Original Research Article

Prevalence of *mycoplasma* and its *neisseria* in asymptomatic women and study of their sensitivity to the aqueous extracts of *tamarindus indica* and *syzygium aromaticum*

Abstract

The female urogenital tract is generally colonized by several bacteria that may be responsible for gynecological infections leading to infertility, including Neisseria and Mycoplasma. In this context, the present work aims to prevent infertility by determining the prevalence of Neisseria and Mycoplasma in asymptomatic women and evaluating the antimicrobial activity of Tamarindus indica and Syzygium aromaticum extracts on these germs. For this purpose, an exhaustive consecutive sample of patients with certain defined characteristics was recruited. Cervico-vaginal and urinary samples were collected using appropriate methods. Neisseria and Mycoplasma strains were isolated on specific media and identified on the basis of their morphological and biochemical characteristics. Plant extracts were obtained by maceration with aqueous, hydroalcoholic and alcoholic solvents, and their antibacterial activity was determined by the agar-well diffusion method. The results show that the prevalence of infection in asymptomatic women was 70% overall, and separately 14.29% for Neisseria and 85.71% for Mycoplasma. Aqueous extracts of Syzygium aromaticum gave inhibition diameters of 43 mm, 40 mm and 32 mm at concentrations of 0.2 mg/mL, 0.1 mg/mL and 0.05 mg/mL respectively. The aqueous extract of Tamarindus indica at different concentrations (0.2 mg/mL, 0.1mg/mL, and 0.05 mg/mL) obtained inhibition diameters of 16 mm, 14 mm and 13 mm respectively. The hydroalcoholic extract resulting from the combination of the two plants obtained for these same concentrations the respective inhibition diameters of 30mm, 25mm and 23mm. As a result, these extracts could help remedy the problem of resistance frequently encountered, and thus combat the infertility caused by these germs.

Key words: Asymptomatic woman, Neisseria, Mycoplasma, Extract, Antimicrobial activity, Infertility

INTRODUCTION

Urogenital infections are infections affecting the urinary and reproductive systems of both men and women. They are caused by microorganisms normally present in the genital tract, or introduced during sexual contact or medical procedures [1]. These urogenital infections are the most common transmissible diseases worldwide, and the number of patients being treated continues to rise every year, as do gonorrhea and mycoplasma infections [2]. The number of new cases of sexually transmitted infections worldwide is estimated at 250 million. Gonorrhea prevalence in international literature ranges from 0% to 1.4% in asymptomatic women [3]. In France, it is estimated that 50% of women are carriers of Ureaplasma urealyticum and 10% of Mycoplasma hominis [4]. With around 63 million cases, Africa is one of the regions with the highest rates [5]. In Cameroon, a case-control study carried out among women with secondary infertility (cases) or an ongoing pregnancy (controls) gave a prevalence of 1.3% versus 2.9% for Neisseria gonorrhoeae [6]. Furthermore, in 2018, the study carried out by Alexandre et al. at the Garoua pasteur annex center showed that the rate of Mycoplasma infection was 61.1% among women. Complications due to sexually transmitted Mycoplasma and gonorrhoeae infections have serious repercussions on sexual and reproductive health, generally leading to infertility [7]. Infertility is defined as the difficulty for a heterosexual couple to conceive a child. Infertility is thus a condition that exists independently of the couple's attempts to achieve pregnancy [8].

Infertility affects 80 million people worldwide [9]. In developing countries such as Cameroon, primary infertility accounts for around 40% of infertility, while secondary infertility is responsible for 60% of total infertility [10]. Despite the many advances made, infertility is still not entirely under control, and the administration of synthetic molecules is often accompanied by adverse effects of varying severity [11].

Sexually transmitted infections (S.T.I.) caused by gonorrhea and mycoplasma have been curable for over 40 years. Despite this, S.T.I.s still pose a public health problem in industrialized and, above all, developing countries, and the antibiotic molecules used are for the most part responsible for the repeated resistance encountered in the germs [12]. Today, people are increasingly turning to natural remedies to treat these infections [13]. Studies carried out by [14] used the dilution method to assess the antibacterial activity of aqueous (EAQ) and methanolic (EME) extracts of the plants Typha angustifolia L and Zingiber officinale Roscoe at different concentrations (0.0031 and 0.05 mg/ml) on Neisseria strains. Thus, despite the development of synthetic drugs, plant medicines in their various forms continue to occupy a place of choice [15]. Among these, Tamarindus indica and Syzygium aromaticum are plants used in traditional Indian medicine for their medicinal properties. In Cameroon, mainly in the Adamaoua region, women are increasingly confronted with problems of conception, and those who do seek medical advice do so too late, when the germs have already acquired a certain resistance and engendered an upper tract genital infection (tubal infertility).

In this context, the aim of the present work is to assess the prevalence of Neisseria and mycoplasma in asymptomatic women in the town of Ngaoundéré, and to highlight the antimicrobial effect of Tamarindus indica and Syzygium aromaticum extracts on the germs isolated.

MATERIALS AND METHODS

Biological material

The strains used in our work are those isolated from biological samples (ECBU and PCV) of 60 asymptomatic women received at the Hôpital Protestant et Régional de Ngaoundéré. *T. indica* and *S. aromaticum* were purchased at the Ngaoundéré city market in November 2022, and transported directly to the Sunshine Microbiology Laboratory.

Methods

- 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].

- 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].

Specimen analysis

- **Cytobacteriological examination of urine (ECBU)**

- Quantitative cytological examination

Enumeration is performed by depositing a precise volume (2 to 5 µl) of urine between a slide and a coverslip, then examining the whole under a fresh microscope with an x40 objective. The number of elements present is reported per ml.

- Qualitative cytological examination

Gram staining is 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).

- 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.

Cervico-vaginal sampling (CVS)

- 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].

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% CO_2 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.

- 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.

Identification and Antibiogram

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

- Gram staining (Gram control)

After spreading the sample on a glass slide, the slide was allowed to air dry, then stained successively with gentian violet for one minute, followed by lugol. Each dye was rinsed thoroughly with clean water before applying the next. The stain was removed with 96% alcohol and the slides rinsed with clean water. Cover the slide with diluted fuchsin (1 ml fuchsin to 9 ml water) for one minute and rinse with clean water. Finally, air-dry and observe the slide under a microscope using oil immersion at 100x objective [19].

- Catalase test

The test was carried out according to the method described by (Reiner, 2013). Using a sterile inoculation loop, a well-isolated colony from a pure culture (18 to 24 hours incubation) was picked and placed on the microscope slide. A drop of 3% hydrogen peroxide was applied to the colony using a Pasteur pipette, and the Petri dish was immediately covered with a lid [19].

- Oxidase test

The test was carried out using the method described by (Reiner, 2013). Using forceps, an oxidase disk was placed on an object slide, then a well-isolated colony representative of the fresh culture to be tested was picked up using a stick or öse. The colony was gently rubbed on the disc until a violet coloration appeared within 30 seconds [19].

Antibiotic susceptibility testing was carried out for mycoplasma directly using the Freeze-Dried test kit, demonstrating sensitivity to 12 antibiotics, namely: Doxycycline, Minocycline, Ofloxacin, Sparfloxacin, Roxithromycin, Azithromycin, Clarythromycin, Josamycin, Spectinomycin, Levofloxacin, Gatifloxacin.

For gonorrhoeae, the antibiogram was performed according to the method described by (Otto et al., 2014). A bacterial suspension was prepared from isolated young colonies, then CHOC media were inoculated by flooding. After drying the agar plates, the antibiotic discs were placed on the plates using sterile forceps, and the whole set was incubated at 37°C with 10% CO2 for 24 hours [20].

Preparation of plant extracts at different concentrations

 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 of extraction solvent in a sterile container (12.5% water / 12.5% ethanol; water 100%; and ethanol 100%). 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. Each Tamarindus and Syzygium extract obtained was dissolved in a 100 mg/ml aqueous, ethanolic and hydro-ethanolic stock solution respectively. Different concentrations were then obtained from this stock solution.

Evaluation of the antimicrobial activity of extracts

The well method was chosen to determine the antimicrobial activity of extracts. The principle of the method is based on the diffusion of the antimicrobial compound in a solid medium in a Petri dish, with the creation of a concentration gradient, after a certain contact time between the product and the target microorganism. The effect of the antimicrobial product on the target is assessed by measuring a zone of inhibition. Depending on the inhibition diameter, the product tested can be classified as sensitive, very sensitive, extremely sensitive or resistant [21].

- **Preparation of culture medium and bacterial inoculum**

Chocolate agar and Mueller Hinton were used to isolate and study the susceptibility of Neisseria and Mycoplasma to plant extracts.

After thawing, the preserved microbial strains were left at room temperature. Using a sterile platinum loop, a few well-isolated, identical colonies were picked from young pre-cultures of each Neisseria gonorrhoeae strain. The loop was then discharged into sterile physiological water to prepare an inoculum with a concentration equivalent to 0.5 Mc Farland [21].

For Mycoplasma, 50 µl were taken from the liquid culture medium using a micropipette and sterile tip, after a positive result of the medium marked by a pink coloration, and then introduced into physiological water.

- Inoculation

Petri dishes containing chocolate or MH medium were aseptically inoculated with the various germs using a sterile swab over the entire surface of the medium, previously soaked in the microbial suspension, and discharged as far as possible, over the entire agar surface in tight streaks [21]. After the plates had dried, wells were cut out using a sterile punch. The bottoms of the cavities thus formed (10mm) were sealed with agar and then filled with extracts (approx. 200 µL per well). The plates were closed and left at room temperature for 15 minutes for pre-diffusion [21], then incubated for 24/48 hours at 37°C with 10% CO_2. Results can be read by measuring the diameter, in mm, of the zone of inhibition for all petri dishes.

- **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 poured and inoculated with a bacterial suspension. The diameter of the inhibition zones around the discs was determined after 24 h incubation at 37°C with 10% CO_2.

Ethical considerations

Research authorization had been obtained from the Dean of the Faculty of Science at the University of Ngaoundéré, the Regional Health Delegate and the various hospitals in the town of Ngaoundéré. Written informed consent was obtained from the participants for the proper conduct of our study.

Statistical analysis

Data were analyzed using Sphinx and EXCEL 2016 software.

RESULTS

- Etiology of urogenital infections in the study population

The overall prevalence of gonorrhoeae and Mycoplasma infections in asymptomatic women is recorded in figure 1. Of the 60 women received, 42 were infected with Neisseria and Mycoplasma, i.e. a prevalence of 70%.

Figure 2 shows the prevalence of Neisseria and Mycoplasma in the target population. Of the 42 women with infections, 6 were infected with gonorrhoeae, i.e. a prevalence rate of 14.29%, and 36 with Mycoplasma, i.e. a prevalence rate of 85.71%.

As far as Mycoplasma is concerned, several species may be responsible, and two of these were investigated. The prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum* is shown in figure 3, which shows that Mycoplasma hominis was the most common species, with a prevalence rate of 22.22%. However, *Mycoplasma hominis* and *Ureaplasma urealyticum* coinfection had a much higher prevalence rate of 58.33%.

- **Antibiotic susceptibility of Neisseria and Mycoplasma**

For Neisseria, several antibiotics were tested on the bacterial strain, and the results are shown in Table 1. This table shows that the isolated germ is resistant to five antibiotics, namely : Ciprofloxacin, Amoxyclav, Azithromycin, Tobramycin, Erythromycin.

In the case of Mycoplasma, Table 1 shows that only two antibiotics were found to be sensitive, namely Josamycin and Minocycline.

 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. All 3 extracts showed activity on *Neisseria gonorrhoeae* isolates, with activity varying according to concentration. The greatest activity was observed with the aqueous extract of *Syzygium aromaticum* (SA), with inhibition diameters of 43 mm, 40 mm and 32 mm respectively for concentrations C1, C2 and C3, followed by 1/1 mixing of the two extracts. The smallest with *Tamarindus indica* aqueous extract. With regard to the activity of extracts on mycoplasma, the same table shows that only the aqueous extract Syzygium aromaticum (SA) at different concentrations has an inhibitory effect on these isolates.

 Effect of Syzygium aromaticum extract combination on antibiotic discs

Table 3 shows the effect of combining Syzygium aromaticum extract with the antibiotics (Fosfomycin, Ceftriaxone, Ofloxacin, Levofloxacin and Chloramphenicol) to which Neisseria was susceptible. At concentrations C1 (0.2 mg/mL) and C2 (0.1 mg/mL), the aqueous extract of Syzygium aromaticum increased the diameter of the zone of inhibition of the various antibiotics.

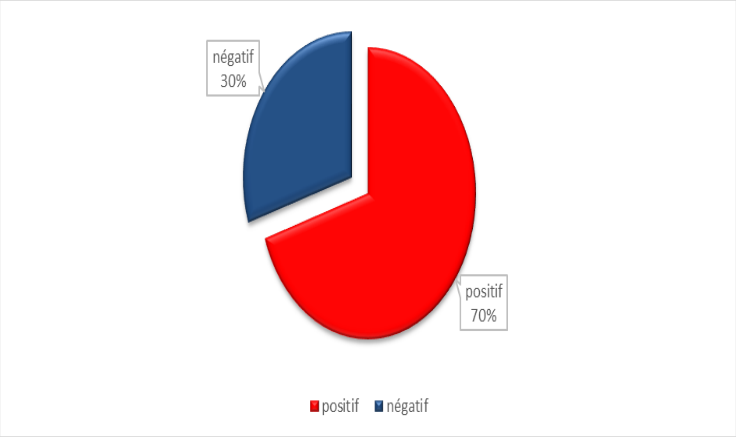

Figure 1 : prevalence of gonorrhoeae and Mycoplasma infections in the study population

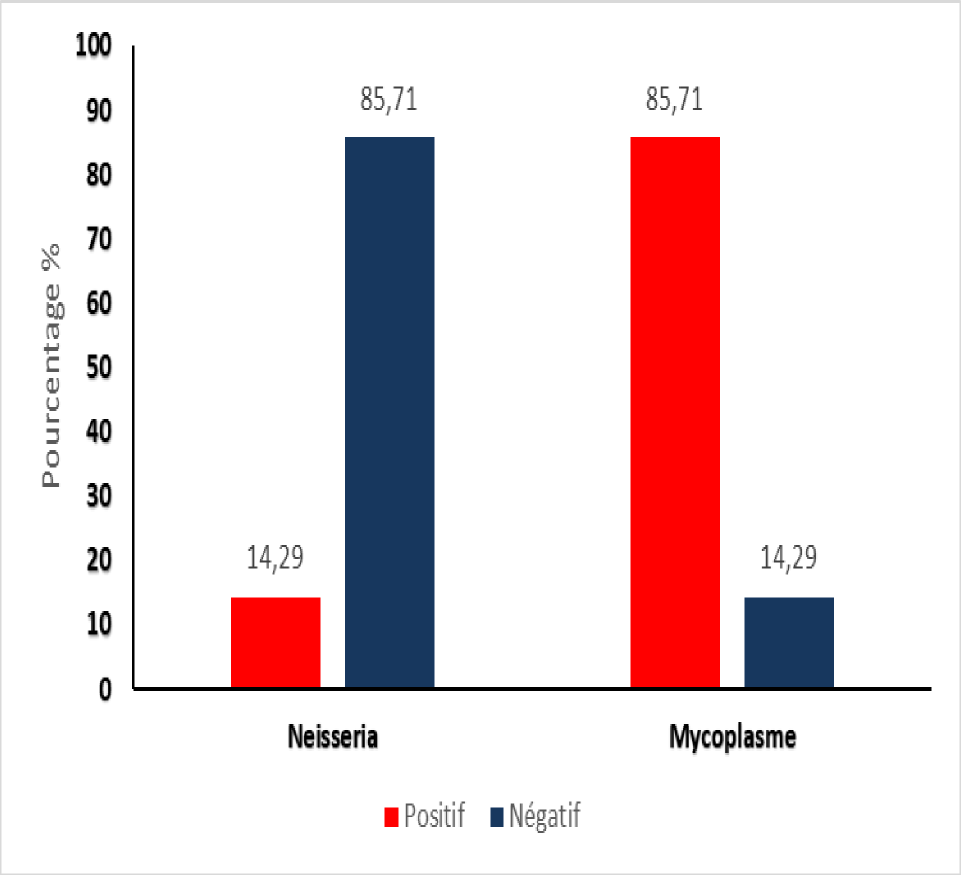

Figure 2 : prevalence of gonorrhoeae and Mycoplasma in the target population

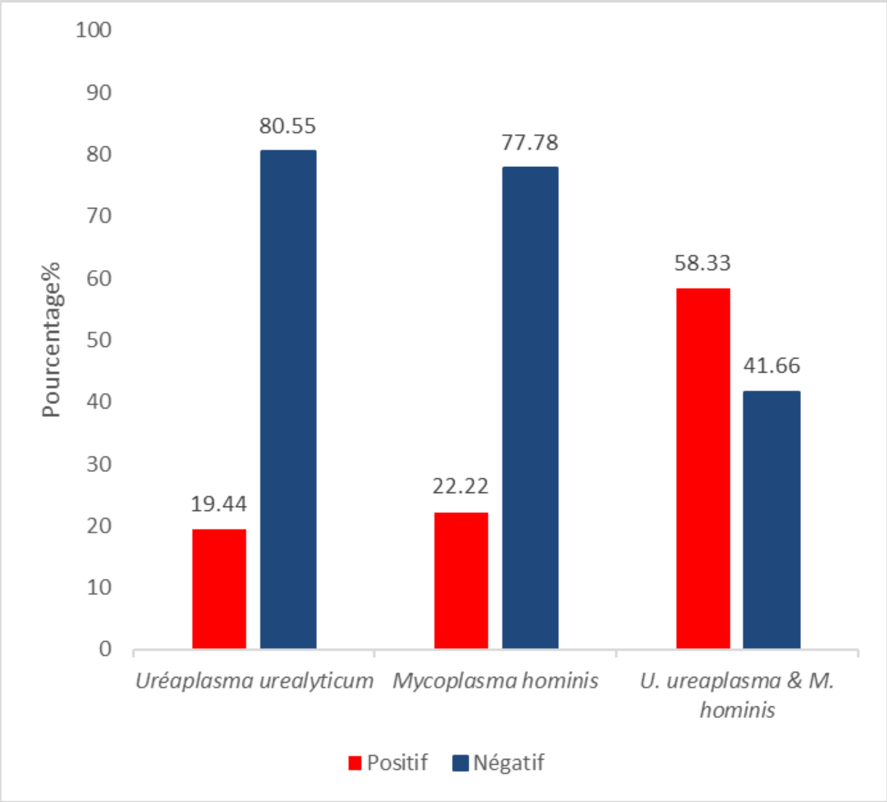

Figure 3 : Prevalence of Mycoplasma hominis and Ureaplasma urealyticum

Table 1 : Antibiogram result

	ATB	Diamètre d'inhibition (mm)
Neisseria	Fosfomycine	18
	Chloramphénicol	23
	Ofloxacine	18
	Ciprofloxacine	-
	Amoxyclav	-
	Lévofloxacine	26
	Ceftriaxone	22
	Azithromycine	-
	Tobramycine	-
	Erythromycine	-
Mycoplasme	Josamycine	a
	Minocycline	a
	Doxycycline	pa

	Ciprofloxacine	pa
	Ofloxacine	pa
	Sparfloxacine	pa
	Roxithromycine	pa
	Azithromycine	pa
	Clarithromycine	pa
	Spectinomycine	pa
	Levofloxacine	pa
	Gatifloxacine	pa

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

Table 2 : Inhibition diameters of Neisseria and Mycoplasma microbial strains

	Extraits	Concentrations (mg/mL)	Diamètre d'inhibition (mm)
	ESA aqueux	0,2	43
		0,1	40
		0,05	32
	ETI aqueux	0,2	16
		0,1	14
		0,05	13
Neisseria	EST hydro	0,2	30

	alcoolique	0,1	25
		0,05	23
	Céfixime	0,2	50
		0,1	36
		0,05	27
	ESA aqueux	0,2	16,5
Mycoplasme		0,1	13
		0,05	10,5
	Ofloxacine	0,2	45
		0,1	38
		0,05	30

ESA = extrait de *Syzygium aromaticum* ; ETI = extrait de *Tamarindus indica* ; EST = extrait de *Syzygium et de Tamarindus.*

Table 3 : Effet synergique extrait-ATB

Echantillons	ATB (mm)	Concentrations (mg/mL) C	Diamètre d'inhibition (mm)
ESA		0,2	36
		0,1	30
		0,05	24
Ofloxacine (5 µg)	18	0,2	21

		0,1	20
		0,05	17
Fosfomycine (50 µg)	18	0,2	33
		0,1	28
		0,05	21
Levofloxacine (5µg)	26	0,2	32
		0,1	27
		0,05	19
Ceftriaxone (30 µg)	22	0,2	22
		0,1	23
		0,5	21
Chloramphénicol (30g)	23	0,2	24
		0,1	23
		0,05	23

C1= 0, 2 mg/mL; C2= 0, 1 mg/ mL; C3= 0, 05 mg/mL

DISCUSSION

 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. 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, 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.

- 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.

- 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.

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 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.

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