

Morphological and biochemical characterization of *Xanthomonas axonopodis* *pv.phaseoli* and Antimicrobial Activity in Vitro of the Essential Oil of *Cinnamomum* *zeylanicum*, *Nigella sativa* and *Syzygium* *aromaticum L.*

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ABSTRACT: The purpose of this research is to propose the alternative solutions based on the use of natural products "Essential oils" to fight the bean's common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* (Xap). This disease, which can cause up to 40% of yield loss, is still considered a major constraint in the production of dry bean in many countries.

To meet this purpose, our work is based on the evaluation of the antibacterial effect in vitro of three plants' essential oils (*C. zeylanicum*, *N. sativa* and *S. aromaticum L.*) on thirteen pathogenic isolates of *Xanthomonas axonopodis* pv. *phaseoli*. The studies were carried out by using the agar diffusion method, for screening the most effective essential oils, and broth dilution method to determine a minimal inhibitory concentration and a minimal bactericidal concentration. The essential oil of *C. zeylanicum* showed a very strong antibacterial activity with a diameter of inhibition zone ranging from 18.00 to 21.00 mm. Its minimal inhibitory concentration was between 0.059-0.12 μ l/ml, while a bactericidal effect was achieved at 0.23 μ l/ml. the comparative tests of the antibacterial activity was performed with antibiotic discs which showed a variable antibacterial activity against the pathogenic isolates.

KEYWORDS: *Xanthomonas axonopodis* pv. *phaseoli*, Antimicrobial Activity, *Cinnamomum zeylanicum*, *Nigella sativa*, *Syzygium aromaticum L.*

I. INTRODUCTION

Dry common bean (*Phaseolus vulgaris L.*) is a widely consumed legume throughout the world and it is recognized as the major source of dietary protein in many Latin-American and African countries [1]. In recent years, bean's common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* (Xap) has caused serious yield losses in several countries. CBB is considered mainly a foliar disease in which symptoms initially appear as small water-soaked spots that then enlarge and become necrotic and usually bordered by a chlorotic zone. CBB seems to be more widespread and causes relatively more yield loss [2]. This disease can cause up to 40% of yield loss [3] and is still considered a major constraint to dry bean production in many countries and in particular Argentina, Brazil, Columbia, Mexico, Uganda, Zambia, Zimbabwe, South Africa, United States and recently Iran [4,5,6,7,8,9].

Several disease management strategies [10] can be used to control common bacterial blight of bean : planting disease-free seed, disease escape by suitable choice of planting date, crop rotation (an 85% reduction of attack was obtained by alternating between bean and maize crops in Romania), sprays and dusts (e.g. with copper compounds or streptomycin), and resistant cultivars [11]. But none is fully effective under the conditions which are highly favorable

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for the disease. For an effective control, the combined use of resistant cultivars and the cultural management is essential. The level of resistance needed in a region depends on the frequency of the favorable conditions for the disease and the effectiveness of other management strategies. The pathogenic variation among the local *X. campestris* pv. *phaseoli* strains, if present, also would determine the level and source of CBB resistance needed [12].

Our work aims at the contribution to the development of new means of biological control based on the use of natural products made with essential oils, which are safe for the environment and for humans, against these pathogenic bacteria for plants. To achieve this objective, we studied the antibacterial effect of the essential oil; of *C.zeylanicum*, *N.sativa* and *S.aromaticum* L.; on isolates of *Xanthomonas axonopodis* pv. *phaseoli*.

II. MATERIAL AND METHODS

A. ESSENTIAL OILS

1.1. Selection of plants

We chose 3 plants in our study due to its known biological effects and their availability in the Moroccan market. The parts used in the extraction of essential oils of such plants are the bark of *Cinnamomum zeylanicum*; the seeds of *Nigella sativa* and the buds of *Syzygium aromaticum* L.

1.2. Essential oil extraction

The used part of each plant was steam distilled in a Clevenger type apparatus [13, 14].

After its extraction, the volume of essential oil obtained in each extraction was measured and stored in glass vials sealed with rubber covers, covered with aluminum foil to protect the contents from light and kept refrigerated at least in 8°C until its use [15].

B. PATHOGENIC STRAINS

1.3. Samples collection

A total of 17 diseased plant samples were collected from different fields located in the area of the study, "Gharb's region". The minimum distance among the fields was approximately 1 kilometer. The leaves of bean were collected in sterile packets. At each time of collection, the precaution was taken to minimize the cross-contamination of samples. The latter were transferred to the Applied Microbiology Laboratory, Faculty of Sciences, Kenitra, for the realization of the isolation and the identification of pathogenic bacteria.

1.4. The Isolation of *Xanthomonas axonopodis* pv. *phaseoli* strains from the Infected Plant Tissue

The plant tissue samples were washed under running tap water to remove surface soil particles, dust and other contaminants, and then were soaked in saline solution (0.85% w/v NaCl) for 30 min to allow the bacterial extraction from the leaf-infected tissue into the solution. 100 µl of the resulting bacterial suspensions per plate were spread on YPGA and incubated from 2 to 6 days at 28°C. The isolated colonies were sub-cultured three times for purification and then transferred to nutrient agar with glucose before performing biochemical and physiological tests.

1.5. Biochemical characterization tests

KOH Test: two drops, approximately 50µl, of a 3% (w/v) solution of potassium hydroxide were placed on a clean glass slide. Bacterial cells were transferred from culture media aseptically with a flat wooden toothpick and placed into the drop of KOH with rapid circular agitation. After 5-8 sec, the toothpick was alternately raised and lowered just off the slide surface to detect to a stringing effect. The KOH test was considered positive if drop viscosity increased and stringing occurred within 15 sec [16].

Gram staining: using bacteriological standard procedures [17].

Presence of catalase: A loopful of bacterial a 24–48 h growth from nutrient agar slope was smeared on a slide and covered by a drop of 20% of hydrogen peroxide [18].

King B medium: The study of fluorescence was performed by seeding the King B medium by the strains isolated, as was described by King et al. in 1954 [19].

Test of Levan production: It shows the ability of the bacterial isolate to produce polysaccharides on rich sucrose medium. Using a steril loop, the levan medium was streaked with the bacteria and incubated at 28°C from 2 to 5 days [20].

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Oxidase test: it was performed by using the disc method [21]. The disks were placed in a clean slide and the selected colonies were transferred onto the disks surface with a Pasteur pipette. The reaction is immediate; the disks become dark purple when the strain is positive oxidase.

Potato rot test: Fresh potato tubers were washed and peeled, alcohol was flamed, and sliced into approximately 7 mm - 1 cm width (1 slice for each isolate and 1 slice for control). The slices were dipped in alcohol and flamed, placed in Petri-dishes, then sterile distilled water was added to a depth of half of the slice. Nicks were formed in the center of the potato with a sterile tool and inoculate with 100 μ l of bacterial suspension. The negative results were indicated by lack of rotting, while the positive results were recorded if most of the slices had been rotted. [22].

The presence of arginine dihydrolase: Thornley's medium was used to detect the formation of alkali from arginine compounds (medium would turn red in four days due to NH₃) under anaerobic conditions. A fresh culture was stabbed into a tube with medium, then covered with sterile paraffine oil and incubated at 27°C [23].

Mannitol motility: With a sterile needle, a well isolated colony was took and picked into the medium till 1 cm of the bottom of the tube, keeping the needle in the same trajectory line in the entry as well as in the output of the agar medium. The incubation was performed at 28°C for 24 h [24].

1.6. Preparation and preservation of pure culture plate

Suspected single colonies of *X. axonopodis* were taken from YPGA plate respectively by sterile loop and inoculated on the NA medium. The plates were then incubated at 28°C for 24 hours after the incubation of the pure culture; the isolates were stored in sterilized 50% of glycerol and were used as stock culture. They were stored at 4°C [25].

1.7. Hypersensitivity reaction

Pathogenicity of the obtained isolates was tested on tobacco plants (*Nicotiana tabacum*) with the method of Klement [26]. Each bacterial isolate was suspended in sterile distilled water and inoculated on the underside of tobacco leaves by injection. Sterile distilled water was used as a negative control. The plants were kept under greenhouse conditions (28°C +/- 1) and the observations for a hypersensitive reaction were conducted after 24 h [24].

1.8. Pathogenicity tests

The tests were performed by immersing the whole trifoliolate leaf of bean at the V3 stage into bacterial suspensions calibrated at 1×10^7 and 1×10^6 CFU/ml. This method allowed the homogeneous and reproducible inoculations and limited inoculum dispersal in the growth chamber. One leaf per plant and three plants per strain were inoculated, incubated at 28°C with 16h of light, 95% of RH / 25°C, 8 h of darkness, 95% of RH. The symptoms were recorded on the 11th day following the inoculation. This method is qualitative, and the results could be positive or negative, depending on the appearance of typical symptoms or not [27].

C. STUDY OF THE ANTIMICROBIAL EFFECT

1.9. Standard disc diffusion method

The screening of antibacterial activity was performed by standard disc diffusion method. Hundred of sterilized discs of filter paper (6 mm diameter) were soaked in 1 ml of each essential oil, separately for 1-2 minutes and then used for screening. The potency of each disc was 10 μ l. Mueller-Hinton agar (MHA) (Merck) was used as base medium and Mueller-Hinton broth (MHB) was used for the preparation of inoculum. Four to five isolated colonies of tested organisms were picked by sterile inoculating loop and inoculated in tubes of MHB (5 ml each). The inoculated tubes were incubated at 28°C for 24 hours and matched with 0.5 McFarland nephelometer turbidity standards. A sterile cotton swab was dipped into the standardized bacterial test suspension to inoculate the entire surface of a MHA plate. The discs of each essential oil were placed on the surface of inoculated plates with the help of sterile forcep. The inoculated plates were incubated at 28°C for 24 hours. After incubation, the inhibition zone diameters' were measured to the nearest millimeter (mm) [28].

The sensitivity towards each essential oil was classified by the diameter of the inhibition zones as follows [29]:

- Not sensitive (-) for total diameter smaller than 8 mm;
- Sensitive (+) for total diameter 9–14 mm;
- Very sensitive (++) for total diameter 15–19 mm;
- Extremely sensitive (+++) for total diameter larger than 20 mm.

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In parallel, the antibiotic disks were tested on various tested strains; it is Amoxicillin + Clavulanic Acid, Cefsulodin, Cefuroxime, Cephalothin, Ciprofloxacin, Clindamycin, Fosfomycin, Imipenem, Oxacillin, Rifampicin and Vancomycin; whose purpose is the comparison of results.

Each assay was performed in duplicates on two separate experimental runs.

1.10. The determination of minimum inhibitory Concentration

600 μ l of the essential oil to be tested are placed in a sterile tube containing 4.4 mL of Mueller-Hinton broth, supplemented with Tween 80 (0.01%, v / v). A cascading dilution ration of 1/2 is carried out in the broth "Mueller-Hinton-Tween 80 (0.01%, v / v)", so as to obtain a concentration range between 120 μ l.ml⁻¹ and 0.0073 μ l.ml⁻¹.

10 μ l of a bacterial inoculum, with an equivalent density standard of 0.5 McFarland (108 UFC.ml⁻¹), were deposited in each tube of the range, which are then placed at 28°C with stirring for 24 hours. A witness to bacterial growth, for which 10 μ l of the standardized inoculum was deposited in the broth "Mueller-Hinton-Tween 80 (0.01%, v / v)," is also performed after the incubation of the tubes are centrifuged at 5000 g for 5 minutes [30]. The minimum inhibitory concentration (MIC) is the lowest concentration of essential oil giving no visible growth in the naked eye [31]. Each experiment was performed three times in three successive experiments.

1.11. The determination of minimum bactericidal concentration

The same concentration range which was carried out by the technical macrodilution in liquid medium, is used to determine the MBC of the essential oil to be tested. The samples are taken in the witness tube and each of tubes was devoid of bacterial pellet visible to the naked eye and then streaked on Mueller-Hinton Agar. The inoculated dishes are incubated for 24 hours at 28°C. The MBC (v/v) essential oils were derived from the first culture plate medium free of bacterium growth [30]. Each experiment was performed three times in three successive experiments.

III. RESULTS AND DISCUSSION

The bacterial strains isolated from bean leaves showing typical symptoms (figure 1.a) were cultured and purified on YPGA plates. The yellow colonies were glistening with an entire margin and a convex elevation (figure 1.b). The shape and the color of these bacterial colonies is compatible with that of *Xanthomonas axonopodis* pv. *phaseoli*. For the rapid determination of the Gram stain of the bacterium a quick test of KOH 3% was adopted (figure 1.c). The confirmation of this test and the determination of the morphology were performed under microscope by Gram staining technique (figure 1.d). The suspect isolates were cultured on nutrient agar (NA) slants and stored in the refrigerator at -4°C. The biochemical characterization of these isolates was performed by the different tests represented in the material and method section.

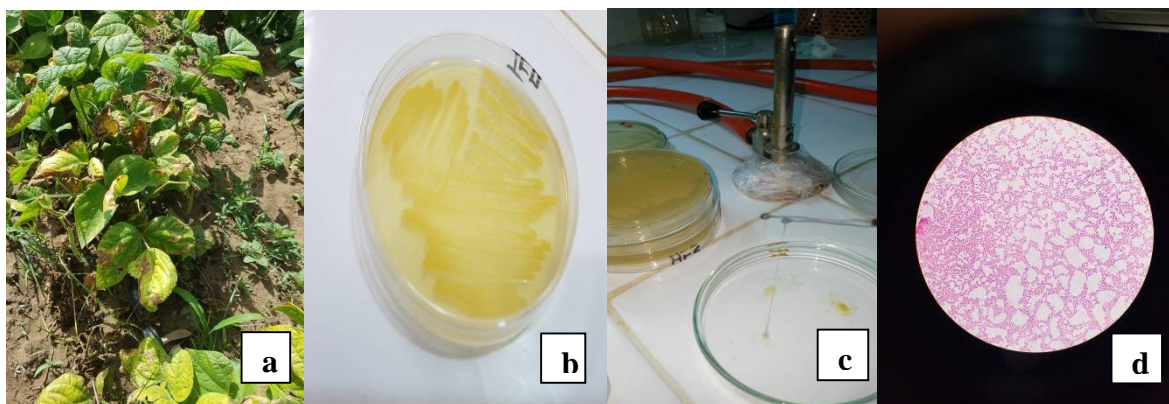


Figure 1: (a) = leaves of bean showing typical symptoms on field; (b) = *Xanthomonas axonopodis* pv. *phaseoli* on YPGA medium; (c) = KOH test; (d) = Gram staining of isolated strains = bacilli Gram-negative.

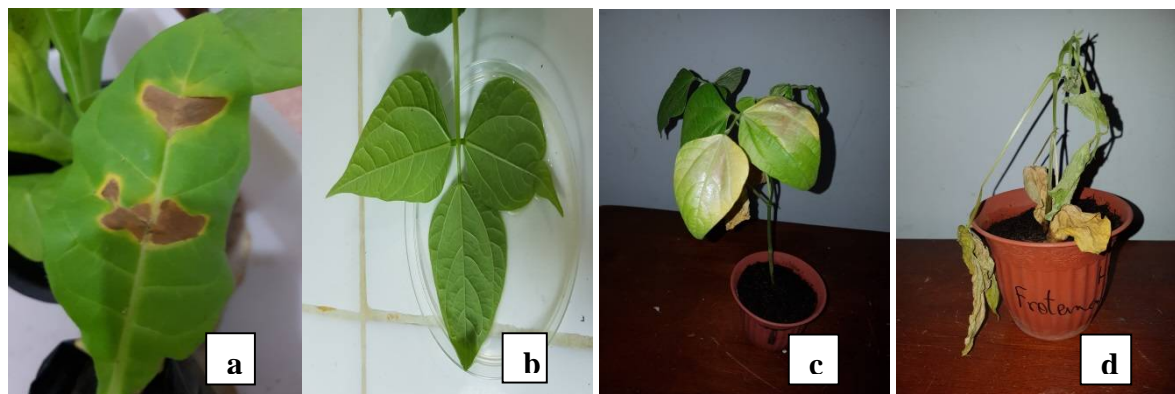


Figure 2: (a) = hypersensitivity reaction of tobacco; (b) = pathogenicity tests by immersing; (c) = spread of small spots and dry leaves; (d) = plant death.

The results of these tests were reported in Table 1. These results showed that the isolated strains correspond to a motile, aerobic, Gram-negative rod, positive levan, negative oxidase, negative pectinolytic activity, and negative arginine dihydrolase. They are consistent with those of *Xanthomonas axonopodis* pv. *phaseoli*, as described by E. Osdaghi et al. [32]. The confirmation of pathogen was also carried out by the reaction of the hypersensitivity of tobacco. This test can only be induced by pathogenic bacteria, which is manifested by tissue collapse and necrosis within 24-48 h [33]. In this study, a total of 13 isolates out of the 17 initially recovered were positive for the hypersensitivity reaction (figure 2.a) as it reported by N. D'Ávila Denardin et al. [34], whereas the 4 remaining isolates were recorded as negative. The positive HR response of 13 bacterial isolates was helpful in screening of these isolates as potential pathogens (Figure 4). The final identification of these 13 strains was carried out by checking the pathogenicity on a susceptible variety of bean. After 9 days of inoculation, it was observed the appearance of a multitude of small spots on the inoculated leaves. Then these spots were spread by causing the dryness and finally the death of plant. This positive result which is reported by J.R. Venette et al. [35], confirms the pathogenicity of these strains against bean (figure 2.b, 2.c and 2.d). All these results were consistent with those of *Xanthomonas axonopodis* pv. *phaseoli*.

Table 2 summarizes the qualitative (diameters of inhibition zones) and quantitative results (minimal inhibitory and bactericidal concentrations) of the antibacterial effect of the essential oil from *Cinnamomum zeylanicum*; *Syzygium aromaticum* L. and *Nigella sativa* on the thirteen assayed pathogenic isolates.

All the essential oils tested in the disc-diffusion method showed an antibacterial activity against the 13 isolates tested. The essential oil of *N. sativa* exhibited the lowest antibacterial activity in the disc-diffusion method with an inhibition zone between 10 to 12 mm of diameters. While the essential oil of *C. zeylanicum* showed a stronger antibacterial activity against these tested 13 isolates and an average antibacterial activity with *S. aromaticum*, their diameters of inhibition were respectively between 18 to 21 mm and 12 to 14 mm (figure 3).

Meanwhile, the results of the sensitivity of these 13 isolates vis-à-vis the eleven tested antibiotics showed an almost total resistance, except for ciprofloxacin, which has shown a medium antibacterial activity with a diameter of inhibition zone of 15mm. Ciprofloxacin showed a lower antibacterial activity than the oil of essential cinnamon, comparable to that of clove essential oil while it is superior to that of nigella. While Tween-80 discs and distilled sterile water, showed a negative antibacterial activity with an area of inhibition 6mm (= disk diameter) (figure 4), the essential oil of *C. zeylanicum* showed a very strong antibacterial activity with MIC at 0.059-0.12µl/ml, and a bactericidal effect was achieved at 0.23µl/ml. The essential oil of *S. aromaticum* showed an average antibacterial activity with MIC at 3.75-7.5µl/ml and MBC at 15µl/ml. The essential oils of *C. zeylanicum* and *S. aromaticum* showed a higher antibacterial activity than that *N. sativa* (MIC 60-120µl/ml and MBC at 600µl/ml).

The antimicrobial activity of cinnamon oil in this study were in confirmity with the results of LU Fei et al. (2001) who found that cinnamon was a promising antibacterial substance against the five bacterial species: three Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus*, and *Staphylococcus aureus*), and two Gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) [36]. The lowest antibacterial activity of the essential oil of *Nigella* against 13 isolates of *Xanthomonas axonopodis* pv. *phaseoli* were consistent with the results obtained by Rym Akloul et

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al. [37], whose results showed that essential oils of *Nigella sativa* are particularly effective against Gram-positive bacteria.

Table 1. Biochemical characterization of various isolates of *Xanthomonas axonopodis* pv. *phaseoli*, collected from Mnasra zone in Morocco during 2016.

| Pathogenic isolates | Basic test | | | | | LOPAT test | | | | |
|---------------------|------------|---------------|---------------|----------|---------------|------------|---------|-----------------|-----------------------|---------------|
| | KOH test | Gram reaction | Catalase test | O/F test | Mobility test | Levane | Oxidase | Potato soft rot | Arginine di-hydrolase | HR of tobacco |
| X.ap01 | + | - | + | O | + | + | - | - | - | + |
| X.ap02 | + | - | + | O | + | + | - | - | - | + |
| X.ap03 | + | - | + | O | + | + | - | - | - | + |
| X.ap04 | + | - | + | O | + | + | - | - | - | - |
| X.ap05 | + | - | + | O | + | + | - | - | - | + |
| X.ap06 | + | - | + | O | + | + | - | - | - | + |
| X.ap07 | + | - | + | O | + | + | - | - | - | + |
| X.ap08 | + | - | + | O | + | + | - | - | - | - |
| X.ap09 | + | - | + | O | + | + | - | - | - | + |
| X.ap10 | + | - | + | O | + | + | - | - | - | + |
| X.ap11 | + | - | + | O | + | + | - | - | - | + |
| X.ap12 | + | - | + | O | + | + | - | - | - | + |
| X.ap13 | + | - | + | O | + | + | - | - | - | + |
| X.ap14 | + | - | + | O | + | + | - | - | - | + |
| X.ap15 | + | - | + | O | + | + | - | - | - | - |
| X.ap16 | + | - | + | O | + | + | - | - | - | + |
| X.ap17 | + | - | + | O | + | + | - | - | - | + |

X.ap.: *Xanthomonas axonopodis* pv. *phaseoli*.

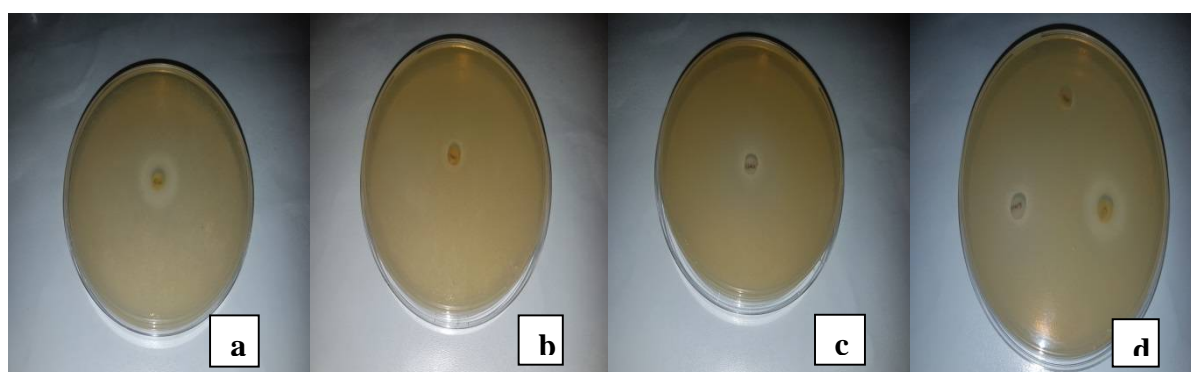


Figure 3: Growth inhibition zone on *Xanthomonas axonopodis* pv. *phaseoli* ((a) = *C. zeylanicum* (b) = *N. sativa*, (c) = *S. aromaticum*, (d) = *C. zeylanicum*, *N. sativa* and *S. aromaticum*).

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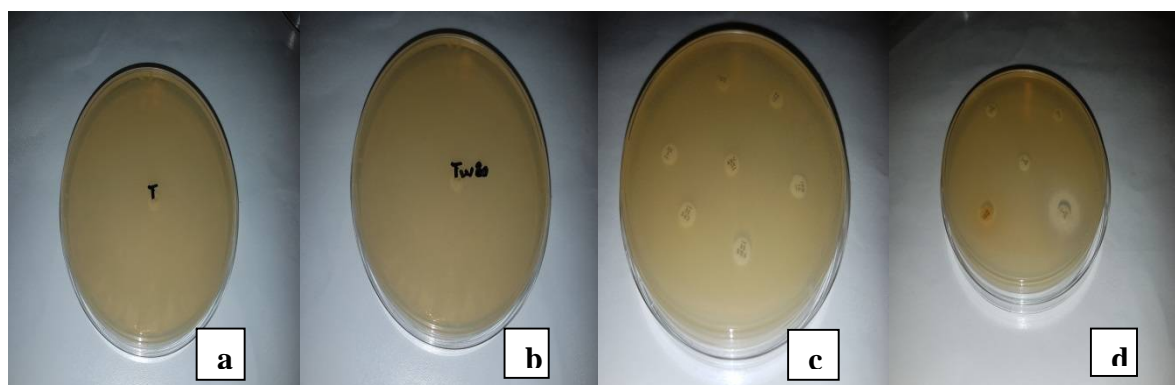


Figure 4: Growth inhibition zone on *Xanthomonas axonopodis pv. phaseoli* ((a) = T: distilled sterile water (b) = Tween-80, (c) = **Antibiotic sensitivity testing 1:** CXM= Cefuroxime, OX= Oxacillin, VA=Vancomycin, RD= Rifampicin and CIP= Ciprofloxacin; (d) = **Antibiotic sensitivity testing 2:** AMC=Amoxicillin + Clavulanic Acid, CFS= Cefsulodin, DA= Clindamycin, FOS= Fosfomycin, IPM= Imipenem, KF= Cephalothin, OX= Oxacillin.).

Table 2. Antibacterial activity of the essential oil from *Cinnamomum zeylanicum*; *Nigella sativa* and *Syzygium aromaticum* L. against pathogenic isolates in solid and liquid media.

| Pathogenic isolates | HE <i>C. zeylanicum</i> | | | HE <i>N. sativa</i> | | | HE <i>S. aromaticum</i> L. | | |
|---------------------|-------------------------|-------------|-------------|---------------------|-------------|-------------|----------------------------|-------------|-------------|
| | IZD (mm) | MIC (µl/ml) | MBC (µl/ml) | IZD (mm) | MIC (µl/ml) | MBC (µl/ml) | IZD (mm) | MIC (µl/ml) | MBC (µl/ml) |
| X.ap01 | 21 | 0.059 | 0.23 | 10 | 60 | 600 | 13 | 7.5 | 15 |
| X.ap02 | 20 | 0.12 | 0.23 | 10 | 60 | 600 | 13 | 7.5 | 15 |
| X.ap03 | 19 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 7.5 | 15 |
| X.ap05 | 19 | 0.12 | 0.23 | 12 | 60 | 600 | 14 | 7.5 | 15 |
| X.ap06 | 19 | 0.12 | 0.23 | 11 | 60 | 600 | 12 | 7.5 | 15 |
| X.ap07 | 18 | 0.12 | 0.23 | 10 | 60 | 600 | 14 | 7.5 | 15 |
| X.ap09 | 20 | 0.059 | 0.23 | 10 | 60 | 600 | 13 | 7.5 | 15 |
| X.ap10 | 19 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 7.5 | 15 |
| X.ap12 | 20 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 3.75 | 15 |
| X.ap13 | 20 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 7.5 | 15 |
| X.ap14 | 19 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 7.5 | 15 |
| X.ap16 | 20 | 0.12 | 0.23 | 12 | 60 | 600 | 13 | 7.5 | 15 |
| X.ap17 | 18 | 0.12 | 0.23 | 10 | 120 | 600 | 12 | 7.5 | 15 |

Final bacterial density was around 10^8 cfu/ml.

IZD: Inhibition zone diameters (mm).

MIC: minimum inhibitory concentration (µl/ml).

MBC: minimum bactericidal concentration (µl/ml).

In addition, Gram-negative bacteria were less sensitive to *Nigella sativa* essential oils with a diameter of growth inhibition ranging from 8.6 (*Enterococcus faecalis*) to 14 mm (*E. coli*) [37]. The average antibacterial activity of the essential oil of clove against 13 isolates tested in this work were in conformity with the results of Md. Mahfuzul Hoque et al. 2008 who showed that Gram-positive bacteria were more sensitive to essential oil of clove than Gram-negative bacteria [41].

The chemical components of cinnamon, nigella and clove oil have been studied in literatures such as Marongiu et al. [39], who found that the main component of the cinnamon (*Cinnamomum zeylanicum*) bark oil was trans-cinnamaldehyde, and the content was 77.1% [42]. The main components of clove were eugenol and β -caryophyllene as

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reported by Alma et al. and Guan et al. [40, 41]. The major components of the nigella are monoterpene hydrocarbons and phenolic monoterpenes as reported by Rym Akloul et al. [37].

The different amounts of specific compounds can affect the antimicrobial activity of essential oils. The presence of the complex chemical structures is constituted of several groups, such as terpenes and terpenoids, aromatic and aliphatic constituents, which are all characterized by low molecular weight. This may be explained by their successful bacteriostatic and bactericidal action.

The phenolic acids such as eugenol are secondary plant metabolites that account for the antimicrobial activity of essential oils such as cinnamon, and clove [42].

Other studies in vitro have shown an antibacterial activity of the essential oil of *N. sativa* on isolated bacterial pathogens of potato [30], and an insecticide effect of the essential oil of *N. sativa* [43], *C. zeylanicum* [44] and *S. aromaticum* [45] on *Tuta absoluta*.

There is a quantitative difference between the data generated from this study as compared with other reported data. This might be explained by the differences in the susceptibility testing conditions, the method of extraction, the species differences, and even the strain to strain differences.

IV. CONCLUSION

These results encourage us to continue our scientific research on the antibacterial activity of essential oil constituents of *C. zeylanicum*, *N. sativa* and *S. aromaticum* against these pathogenic isolates in vivo. Its final objective is the contribution to research with an exploitable natural product in the field of fighting against the pathogenic bacteria of plants.

Though, the antimicrobial activities of the *N. sativa* and *S. aromaticum* essential oils are less effective than the *C. zeylanicum* oils, their antimicrobial activities are not regarded as useless.

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