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Antimicrobial activity of *Anthostomelloides leucospermi* TBT10, an endophytic fungus isolated from *Taxus baccata* stems against human pathogens

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Abstract

The objective of this study is to identify and evaluate the antimicrobial activity of the endophytic fungus TBT10 isolate from *Taxus baccata* stems. Morphological and molecular identification allowed us to identify this isolate as belonging to the *Anthostomelloides leucospermi* species. After preliminary screening by agar diffusion method which showed the inhibitory potential of TBT10 against the test bacteria, an optimization of the culture medium, the type of fermentation as well as the extraction solvent was carried out. The Potato Dextrose Agar was the best medium giving the highest production of secondary metabolites with average inhibition zones of 23.25 mm; the liquid culture was the one that gave the best activity (10.13 mm). Regarding the solvent, ethyl acetate extract was the solvent allowing the best extraction of the active molecules with average inhibition zones of 15.88 mm. Ethyl acetate extract was active against all used Gram positive bacteria and the largest inhibition was obtained against *Micrococcus luteus* (28.5 mm), but Gram negative bacteria were all resistant. MICs were ranged from 0.5625 to 4.50 mg/mL, while the MBCs were between 0.5625 to >4.50 mg/mL and the effect of extract was bactericidal against all bacteria except against *Enterococcus faecalis* 4 and *Enterococcus faecium* 1 where it was bacteriostatic. Time kill kinetic of ethyl acetate extract was bactericidal against *Enteroccocus faecium* 2 after 1 and 24 hours for the MIC×4 (4.50 mg/mL) and MIC×2 (2.25 mg/mL) concentrations respectively. Whereas against *Streptococcus pyogenes*; the bactericidal effect was observed after 1, 4and 12 hours of contact with MIC×4 (2.25 mg/mL), MIC×2 (1.125 mg/mL) and MIC (0.5625 mg/mL) respectively. TBT10 ethyl acetate extract can be a promising source for production of biologically active molecules.

Key words – Biological activities – endophytes – medicinal plant – secondary metabolites – time kill

Introduction

The word endophyte refers to any microorganisms living at least for a portion of their lives asymptomatically within plant tissues (Liu et al. 2019), (Kaul et al. 2012). Endophytic fungi are

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ubiquitous and have been found in all the plants studied so far (Liu et al. 2019). This unique habitat allows them to produce different types of natural products with significant bioactivity such as antimicrobial, antitumor, cytotoxic, anti-inflammatory, antiparasitic, anti-oxidant and neuroprotective activities (Liu et al. 2019). These natural products are very highly coveted, especially in recent years where resistance to antibiotics is increasing (Baba Ahmed-Kazi Tani & Arlet 2014).

Algeria is one of the richest Arab countries in medicinal plants with 3164 species (Benarba 2016); among them *Taxus baccata* a tree belonging to the class of Pinopsida, order of taxales and to the family of Taxaceae, used in traditional medicine as abortive, antimalarials, antirheumatic, as well as to treat bronchitis (Patel et al. 2011, Kashani et al. 2018). The yew produces a diterpenic alkaloid, taxol or paclitaxel, it is recognized as the most important natural anti-cancer agent.

For the first time in this study, *Taxus baccata* growing in the mountains of Babors in Algeria was used to isolate and evaluate the antimicrobial activity of their endophytic fungi.

Materials & Methods

Collection of plant sample

Healthy leaves; stems and fruits of medicinal plant *Taxus baccata* were collected from the Babors mountains, Setif, Algeria in June 2014; The plant has been identified by botanists of the faculty, and all parts were brought to the laboratory in pre-sterilized bags and processed within 24 hours after the sampling.

Isolation of endophytic fungi

Endophytic fungus TBT10 was isolated using a modified method described by Pimentel et al.(2006). First, the samples were rinsed under running tap water to remove the dust, and then cut into small pieces. Surface sterilization was then done by soaking in 70% ethanol for 1 minute, followed by sodium hypochlorite 3% for 4 minutes and in 70% ethanol for 1 minute. Finally, the samples were rinsed using sterile distilled water three times separately and drying on sterile filter paper. The surface sterilized segments were aseptically cut into 5 mm pieces and placed into Petri dishes containing Potato Dextrose Agar (PDA) amended with 100 mg/mL of Gentamicin. The dishes were incubated at 28°C and monitored every day. The fungi that grown out from the samples were isolated, purified and conserved on PDA slants at 4°C till further use.

Identification of the endophytic fungus

Morphological and multi-locus gene identification

The endophytic fungus was initially identified phenotypically based on microscopic and macroscopic characteristics (Qiu et al. 2010, Jiaojiao et al. 2016). For molecular identification, the genomic DNA was extracted from the pure culture using a microbial DNA isolation kit (Ultraclean Microbial DNA isolation Kit, Mobio Laboratories, Inc., USA) following the manufacturer's instructions.

The internal transcribed spacer (ITS) region (ITS1F, CTT GGT CAT TTA GAG GAA GTA A; ITS4, TCC TCC GCT TAT TGA TAT GC), large subunit (LSU) region (LR0R, ACC CGC TGA ACT TAA GC; LR5, TCC TGA GGG AAA CTT CG) (Demirel 2016), β-tubulin (*BenA*) (Bt2a, GGT AAC CAA ATC GGT GCT GCT TTC; Bt2b, ACC CTC AGT GTA GTG ACC CTT GGC) (Raja et al. 2017) and RNA polymerase II second largest subunit (RPB2) (5F, GAY GAY MGW GAT CAY TTY GG; 7CReur, CCC ATR GCY TGY TTR CCC AT) (Visagie et al. 2014) were amplified by the polymerase chain reaction (PCR). PCR was conducted in 25 µL final reaction volume, where each tube contained: 1 µL of genomic DNA, 2.5 µL of 2.5 µM forward and reverse primers, 2.5 µL of 10× Taq buffer+ Kcl-$MgCl_2$, 2.5 µL of 25 mM $MgCl_2$, 2 µL of 2.5 mM dNTP mix, 0.25 µL of 5 U: µL Taq DNA polymerase and 11.75 µL of sterile deionized water (Kadaifciler & Demirel 2017).

For ITS, LSU and *BenA*, the amplification conditions consisted of an initial denaturation step at 94°C for 5 min (ITS and *BenA*)/10 min (LSU), followed by 35 cycles at 94°C for 45 s (ITS and *BenA*)/ 15s (LSU), 55°C for 45s (ITS and *BenA*)/ 48°C for 30s (LSU), 72°C for 60s (ITS and *BenA*)/90s (LSU) and final extension at 72°C for 7 min (Visagie et al. 2014, Demirel 2016). For RPB2, the initial denaturation was at 94°C for 5 min was followed by 5 cycles (94°C for 45s, 50°C for 45s, 72°C for 60s), 5 cycles (94°C for 45s, 52°C for 45s, 72°C for 60s) and 30 cycles (94°C for 45s, 55°C for 45s, 72°C for 60s) (Visagie et al. 2014). PCR products were separated by agarose gel electrophoresis (1% w/v in 1×TAE), cleaned using EXOSAP-IT (Affimetrix) and sequenced with the CEQ 8000 Genetic Analysis System using the same primers used during the amplification for ITS, *BenA* and RPB2. For LSU, two additional primers (LR3, GGT CCG TGT TTC AAG AC; LR3R, GTC TTG AAA CAC GGA CC) were used (Visagie et al. 2014, Raja et al. 2017).

Phylogenetic analyses

The sequences of the different DNA regions obtained after sequencing were compared with sequences recorded on National Center for Biotechnology Information (NCBI) and aligned with those morphologically and phylogenetically related species (Table 1) by Muscle in MEGA7 software (Kumar et al. 2016). The set of sequences of the different aligned regions were then concatenated using the Mesquite 3.6 software, then analyzed by Maximum likelihood (ML) based on the "Tamura Nei" model (Tamura & Nei 1993) using MEGA7 with 1000 Boostrap replicates. *Sordaria fimicole* CBS 72396 was used as out group.

Table 1 Sequences of the species used in phylogenetic analysis. TBT10 isolate of this study is in bold.

Species	Strain number	GenBank Accession Number			
		ITS	**LSU**	***BenA***	**RPB2**
Anthostomelloides leucospermi	**TBT10**	**MN611120**	**MN611121**	**MN630033**	**MN630032**
Xylaria longipes	CBS 148.73	KU683768	KX533452	KU684204	KX789491
Xylaria longipes	CBS 347.37	MH855925	MH867427	-	-
Xylaria acuta	5220	JQ862676	JQ862637	JX868537	-
Xylaria badia	5256	JQ862687	JQ862643	JX868543	-
Xylaria plebeja	HAST 91122401	GU324740	-	GQ502689	GQ848353
Xylaria intracolorata	HAST 90080402	GU324741	-	GQ502690	GQ848354
Xylaria grammica	5151	JQ862665	JQ862626	JX868535	-
Xylaria polymorpha	CBS 162.22	MH854735	MH866242	-	-
Xylaria hypoxylon	CBS 126417	MH864103	MH875562	-	-
Xylaria bambusicola	MFLUCC 11-0606	KU940160	KU863148	-	KU940183
Xylaria enteroleuca	CBS 128357	MH864898	MH876349	-	-
Xylaria berteri	JDR 256	GU324750	-	GQ502698	GQ848363
Xylaria xylarioides	CBS 127883	MH864741	MH876177	-	-
Xylaria arbuscula	CBS 126416	MH864102	MH875561	-	-
Anthostomelloides leucospermi	CBS 110126	EU552100	EU552100	-	-
Anthostomelloides brabeji	CBS 110128	EU552098	EU552098	-	-
Anthostomelloides proteae	CBS 110127	EU552101	EU552101	-	-
Anthostomella eucalyptorum	CBS 120036	DQ890026	DQ890026	-	-
Anthostomella helicofissa	MFLUCC 14-0173	KP297406	KP297406	KP406617	KP340534
Anthostomella rubicola	MFLUCC 16-0479	KX533455	KX533456	KX789494	KX789493

Table 1 Continued.

Species	Strain number	GenBank Accession Number			
		ITS	**LSU**	***BenA***	**RPB2**
Anthostomella Formosa	MFLUCC 14-0170	KP297403	KP340544	-	KP340531
Anthostomella obesa	MFLUCC 14-0171	KP297405	KP340546	-	KP340533
Biscogniauxia petrensis	LC5697	KU746669	KU746715	KU746761	-
Biscogniauxia marginata	MFLUCC 12-0740	KJ958407	KJ958408	KJ958406	KJ958409
Biscogniauxia mediterranea	CBS 129073	MH865188	MH876625	-	-
Rosellinia mammiformis	CBS 445.89	KF719200	KF719212	-	-
Rosellinia nectrioides	CBS 449.89	FJ175181	KF719213	-	-
Rosellinia necatrix	CBS 349.36	AY909001	KF719204	KY624310	KY624275
Lopadostoma dryophilum	CBS 133213	KC774570	KC774570	MF489023	KC774526
Lopadostoma turgidum	CBS 133207	KC774618	KC774618	MF489024	KC774563
Hypoxylon carneum	MUCL 54177	KY610400	KY610480	KX271270	KY624297
Sordaria fimicola	CBS 723.96	MH862606	MH874231	DQ368618	DQ368647

Screening of antimicrobial activity

The preliminary screening of antimicrobial activity of endophytic fungus was carried out by the agar diffusion method against four pathogenic bacteria, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC27853 and E*scherichia coli* ATCC 25922 and one yeast, *Candida albicans* ATCC 1024. Agar plugs (6 mm) were cut from the PDA plate of actively growing endophytic fungus and inoculated on the surface of Muller-Hinton Agar (MHA) and PDA Petri dishes previously spread with 100 µL of bacteria and yeast suspensions respectively. Plates were kept in a refrigerator at 4°C for 6 hours for diffusion of metabolites from the fungal disks. After incubation at 37°C for 24 hours for bacteria and 48 hours for yeast, the zones of inhibition were measured (Powthong et al. 2013, Sahani et al. 2017).

Choice of the best culture medium for fermentation

Different culture media such as PDA, Sabouraud's Dextrose Agar (SDA), Yeast Extract Agar (YEA), Malt Extract Agar (MEA) and Yeast Malt Extract Agar (YMEA) were used to choose the one that allows the best production of bioactive molecules by TBT10 isolate. After culturing the endophytic fungus on the culture media for 14 days at 28°C, the agar diffusion method described above was performed against four Gram positive bacteria *Bacillus cereus* ATCC 10876, *Enterococcus faecalis* ATCC 49452,*Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* ATCC 43300 and four Gram positive bacteria *Citrobacter freundii* ATCC 8090, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC27853, E*scherichia coli* ATCC 25922 and one yeast, *Candida albicans* ATCC 1024.

All Petri dishes were incubated for 24 hours at 37°C for bacteria and 48 hours for yeast. The antimicrobial activity of the endophytic fungus TBT10 isolate was detected by the appearance of the inhibition zones around the agar plugs.

Cultivation of endophytic fungus for metabolites production

Liquid and solid cultures of the endophytic fungus TBT10 were prepared following the modified protocol of Kim et al. (2016) and Son et al. (2018).

For liquid cultures; 100 mL of Potato Dextrose Broth (PDB) medium selected in the previous step in Erlenmeyer flasks was inoculated by the endophytic fungus. For solid cultures; the isolate

was inoculated into five 9 cm diameter Petri dishes containing 20 mL of PDA medium. After incubation of 21 days at 28°C with agitation at 200 rpm, the liquid medium was separated from the mycelium using Whatman filter paper and then centrifuged at 4000 rpm for 5 min, while the solid cultures were removed from the Petri dishes and then ground.

Extraction of metabolites from endophytic fungus cultures

In order to choose the best solvent for the maximum extraction of the secondary metabolites, extraction with three solvents was carried out following the method of Saraswaty et al. (2013) and Son et al. (2018) with some modifications. To the filtrate, equal volume of n-Hexane was added, mixed for 2 hours at 200 rpm and allowed to stand for 1 hour, the upper layer of solvent containing the extracted compounds was collected and the filtrate was re-extracted with the other two solvents dichloromethane and ethyl acetate successively. On the other hand, the metabolites of solid cultures were extracted by adding 100 mL of n-Hexane to the crushed medium followed by agitation for 24 hours at 200 rpm, the layer of n-Hexane was then collected and the same steps were repeated for the other two solvents.

All recovered organic layers were evaporated under vacuum using a rotary evaporator (BÜCHI), the crude extracts were then dissolved in dimethyl sulfoxide (DMSO) and kept at 4°C.

Determination of antimicrobial activity of fungal extracts

Antimicrobial activity of the various extracts of TBT10 was performed using the agar well diffusion method against *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Citrobacter freundii* ATCC 8090, *Salmonella typhimurium* ATCC 13311, *Escherichia coli* ATCC 25922 and the yeast *Candida albicans* ATCC 1024.

After inoculation of the MHA and PDA agar plates with 100 µL of the bacterial (10^8 CFU/ mL) and yeast (5×10^6 cells/ mL) suspensions respectively, wells of 6 mm diameter were formed and filled with 25 µL of each extract, DMSO was used as negative control. Refrigeration for 6 hours at 4°C is necessary to allow the diffusion of bioactive molecules followed by incubation at 37°C for 24 hours for bacteria and 48 hours for yeast. The diameters of the inhibition zones were measured in millimeters (Fatima et al. 2016).

Antibacterial activity of ethyl acetate extract against multi-drug resistant bacteria

Since the ethyl acetate extract is the one that gave the best activity against the bacteria tests, it was chosen to test its activity against a wide range of pathogenic and multi-drug resistant bacteria (Fig. 6) following the method previously described.

Determination of minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

MICs and MBCs of ethyl acetate extract of TBT10 against Gram positive bacteria listed in the table 3 were determined using microdilution technique according to the standard reference method M07A8 from Clinical and Laboratory Standards Institute (CLSI 2012. The assay was carried out in 96-well microplates containing 180 µL of Muller-Hinton Broth (MHB). Initially, the ethyl acetate extract was dissolved in DMSO, and then, its dilutions were prepared in wells of microplates to get final concentrations ranging from 0.0044 to 4.5 mg/mL. After this step, each well received 20 µL of bacterial suspension (10^6 CFU/mL). DMSO and the Gentamicin were used as negative and positive control respectively. Plates were then incubated at 37°C for 24 hours. The MICs of the extract were detected after addition 20 µL of 2, 3-5 Triphenyltetrazolium chloride aqueous solution and incubation at 37°C for 30 min. the lowest concentration of extract showing no visual color change and therefore no growth was considered as MIC.

Using the results of the MIC assay, the concentrations showing a complete absence of visual growth of bacteria were identified and subcultured onto agar plates and incubated at 37°C for 24 hours. The complete absence of growth on the agar surface at the lowest sample concentration was

defined as MBC.

Time-kill kinetics assay

Time-kill assay was conducted against *Enterococcus faecium* 2 and *Streptococcus pyogenes* as described by CLSI (1999). This involved the monitoring of the decrease of bacteria cell growth, due to the effect of a definite extract concentration over time. Concentrations equal to 1xMIC, 2xMIC and 4xMIC of the ethyl acetate extract were prepared, and then the bacterial suspension was transferred to all the tubes in order to obtain a final inoculum of 5×10^5 CFU/mL. For the negative control, the extract was not added and has been replaced by the broth. The cultures were then inoculated in a shaker at 37°C and aliquots of 100 µL were withdrawn from the different tubes at different time intervals (0, 1, 2, 3, 4, 6, 12 and 24 hours), diluted and spread on the surface of MHA. Colony count was performed after 24 hours incubation at 37°C. A graph of the log CFU/mL was then plotted against time. The percentage of lethality was calculated according to the following equation:

$$\text{Reduction (\%)} = V_0 - V_z/V_0 \times 100$$

Where, V_0 is the initial viable cell count at T= 0, V_Z is the viable cell count at time Z.

The extract is considered bactericidal when the percentage of lethality reached 99.99% after 24 hours of contact.

Statistical analysis

All experiments were performed in duplicates and statistical analysis was carried out using SAS/STAT® 9.2 software. Comparison of groups was performed using the one-way ANOVA followed by Student-Newman-Keuls multip-rang test. Results are represented as mean ± standard deviation (SD) and significant effects of treatments were determined by F values ($P \leq 0.05$).

Results & Discussion

Isolation and identification of endophytic fungus

The fungus TBT10 isolated as endophyte from healthy stems of *Taxus baccata* grew as cottony circular colonies, fast growing with 46 mm diameter on the PDA and 45 mm on MEA after 7 days of incubation at 28°C. On PDA as well as on MEA, the colony is white with a white lapel; however, on MEA the colony is denser. The mycelium is transparent, very dense, fine and sterile (Fig. 1). For the molecular identification, phylogenetic tree (Fig. 2) was conducted using combined ITS, LSU, *BenA* and RPB2 sequences of TBT10 isolate and those downloaded from GenBank, of which *Sordaria fimicola* CBS 72396 was used as an out-group. The analyses showed that TBT10 was closely related to the *Anthostomelloides leucospermi* CBS110126 species previously named *Anthostomella leucospermi* (Daranagama et al. 2016), with which it formed a single clade (boostrap of 77%). The sequences of the different regions ITS, LSU, *BenA* and RPB2 were deposited in GenBank database under accession numbers MN611120, MN611121, MN630033 and MN630032 respectively.

Several studies in different regions of the world such as those of Jam Ashkezari & Fotouhifar (2017) in Iran, Tayung & Jha (2010) in India and Rivera-Orduña et al. (2011) in Mexico were isolated of endophytic fungi from *Taxus baccata.* However, none have been worked on *Taxus baccata* growing in Algeria and none of them have been isolated of *Anthostomelloides leucospermi*. This species has been isolated from other plants such as deciduous leaves belonging to the Betulaceae family in Japan (Osono & Masuya 2012) and from leaves of *Oryza sativa* (Lapuz et al. 2018).

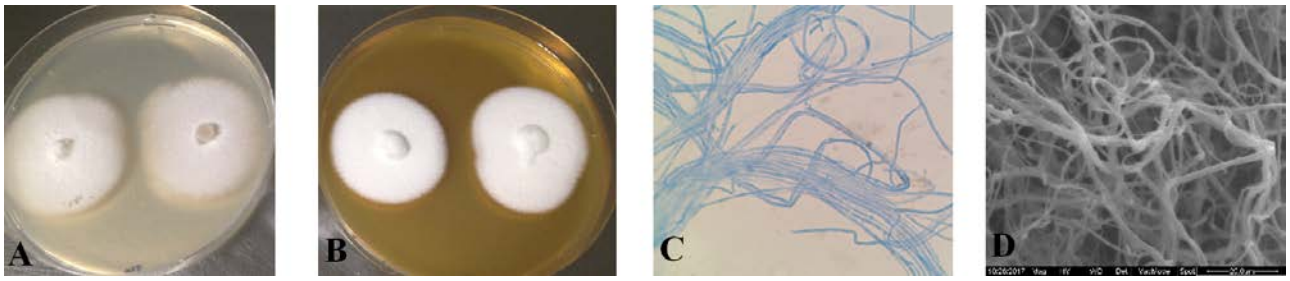

Fig. 1 – Morphological characteristics of TBT10 isolate. A Colony on PDA. B Colony on MEA. C, D Microscopic appearance under optical and electronic microscope respectively.

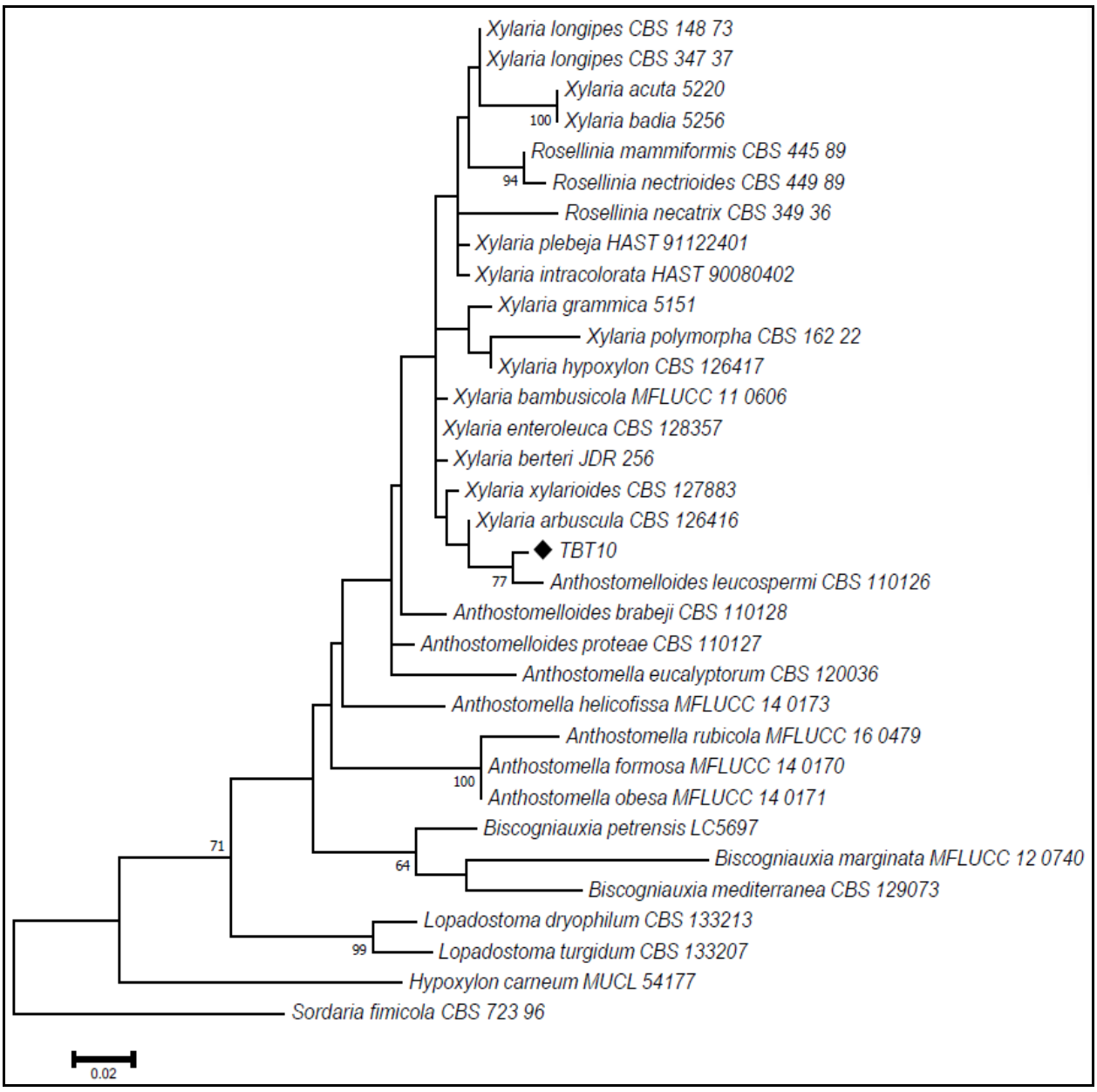

Fig. 2 – Phylogenetic tree generated by Maximum Likelihood (ML) analysis based on the Tamura-Nei model calculated using MEGA7 based on the combined alignment of ITS, LSU, *BenA* and RPB2 sequences, showing the relationships of the TBT10 sequences with previously known taxa in the NCBI GenBank. The ML boostrap rates (expressed as a percentage of 1000 replications), greater than 50% are represented at the node level. The tree is rooted with *Sordaria fimicola* CBS 72396.

Screening of antimicrobial activity

TBT10 isolate was submitted to an antimicrobial screening on solid medium. The isolate showed a good activity against three pathogenic bacteria with a maximum activity of 30 mm observed against *Staphylococcus aureus* ATCC 25923; whereas against *Candida albicans* 1024 no activity was observed (Table 2).

Table 2 Results of preliminary screening obtained against pathogenic microorganisms.

	Inhibition zones (mm)				
	***Bacillus cereus* ATCC 10876**	***Staphylococcus aureus* ATCC 25923**	***Pseudomonas aeruginosa* ATCC 27853**	***Escherichia coli* ATCC 25922**	***Candida albicans* ATCC 1024**
TBT10	15.5	30	13.5	00	00

One of the most important properties of endophytic fungi is the production of a wide variety of bioactive molecules that inhibit a wide range of pathogenic microorganisms. The agar diffusion method allows the diffusion of these bioactive molecules previously secreted by the endophytic fungi to the agar seeded with the test bacteria, thus producing zones of inhibition (Balouiri et al. 2016). This allows rapid and qualitative selection of active endophytic fungi (Santos et al. 2015). Several studies have allowed the isolation of several endophytic fungi as well as the determination of their antimicrobial activities on agar medium. Twelve endophytic fungi isolated from *Cinnamomum mercadoi* bark were all active against at least one of the four pathogenic bacteria used in our study (Marcellano et al. 2017). From 65 endophytic fungi isolated from the leaves of *Indigofera suffruticosa* Miller, 18 showed antagonistic activities against at least two pathogenic bacteria (Santos et al. 2015).

Choice of the best culture medium for fermentation

In order to optimize the fermentation conditions, the choice of the culture medium allowing the best production of the active molecules is an important step. For this, TBT10 isolate was grown on five different culture media, and the agar diffusion method was used to estimate the antimicrobial activity. The results presented in figure 3 shows that PDA and SDA were in first position with means of inhibition zones of 23.25 and 22.50 mm respectively, followed by YMEA (14.25 mm), YEA (14.00 mm). On the other hand, and for the MEA no activity has been obtained with this medium.

According Devaraju & Satish (2011), the production of the secondary metabolites is not only influenced by the species and genetic base of the microorganism, the physicochemical conditions of her environment but also depends on specific media and kinds of nutrients available. Consequently, the culture medium can affect the presence or absence of secondary metabolites and/or their level of production by endophytic fungi (Paterson & Bridge 1994). Several studies such as that of Devaraju & Satish (2011), Deka & Jha (2018) and Samae et al. (2019) who found that after growth on the PDA, endophytic fungi showed greater antimicrobial activity. However, Zerroug et al. (2018) and Chatterjee et al. (2019)found that the culture medium which gave the best activity was the MEA.

Determination of antimicrobial activity of fungal extracts

After selection of the optimum culture medium, the choice of the type of fermentation and solvent permitting the highest production as well as the maximal extraction of the secondary metabolites was carried out using the agar well diffusion method. The average of the inhibition zones was 10.13 mm for liquid medium, whereas on the solid medium, the average was of 5.30 mm (Fig. 4).

Regarding the extraction solvents, ethyl acetate was the best solvent for extraction with an average of inhibition zones of 15.88 mm, followed by dichloromethane extract (14.50 mm). However, the n-Hexane extract was not active (Figs 5, 6).

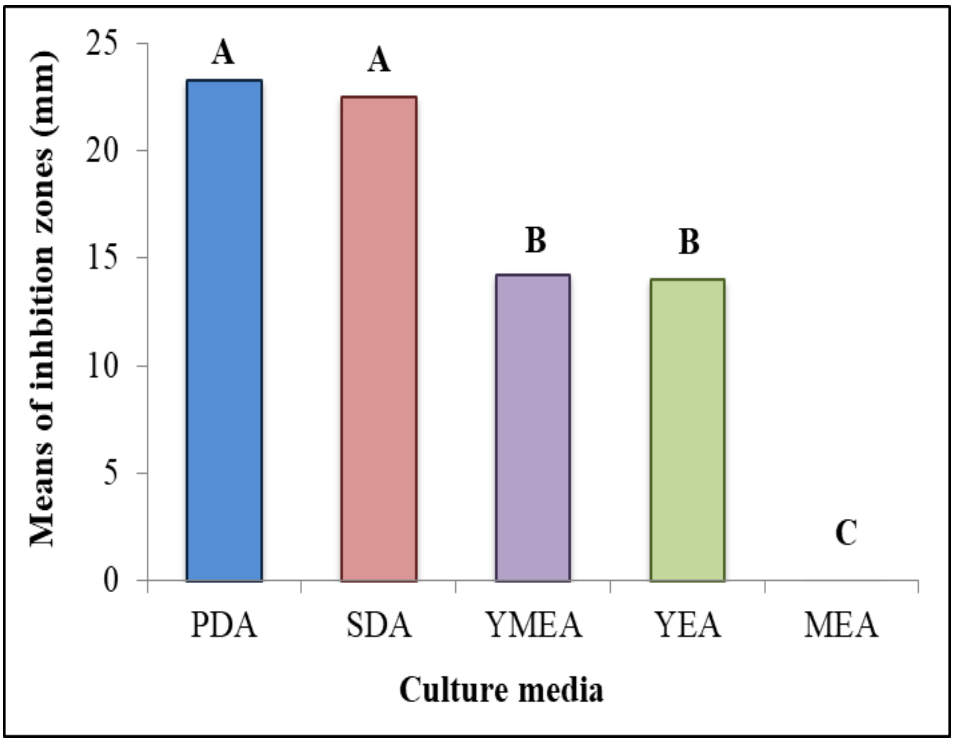

Fig. 3 – Antimicrobial activity of TBT10 isolate after growth on different culture media. Means with the same letter are not significantly different ($P < 0.05$).

According to Son et al. (2018), the two types of fermentation on solid or liquid medium have advantages. However, according to Subramaniyam (2012), the fermentation on solid and liquid medium shows that the obtained results depends more on the microorganisms used than on the fermentation process. Due to this reason, it is important to test both types of fermentation for each isolate. The study realized by Hamzah et al. (2019) showed that several *Penicillium* isolates have antibacterial activity after fermentation on both of solid and liquid media, but the fermentation on liquid medium gave the best activity. Whereas, another study done on *Aspergillus oryzae* showed that the extract from solid medium gave the greater activity than the extract from liquid fermentation (Son et al. 2018).

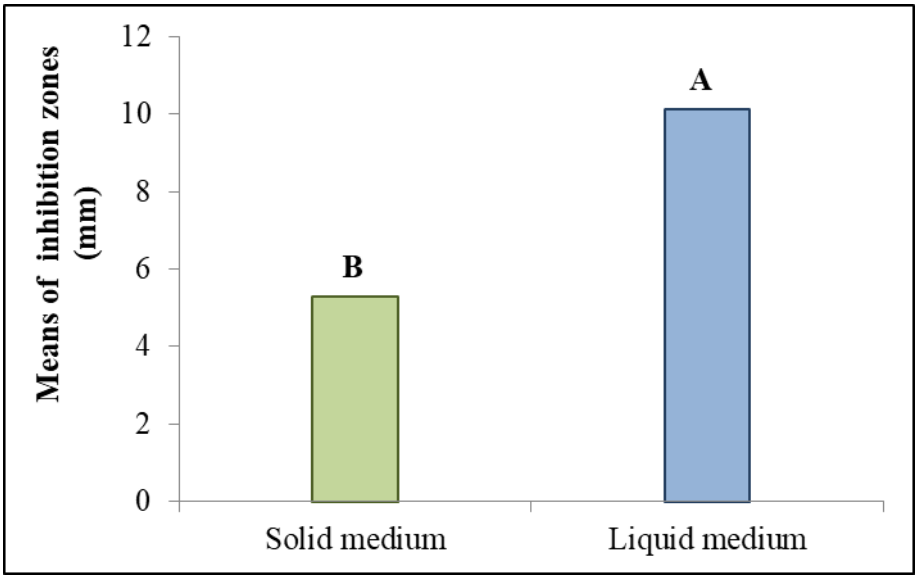

Fig. 4 – Effect of fermentation type on antimicrobial activity of TBT10 isolate against all pathogenic microorganisms. Means with the same letter are not significantly different ($p < 0.05$).

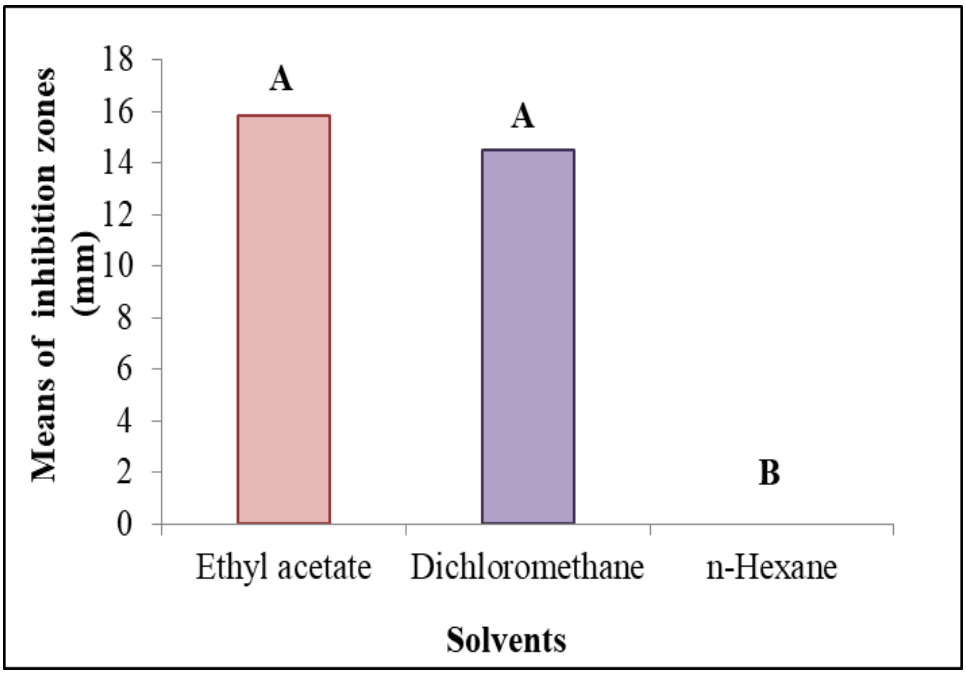

Fig. 5 – Effect of different solvents on antimicrobial activity of TBT10 isolate against all pathogenic microorganisms. Means with the same letter are not significantly different ($p < 0.05$).

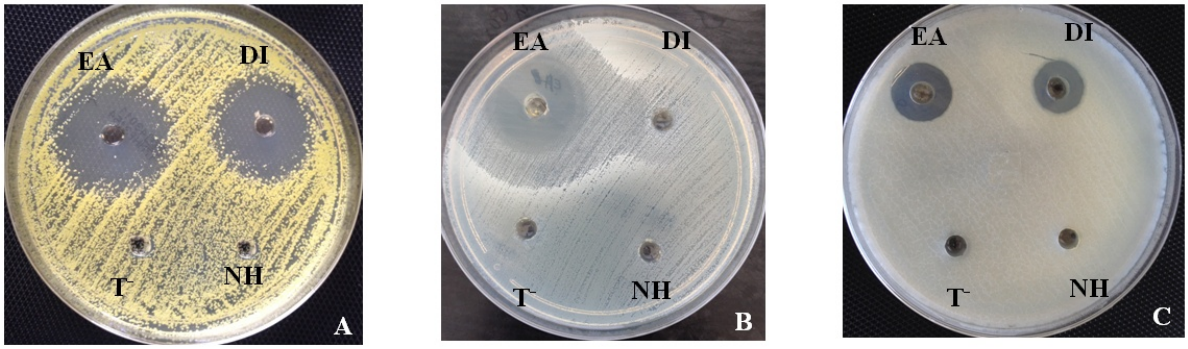

Fig. 6 – Antibacterial activity of TBT10 extracts against pathogenic bacteria. A *Micrococcus luteus* NRLL B-4375. B *Bacillus cereus* ATCC. C *Staphylococcus aureus* ATCC 25923, EA Ethyl acetate extract, DI Dichloromethane extract, NH n-Hexane extract, T: Negative control.

The choice of the appropriate solvent for the maximal extraction of secondary metabolites depends on the nature of the bioactive compounds present in the medium after fermentation (Goutam et al. 2016). Since endophytic fungi generally produce semi-polar molecules (Sugijanto & Dorra 2016), and dichloromethane and ethyl acetate allow the extraction of semi-polar compounds (Djamaan et al. 2018), the obtained results are explained. The results of this study are similar to those found by Musavi & Balakrishnan (2014) where the ethyl acetate extract of the endophytic fungus *Fusarium oxysporum* NFX06 was the most active. The same for those of Mu'azzam et al. (2015) which also found that the two most active fungi (TLBML-M1 and TLBML-PHP2) among those isolated from *Ocimum citriodorum* Vis. showed the better activity after extraction with ethyl acetate and dichloromethane.

Antibacterial activity of ethyl acetate extract against multi-drug resistant bacteria

The ethyl acetate extract of TBT10 has been tested on a wide range of pathogenic and multi-drug resistant bacteria; the latter has been active only on Gram positive bacteria. Of these, all strains of *Staphylococcus aureus* were weakly inhibited with means of inhibition zones ranging

from 10 mm to 14 mm, the other strains were moderately inhibited with means of inhibition zones reaching 28.5 mm obtained against *Micrococcus luteus* (Fig. 7).

The resistance of Gram negative bacteria is due either to the complexity of the liquid bilayer of their outer membrane, or to the highly negatively charged lipopolysaccharides that it contains, which prevents the attachment of anionic and neutral molecules due to the absence of attraction of charges, thus preventing their penetration and inactivation of these bacteria (Tang et al. 2007, Sadrati et al. 2013).

The Infectious Disease Society of America recognizes that antibiotic resistance is one of the greatest threats to human health in the world (Van Duin & Paterson 2016). In the face of this growing bacterial resistance observed in the world and recently in Algeria (Baba Ahmed-Kazi Tani & Arlet 2014), natural bioactive molecules are more in demand. Several studies have shown that endophytic fungi are a good reservoir for these molecules; for example, the ethyl acetate extract of *Chaetomium* sp. NF15, an endophytic fungus isolated from *Justicia adhatoda* L., inhibited the growth of multi-drug resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria (Fatima et al. 2016). Another endophytic fungus, *Aspergillus rhizopodus* isolated from seaweeds, showed an inhibitory effect against multi-drug resistant bacteria such as *Staphylococcus aureus*, *Salmonella weltevreden* and *Escherichia coli* (Samae et al. 2019).

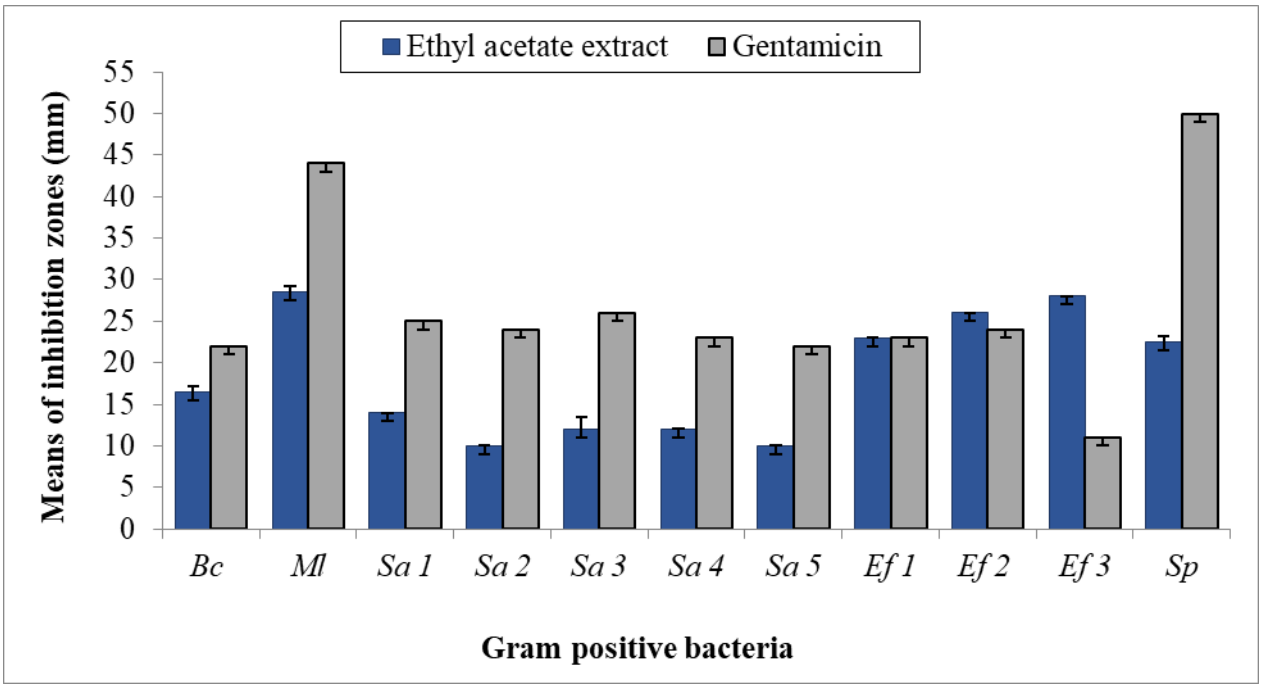

Fig. 7 – Antibacterial activity of TBT10 ethyl acetate extract obtained against Gram positive bacteria.– Data were presented as mean± SD (n=2). Bc *Bacillus cereus*, Ml *Micrococcus luteus*, Sa 1 *Staphylococcus aureus* 1, Sa 2 *Staphylococcus aureus* 2, Sa 3 *Staphylococcus aureus* 3, Sa 4 *Staphylococcus aureus* 4, Sa 5 *Staphylococcus aureus* 5, Ef 1 *Enterococcus faecalis* 1, Ef 2 *Enterococcus faecalis* 2, Ef 3 *Enterococcus faecalis* 3, Sp *Streptococcus pyogenes*.

Determination of MICs and MBCs of ethyl acetate extract

TBT10 ethyl acetate extract has antimicrobial activity against Gram positive bacteria with MICs ranging from 0.5625 to 4.5 mg/mL. While, the MBCs were between 0.5625 to > 4.5 mg/mL, the most sensitive bacteria were *Streptococcus pyogenes* (Table 3).

According to Appiah et al. (2017), when the MBC/MIC ratio is less than or equal to 4, the extract is bactericidal, and conversely, if it is greater than 4, the extract is bacteriostatic. After calculating this ratio for the TBT10 extract (Table 3), the latter is considered bactericidal against all bacteria except against *Enterococcus faecalis* 4 and *Enterococcus faecium* 1 where it was bacteriostatic.

No studies have been made on the antibacterial activity of the species *Anthostomelloides leucospermi*. However, another study done by De amorim et al. (2016), has tested the bioactive molecules produced by another species *Anthostomelloides brabeji* formerly named *Anthostomella brabeji*, an endophytic fungus isolated from *Paepalanthus planifolius*. Two of the four molecules produced by this fungus were the most active; the siccayne was active against *Staphylococcus aureus* with MIC of 62.5µg/mL, whereas the eutypinol was active against *Staphylococcus aureus* and *Salmonella setubal* with MIC of 31.25 µg/mL; The other two molecules showed the lowest activity with MICs ranging from 250 µg/mL to values above 1000 µg/mL.

Table 3 MICs and MBCs values (µg/mL) obtained by TBT10 ethyl acetate extract against the Gram positive bacteria.

Pathogenic bacteria	Ethyl acetate extract			Gentamicin
	MIC	**MBC**	**MBC/MIC**	**MIC**
Bacillus cereus	2.25	2.25	1	0.00025
Micrococcus luteus	4.50	4.50	1	0.000063
***Staphylococcus aureus* 1**	2.25	4.50	2	0.0005
***Staphylococcus aureus* 2**	2.25	4.50	2	0.00025
***Staphylococcus aureus* 3**	2.25	4.50	2	0.00025
***Staphylococcus aureus* 4**	2.25	4.50	2	0.0005
Staphylococcus aureus 5	4.50	>4.50	Nd	0.00025
***Enterococcus faecalis* 1**	1.125	4.50	4	0.008
***Enterococcus faecalis* 2**	1.125	4.50	4	0.004
***Enterococcus faecalis* 3**	1.125	1.125	1	0.256
Enterococcus faecalis 4	1.125	>4.50	>4	0.001
Enterococcus faecium 1	1.125	>4.50	>4	0.256
Enterococcus faecium 2	1.125	1.125	1	0.004
Streptococcus pyogenes	0.5625	0.5625	1	0.001
Streptococcus australis	2.25	2.25	1	0.000063

Nd: Not determined

Time-kill kinetics assay

The killing growth profile of TBT10 ethyl acetate extract against *Enterococcus faecium* 2 (Fig. 8) and *Streptococcus pyogenes* (Fig. 9) show that for *Enterococcus faecium* 2, the bactericidal effect was observed after 1 and 24 hours for the concentrations MIC×4 (4.50 mg/mL) and MIC×2 (2.25 mg/mL) respectively. Whereas for *Streptococcus pyogenes*, all the concentrations used (MIC= 0.5625 mg/mL, MIC×2=1.125 mg/mL and MIC×4=2.25 mg/mL) had a bactericidal activity after 12, 4 and 1 hours of contact respectively. These results confirm what was obtained in table 3, where the effect of the TBT10 ethyl acetate extract was bactericidal against most bacteria including *Enterococcus faecium* 2 and *Streptococcus pyogenes*.

It is also observed that the time of complete elimination of the two bacteria tested decrease by increasing the concentration of the ethyl acetate extract, which indicates that bactericidal activity is concentration dependent.

The time kill test is used in microbiology to evaluate *in vitro* the antimicrobial activity of an agent in relation to time, and it is the most appropriate method for determining the bactericidal effect (Taufiq & Darah 2018). Similarly, and with results very close to those obtained in this study, the bactericidal effect has been observed with several endophytic fungal extracts, such as the extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 isolated from *Ocimum sanctum* LINN leaves which demonstrated a bactericidal activity after 20-24 hours of contact at MIC (0.125 mg/mL) and 16-20 hours at 2×MIC (0.25 mg/mL) against both bacteria, methicillin-resistant *Staphylococcus aureus* (Taufiq and Darah 2018) and *Streptococcus mutans* (Taufiq and Darah 2019). A concentration of 3.125 mg/mL of *Polycephalomyces nipponicus* Cod-MK1201 extract was required to kill 99.9% of methicillin-resistant and susceptible *Staphylococcus aureus* after 24 hours of contact (Thammawat et al. 2017).

According to the obtained results in this study, the bioactive secondary metabolites produced by the endophytic fungus TBT10 appear to be of great importance in the pharmaceutical field as well as in the fight against pathogenic bacteria, this is encouraging carrying out other in-depth studies.

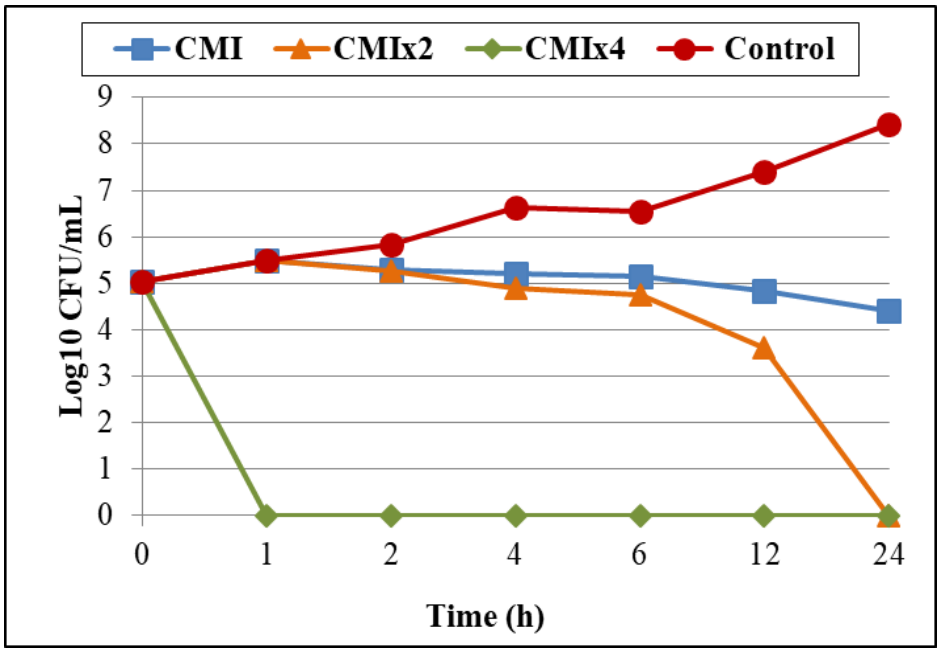

Fig. 8 – Time kill curve of TBT10 ethyl acetate extract against *Enterococcus faecium* 2.

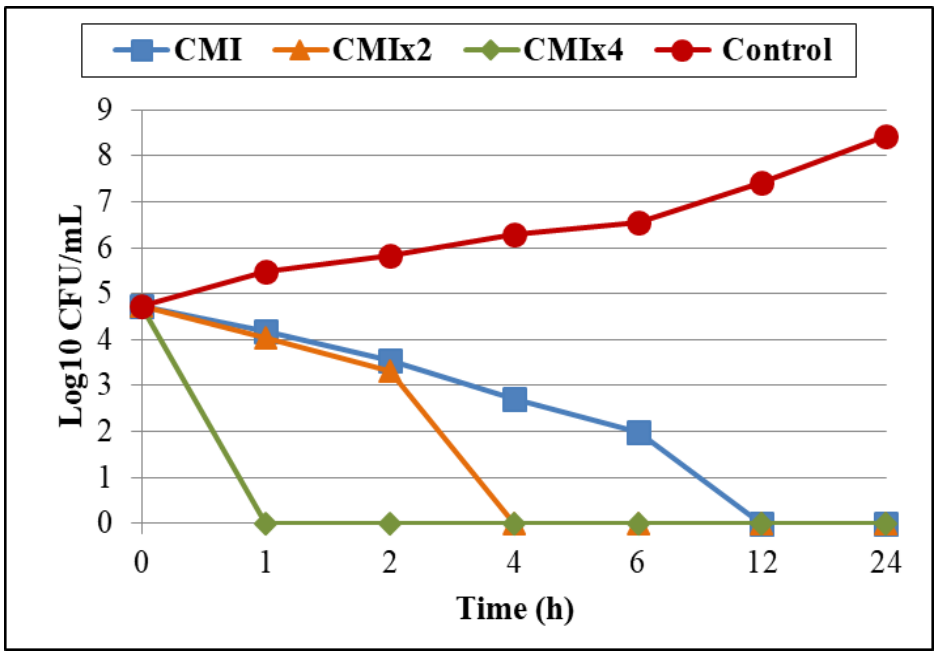

Fig. 9 – Time kill curve of TBT10 ethyl acetate extract against *Streptococcus pyogenes.*

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