Antimycoplasmal, proximate composition, and phytochemical evaluation of selected fruit juices

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Abstract:

Background: Sexually transmitted infections (STIs) represent a significant global public health challenge. Among the bacterial pathogens, *Mycoplasma genitalium* and *Mycoplasma hominis* are increasingly recognized for their involvement in STIs. The development of drug resistant strains of *Mycoplasma* have directed for new, efficacious and safer antibiotics. The plant-based herbal therapies have emerged as a promising alternative for treating genital *Mycoplasma* infections.

Methods: *Mycoplasma* spp. were cultured from endocervical/vaginal swab samples, previously collected from women suffering from STD. AST for different antibiotics was performed using the Kirby-Bauer disk diffusion method. Fresh fruit juices of *Emblica officinalis* (Amla), *Vitis vinifera* (Black grapes and green grapes), *Ziziphus jujube* (Ganga ber), *Citrus limon* (Lemon), *Citrus limetta* (Mosambi), *Citrus sinensis* (Orange), *Ananas comosus* (Pineapple), *Punica granatum* (Pomegranate), and *Tamarindus indica* (Tamarind) were evaluated for their efficacy against MDR *Mycoplasma* spp. The MIC, biofilm inhibition activity as well as phytochemical & nutritional analysis of fresh juices were also performed.

Results: Out of the 20 endocervical/vaginal swab samples, 6 were found culture-positive for presence of *Mycoplasma* spp. Among the tested fruit juices, pomegranate exhibited the highest activity with an inhibition zone and MIC (24 mm: 10.41 µg/mL respectively), followed by lemon (18 mm; 20.8 µg/mL) and tamarind (16 mm; 83.3 µg/mL), while the remaining juices showed minimal or no activity. Phytochemical screening confirmed the presence of tannins, polyphenols, and flavonoids in all juices, with tamarind having the lowest pH and amla the highest ascorbic acid content. Biofilm inhibitory activity was found to be concentration-dependent, with lemon showing the greatest effect (72%), followed by tamarind (48%) and pomegranate (34%), compared to ofloxacin, which demonstrated 62% inhibition at ½ MIC.

Conclusion: Fruit juices of lemon, pomegranate, and tamarind demonstrated significant antibacterial and antibiofilm activities against MDR *Mycoplasma* spp. These findings highlight their potential as natural therapeutic alternatives for managing drug-resistant genital *Mycoplasma* infections.

Keywords: *Mycoplasma* spp., MDR, fruit juices, MIC, antibacterial activity, biofilm Inhibition, phytochemicals

1. Introduction

Sexually Transmitted Infections (STIs) occur worldwide and are an important public health concern that needs to be addressed. STIs in developing countries are among the five most significant causes for seeking public health services [1]. According to the WHO, an estimated 448 million new cases of sexually transmitted infections are acquired worldwide annually [2]. A variety of pathogens, including bacteria, viruses, and fungi, cause STIs. Bacterial pathogens, such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and various species of *Mycoplasma* and *Ureaplasma*, are particularly significant among these due to their prevalence and potential to cause long-term reproductive health complications [3]. In recent studies, the bacterial agents such as *Mycoplasma genitalium* and *Mycoplasma hominis* are gaining recognition for their roles in urogenital tract infections. *M. genitalium*, is the causative agent of non-gonococcal urethritis (NGU), cervicitis, pelvic inflammatory disease (PID), and adverse pregnancy outcomes [4, 5, 6]. In contrast, *M. hominis* is frequently detected in the urogenital tract of sexually active individuals and has been implicated in various reproductive tract disorders, including bacterial vaginosis, postpartum endometritis, infertility, and, in rare cases, systemic infections such as neonatal sepsis and extragenital abscesses [7, 8]. Globally, the direct mortalities linked to antimicrobial resistance (AMR) exceeded 1.2 million in 2019, with a projected escalation to approximately 10 million deaths annually by 2050, if inadequate measures are not implemented to combat AMR [9,10]. Mycoplasmas are intrinsically resistant to antimicrobials that attack the cell wall, such as fosfomycin, glycopeptides, or β-lactam antibiotics, as well as sulphonamides, first-generation quinolones, trimethoprim, polymixins, and rifampicin. Recent studies highlight growing concerns over antibiotic resistance in *Mycoplasma genitalium* and *Mycoplasma hominis*. A global meta-analysis of *M. genitalium* infections revealed macrolide resistance prevalence had risen to 33.3%, with fluoroquinolone resistance at 13.3%, and dual resistance (both macrolide and fluoroquinolone) present in 6.5% of cases.

Recent studies have shown that various plant extracts/ purified phytochemicals possess promising activity against genital *Mycoplasma* infections. It has been reported that extracts from turmeric, garlic, ginger, cloves, and bitter kola exhibited significant antibacterial effects against genital *Mycoplasma* spp. [11]. Therefore, the present study assessed the antibacterial and antibiofilm activities of nine fruit juices against multidrug-resistant (MDR) *Mycoplasma* spp.

2. Materials and methods

2.1. Selection and revival of sample

Twenty Endocervical/Vaginal swab samples previously collected women (age 20 to 55), symptomatic for STD and visiting to the OPDs of District Hospital Anuppur, Madhya Pradesh, India were used in present study.

2.2. Ethical Approval

The study received ethical clearance from the Institutional Ethics Committee (Ref. No. IGNTU/IEC/01/2019) and Birsa Munda Government Medical College, Shahdol (Ref. No. IERC/22/06/001). Written informed consent was obtained from all participants.

2.3. Preparation of fruit juices

Fresh, undamaged, and ripened seasonal fruits of lemon (*Citrus limon*), amla (*Phyllanthus emblica*), pineapple (*Ananas Comosus*), mosambi/sweet lime (*Citrus limetta*), orange (*Citrus Sinensis*), black and green grapes (*Vitis vinifera*), and tamarind (*Tamarindus indica*), gangaber (*Ziziphus jujube*) and pomegranate (*Punica Granatum*) were purchased from the local market. The fruits were thoroughly washed with distilled water, disinfected using 70% ethanol, and air-dried until the smell of ethanol disappears. Juice was extracted from fruits, using a mortar and pestle. The juice was filtered through sterile muslin cloth and Whatman No. 1 filter paper to remove pulp and solids, and stored in sterile glass bottles at 4°C until further use.

2.4. Culture of *Mycoplasma* spp.

The revived samples were cultured on A-7 agar selective media with *Mycoplasma* urogenital selective supplement, enriched with vitamin growth supplement, Horse serum, and urea and incubated overnight at 37 °C at 5% CO2.

2.5. Biochemical test

The arginine test was performed to confirm the presence of *Mycoplasma* spp. Specifically, *Mycoplasma hominis* metabolizes arginine, producing a reaction that is indicated by a visible color change in the medium.

2.6. Antimicrobial susceptibility test (AST)

AST of *Mycoplasma* isolates was performed using the Kirby-Bauer disk diffusion method. The antibiotics tested include tetracycline (TE), ceftriaxone (CTR), azithromycin (AZM), ciprofloxacin (CIP), levofloxacin (LE), norfloxacin (NX), erythromycin (E), doxycycline (DO), and ofloxacin (OF). The isolates were classified as sensitive, intermediate, or resistant based on inhibition zone diameters according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Isolates showing resistance to three or more antibiotic classes were designated as multidrug-resistant (MDR) and selected for further studies.

2.7. Antibacterial activity of fruit juices

The antibacterial activity of fruit juices was carried out using the agar well-diffusion method on Mueller-Hinton Agar [12]. Briefly, 100 µL of freshly revived bacterial suspensions adjusted to 0.5 McFarland standard were evenly spread onto MHA plates. Wells were created in MHA plates using a sterile cork borer. Subsequently, 100 µL of each fruit juice was added to the wells, and a standard antibiotic disc was placed on each plate to allow comparative assessment of the antibacterial activity of fruit juices and antibiotic. The plates were sealed and incubated overnight at 37°C under 5% CO_2 . Zones of bacterial growth inhibition were measured using the HiAntibiotic Zone Scale.

2.8. Minimum inhibitory concentration (MIC)

MIC of selected fruit juices and antibiotic was determined using the broth microdilution method. MDR *Mycoplasma* spp. strains were inoculated into 96-well plates containing serial dilutions of the fruit juices, ranging from 333.3 to 0.19 µg/mL. The MIC was defined as the lowest concentration that visibly inhibited bacterial growth.

2.9. Biofilm inhibition assay

The effects of sub-MIC concentrations of fruit juices on biofilm formation were evaluated using a modified microtiter plate assay. Briefly, biofilms were allowed to develop in the presence of various sub-MIC levels of fruit juices by incubating the plates at 37°C for 48–72 hours. Following incubation, non-adherent cells were removed by gently aspirating the wells and washing three times with sterile phosphate-buffered saline (PBS). Biofilms were stained by adding 150 µL of 0.1% crystal violet solution to each well and incubating at room temperature for 15 minutes. Excess stain was removed by washing with PBS, and plates were air-dried. The bound dye was then solubilized with 150 µL of 33% (v/v) glacial acetic acid, and the extent of biofilm inhibition was quantified by measuring the optical density at 560 nm, comparing treated samples to untreated controls.

2.10. Phytochemical & nutritional analysis of fruit juices

Phytochemical and nutritional analyses of the fruit juices showing promising antibacterial activity, were carried out. The pH of the selected juices was measured using a Cyber Scan 2100 pH/MV/ION benchtop pH meter. Moisture content was determined by the gravimetric method [13], protein content was estimated using the Kjeldahl method with a nitrogen-to-protein conversion factor of 6.25 [14], and total fat content was measured by Soxhlet extraction [15]. Crude fiber was assessed through acid–alkali digestion [16], while carbohydrate content was quantified using the Anthrone method [17]. Ascorbic acid levels were determined by the 2,6-dichlorophenolindophenol (DCPIP) titrimetric method [18]. Phytochemical screening was conducted using standard assays: Mayer's test for alkaloids, alkaline reagent test for flavonoids, ferric chloride test for tannins, froth test for saponins, Keller–Kiliani test for glycosides, and Folin–Ciocalteu assay for total polyphenols (expressed as gallic acid equivalents, GAE) following established protocols [19].

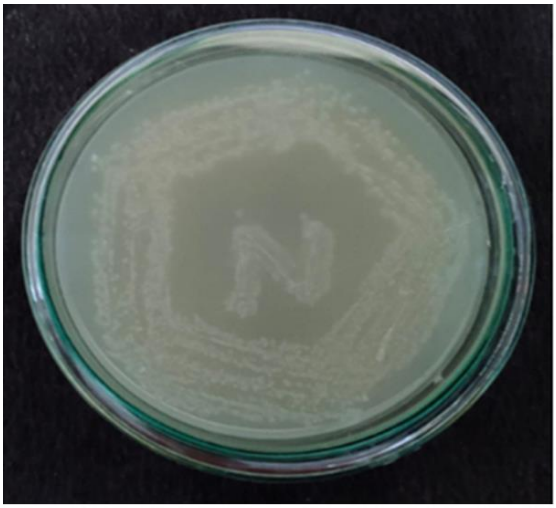
2.11. Statistical Analysis

The mean, standard deviation, and p-values with 95% confidence intervals (CIs) were calculated for MIC and biofilm inhibition data using SPSS version 20. One-way ANOVA was employed to compare MIC values across the groups treated with selected fruit juices, while biofilm inhibition percentages were analyzed using two-way ANOVA.

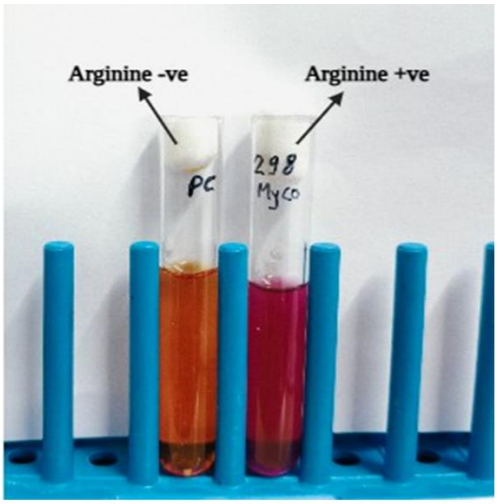
3. Results and Discussion

3.1. Identification of *Mycoplasma* spp.

Out of the 20 swab samples collected, 6 (30%) were culture-positive for genital *Mycoplasma* spp., while the remaining 14 (70%) were culture-negative. All six culture-positive isolates tested positive for arginine utilization (Fig. 1). The presence of *Mycoplasma* spp. in these clinical samples highlights the ongoing issue of genital mycoplasmosis, which often remains underdiagnosed due to fastidious growth requirements. The arginine-positive nature of all isolates indicates their metabolic profile and supports identification.



Off-white colony of *Mycoplasma* spp. on A7 agar



Arginine test shows colour change from yellow to dark pink

Fig. 1: Growth of *Mycoplasma* spp. and confirmatory arginine test

3.2. AST profile of *Mycoplasma* spp. isolates

All six culture-positive *Mycoplasma* spp. isolates exhibited resistance to nine different antibiotics, indicating a multidrug-resistant (MDR) profile (Fig. 2). This result aligns with the global trend of rising antimicrobial resistance among *Mycoplasma* spp., which complicates conventional therapeutic approaches and necessitates the exploration of novel antimicrobial strategies [20,21,22]. In this context, fruit-derived phytochemicals have gained attention due to their wide availability, safety, and broad-spectrum bioactivity. The present study evaluated the antibacterial and antibiofilm activities of six fruit juices against MDR *Mycoplasma* spp., with results indicating promising alternative or adjunctive therapeutic potential.

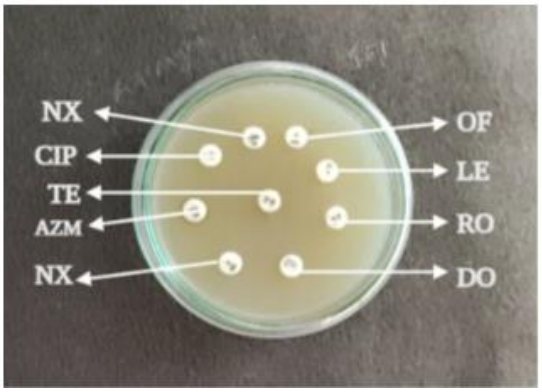


Fig. 2: AST against *Mycoplasma* spp. with all 9 antibiotics resistant

3.3. Antibacterial activity, MIC, phytochemical and nutritional analysis of fruit juices

The selected fruit juices demonstrated varied activity against against six MDR *Mycoplasma* spp. isolates. Pomegranate showed the highest activity with an inhibition zone of 24 mm, followed by lemon (18 mm) and tamarind (16mm). Moderate inhibition was recorded for green grapes (15 mm), black grapes (13mm), and amla (12mm). In contrast, the remaining fruits and the standard antibiotic ofloxacin exhibited no measurable antibacterial effect against MDR *Mycoplasma* spp. (Table 2 & Fig. 3).

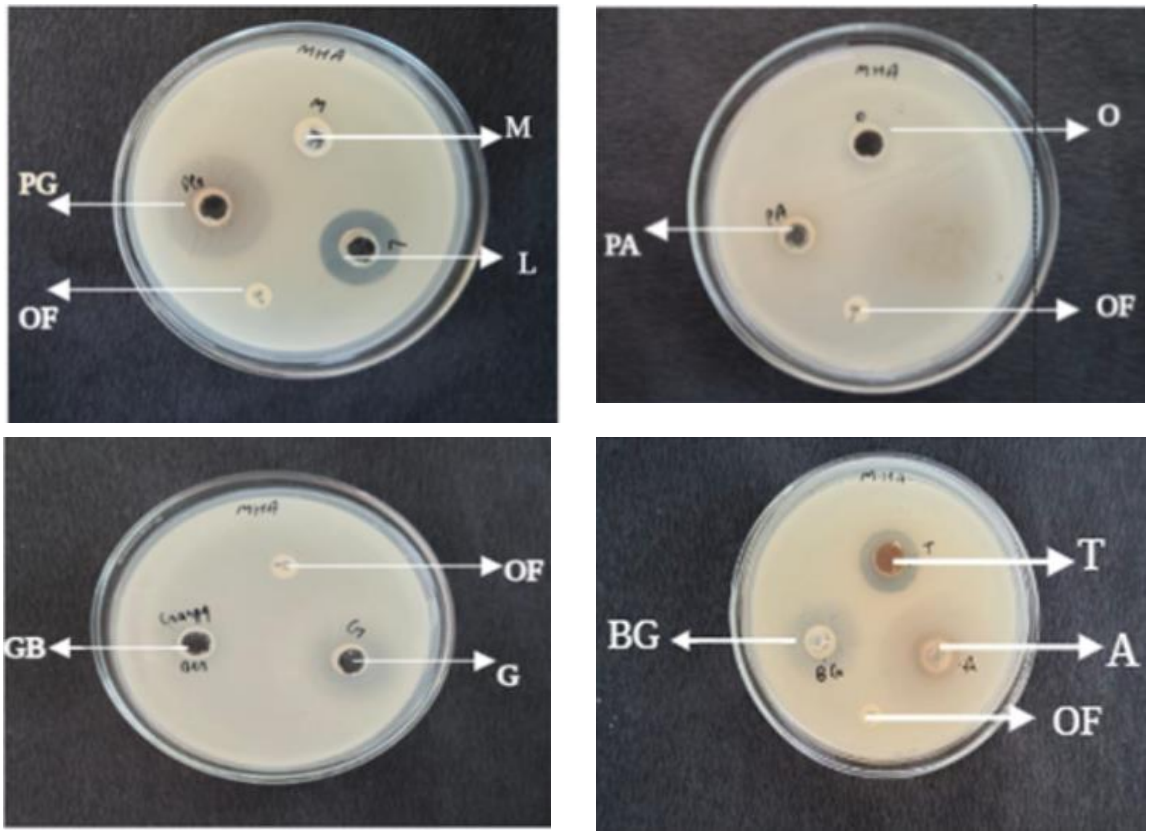


Fig. 3: Antibacterial activity of fruit juices
PG-pomegranate, L- lemon, M-mosambi, PA-pineapple, O- orange, GB-ganga ber, G-green grapes, T-tamarind, A-amla, BG-black grapes, OF- ofloxacin

Table 2: Antibacterial activity and MIC of fruit juices

Antibacterial Compounds	**Zone of Inhibition diameter (mm+ SEM)**	**MIC (µg/ml ± SEM)**
Pomegranate (PG)	24±2.1	**10.41 ± 0.2**
Mosambi (M)	NZ	-
Lemon (L)	18 ±0.8	**20.8 ± 0.6**
Pineapple (PA)	NZ	-
Orange (O)	NZ	-
Ganga ber (GB)	NZ	-
Green grapes (GG)	15 ±0.7	**41.6 ± 0.6**
Amla (A)	12±0.5	166.6 ± 0.4
Tamarind	16±0.5	83.3 ± 0.6
Black Grapes (BG)	13± 0.6	83.3 ± 0.6
Ofloxacin	NZ	-

Pomegranate juice exhibited the lowest MIC value (10.41 µg/ml), indicating the strongest inhibitory activity among all tested fruit juices. This was followed by lemon (20.8 µg/ml), green grapes (41.6 µg/ml), and both black grapes and tamarind (83.3 µg/ml). Amla juice demonstrated the weakest activity with the highest MIC value of 166.6 µg/ml (Table 2 & Fig.4).

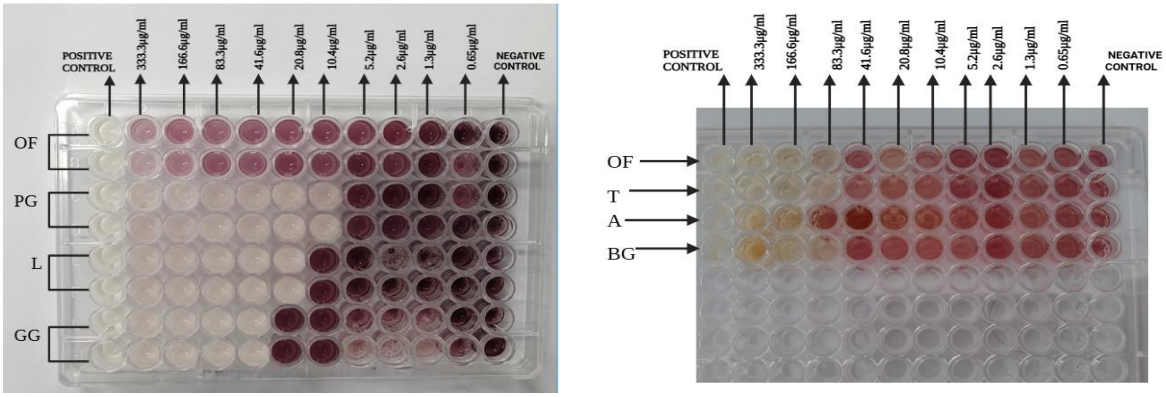


Fig. 4: MIC of fruit juices and antibiotic

Phytochemical analysis showed presence of tannins, flavonoids, and polyphenols in all juices, while saponins and glycosides were absent in amla, and alkaloids were lacking in tamarind (Table 3). Proximate analysis showed highest carbohydrate (69.8g), protein (3.2 g) fiber (5.3g) in 100 g tamarind juice, highest ascorbic acid (307mg/100g) in India gooseberry and fat (0.4g/100g) and moisture (92.2%) in lemon juice. All fruit juices were acidic in nature. Tamrind juice has lowest (2.1) and black grapes (3.6) have highest pH (Table 4).

Table 3: Phytochemical analysis of fruit juices

Phytochemical	Lemon	Pomegranate	Green Grapes	Black Grapes	Tamarind	Amla
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	-
Glycosides	+	+	+	+	+	-
Flavonoids	+	+	+	+	+	+
Alkaloids	+	+	+	+	-	+
Polyphenols	+	+	+	+	+	+

Table 4: Nutritional analysis of fruit juices

Nutritional parameters	Lemon	Pomegranate	Green Grapes	Black Grapes	Tamarind	Amla
Moisture (%)	92.2	85.3	81.7	85.3	24	86.02
Protein (g)	0.5	0.95	0.78	0.98	3.2	0.4
Fat (g)	0.4	0.5	0.23	0.29	0.13	0.17
Carbohydrates (g)	6.3	11.02	15.3	17.9	69.8	3.7
Crude Fiber (g)	0.2	3.2	1.15	1.14	5.3	3.7
Ascorbic Acid (mg)	50.85	9.96	20.40	24.20	3.3	307
pH	2.2	3.2	3.2	3.6	2.1	2.7

Among the tested fruits, pomegranate and lemon demonstrated the strongest antibacterial effects, as evidenced by their large inhibition zones (24 mm and 18 mm, respectively) and low MICs (10.41 µg/mL and 20.8 µg/mL). These findings support earlier reports describing the potent antimicrobial activity of pomegranate polyphenols, particularly punicalagins and ellagitannins [23,24], and the inhibitory role of citric and ascorbic acid in lemon [25]. Tamarind also showed considerable efficacy (zone of inhibition: 16 mm; MIC: 83.3 µg/mL), which is likely mediated by tartaric acid and organic acids, consistent with the antimicrobial effects described by Nwodo et al. [26]. Green and black grapes displayed moderate activity (zones of inhibition: 15 mm and 13 mm; MICs: 41.6 µg/mL and 83.3 µg/mL), aligning with reports on grape-derived polyphenols against *Staphylococcus aureus*

[27,28]. Amla, despite its remarkably high ascorbic acid content, exhibited the weakest effect (MIC: 166.6 µg/mL), suggesting that the absence of key phytochemicals such as saponins may restrict its direct antibacterial potential, though antioxidant properties may still contribute to host protection [29].

3.4. Biofilm inhibition potential of active fruit juices

The antibacterial fruit juices inhibited biofilm formation in a concentration dependent manner. Lemon juice showed the strongest effect, achieving 71.99% inhibition at 1/2 MIC, which declined to 54.01% at 1/4 MIC and 36.68% at 1/8 MIC. Tamarind juice exhibited moderate activity with 48.25% inhibition at 1/2 MIC, 35.11% at 1/4 MIC, and 22.68% at 1/8 MIC. Pomegranate juice also showed inhibitory potential, with 33.54% at 1/2 MIC, 23.86% at 1/4 MIC, and 14.20% at 1/8 MIC. The reference antibiotic, ofloxacin, displayed 61.9% inhibition at 1/2 MIC, which decreased to 23.86% at 1/4 MIC and 5.32% at 1/8 MIC (Fig. 5).

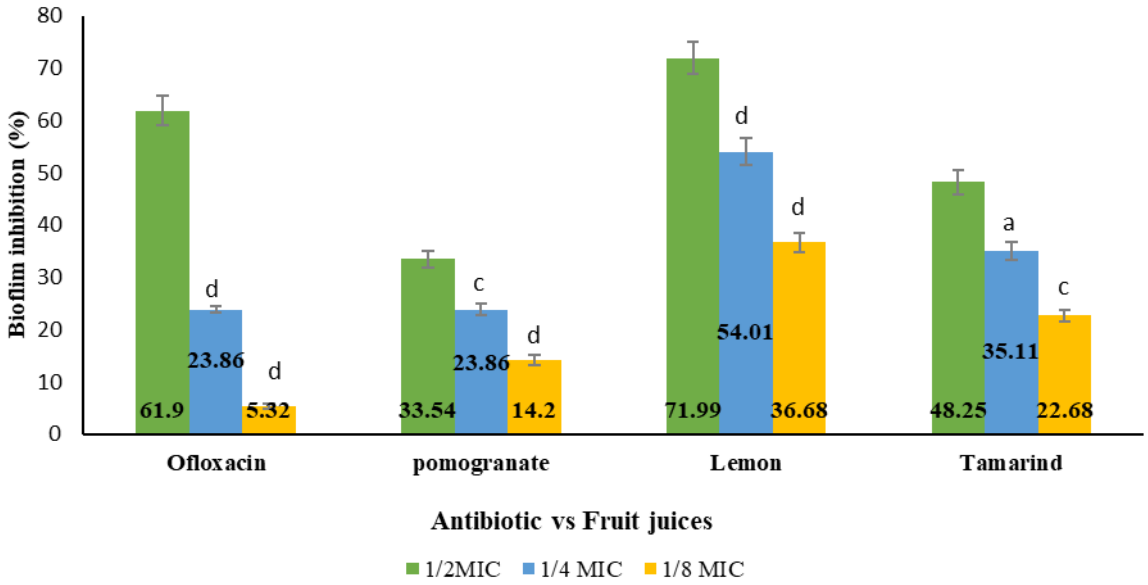


Fig. 5: - Biofilm inhibition activity of active fruit juices and antibiotic
Data are expressed as mean+ SEM, a=p>0.05, c=p<0.01 and d=p<0.001 as compared to ½ MIC

Pomegranate, tamarind, and lemon significantly inhibited biofilm formation, possibly through the actions of punicalagins, tartaric acid, and citric acid. These compounds are known to disrupt microbial adhesion, quorum sensing, and extracellular polymeric substance (EPS) synthesis [30,31,32]. The observed inhibition is consistent with previous studies demonstrating the antibiofilm potential of organic acids and polyphenols [33,34,35].

Mycoplasma spp. evade antibiotic action by producing lipid-associated membrane proteins (LMPs) that adhere to epithelial cell receptors, initiating colonization, immune evasion, and subsequent biofilm formation. Fruit-derived phytoconstituents including alkaloids, flavonoids, tartaric acid, ascorbic acid, citric acid, polyphenols, and tannins interfere with this process by competitively binding to LMPs or blocking their interaction with host receptors. This disruption impedes bacterial attachment and extracellular polymeric substance (EPS) synthesis, ultimately preventing biofilm establishment. Such multitarget activity highlights the potential of natural fruit juices as promising antibiofilm agents against MDR *Mycoplasma* spp (Fig. 6).

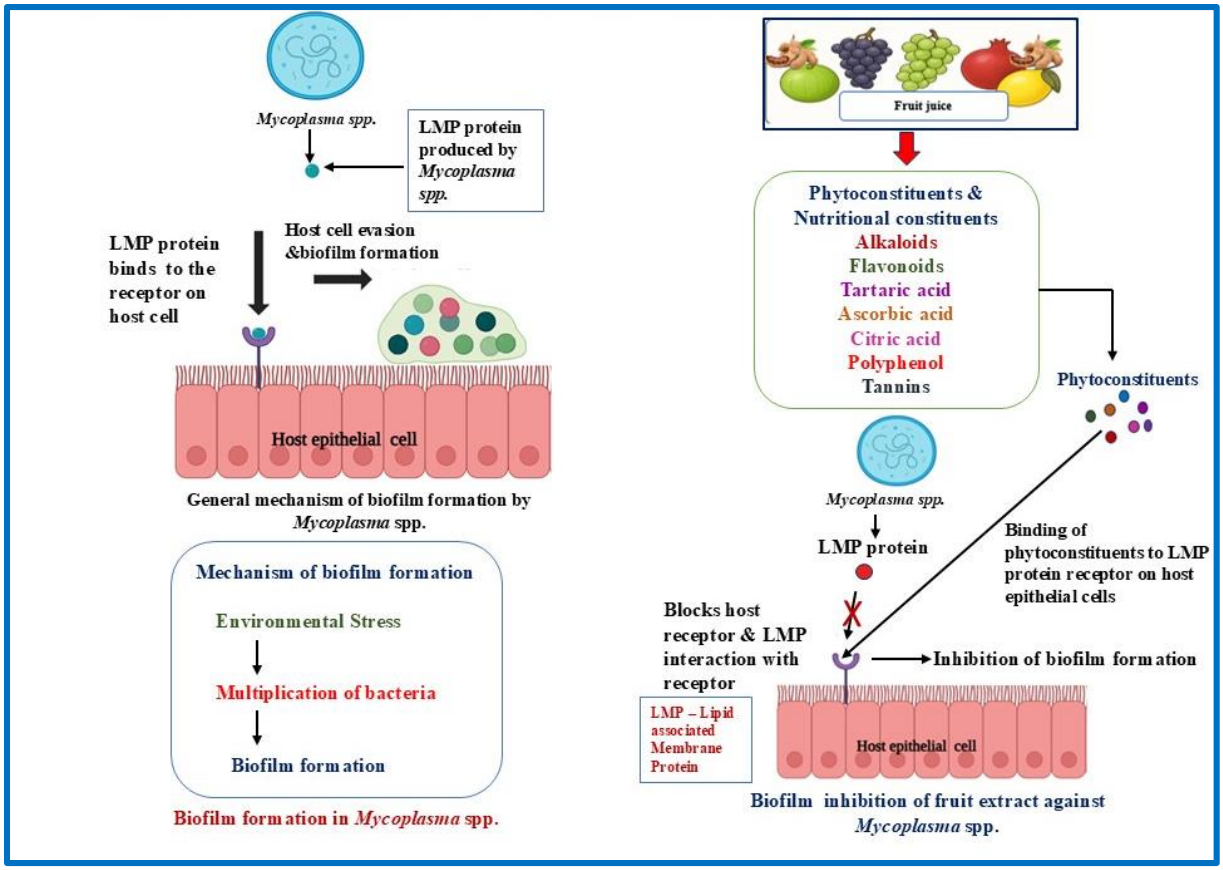


Fig. 6. Possible mechanism of biofilm inhibition activity of fruit juices against *Mycoplasma* spp.

4. Conclusion

This study demonstrates the antibacterial activity of selected fruit juices against MDR *Mycoplasma* spp. The high antibacterial and antibiofilm activity of pomegranate and lemon juices is largely due to presence polyphenols and acidic. These findings suggest that fruit juices represent promising natural, multitarget alternatives to combat antibiotic resistance and support plant-based therapeutic strategies against persistent *Mycoplasma* infections.

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Declaration of conflict of interest

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