. This is explained by the fact that Mycoplasmas are commensal germs of the genital tract, which explains their presence in greater numbers. These results are higher than those obtained by Hassan at the Centre Médical EXACT in Garoua (2021), which were 48.38%. Speaking of the Mycoplasma species responsible for infections, two were the subject of our study, namely *Uréaplasma urealyticum* and *Mycoplasma hominis*. Of the 85.71% of Mycoplasmas obtained, 19.44% represented the prevalence of *Ureaplasma urealyticum*. *Mycoplasmahominis*, 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, 2008) 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., 2021) 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* (TI) and *Syzygium aromaticum* (SA) extracts.

The phenomenon of antibiotic resistance observed in microbial strains is due to the frequent use of antibiotics, generating 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 3 extracts were active on 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 with the aqueous extract of *Tamarindus indica* (16 mm). Our study differs from that carried out by (Bashige et al., 2020), which shows an antimicrobial effect of aqueous and alcoholic extracts on Neisseria strains.

As far as Mycoplasma is concerned, 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. We can therefore conclude from this result that S. aromaticum has antimicrobial activity against mycoplasma and not T. indica.

4.3.Effect of *Syzygium aromaticum* extract combination on antibiotic discs

The inhibitory action of Syzygium aromaticum aqueous extract on germs enabled us to highlight the synergistic effect of this extract with antibiotics that had an inhibitory action on Neisseria strains. The results obtained showed that the various antibiotics tested, namely Fosfomycin,

Ceftriaxone, Ofloxacin, Levofloxacin and Chloramphenicol at different concentrations C1= 0.2 mg/ml; C2= 0.1 mg/ml; C3= 0.05 mg/ml in combination with the aqueous extract of S. aromaticum had a synergistic effect on the Neisseria strains tested. The most significant effect was observed with Fosfomycin (18-33 mm), a broad-spectrum antibiotic that inhibits the bacterial wall. The aqueous extract would therefore have greater affinity with the latter, enhancing its mechanism of action. This could be explained by the similarity of the mechanism of action of these antibiotics and the extract. Our study differs from that carried out by (Science & In, 2020), which demonstrated the synergistic effect of combining methanolic plant extract with seven antibiotics on Gram-negative bacterial strains, including Neisseria.

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%. *Neisseriagonorrhoeae* was 14.29%. Two Mycoplasma germs are responsible for Mycoplasma infections, and the most frequently encountered species 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. On the basis of the results obtained, we can say that *Syzygium aromaticum* and *Tamarindus indica* extracts are effective on the microorganisms tested, so they can be used as alternative molecules for the treatment of gonorrhoeae and Mycoplasma infections.

Ethical Approval and consent

The several hospitals in the town of Ngaoundéré, the regional health delegate, and the dean of the University of Ngaoundéré's faculty of science had all granted permission for the research. For the proper conduct of our study, we got written informed consent from the participants.

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