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Characterization and evaluation of antimicrobial activity of Algerian *Aloysia triphylla* essential oil against clinical female genital pathogens

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ABSTRACT

The aim of the study is to investigate *in vitro* the antimicrobial activity of *Aloysia triphylla* essential oil EO on ten pathogens isolated from women with vaginitis. Essential oils from aerial parts of medicinal plant were obtained by steam distillation using a Clevenger-type system. The antimicrobial effects against *Escherichia coli*, *Salmonella* sp., *Proteus* sp., *Eterobacter* sp., *Shigella* sp., *Streptococcus* sp., *Staphylococcus aureus*, *Candida albicans*, *Lactobacillus* sp., and *Bifidobacterium* sp. were tested using paper disk diffusion method, followed by determination of minimum inhibitory (MIC) concentration of *A. triphylla* EO. Standard discs of antibiotics piperacillin, streptomycin, cloxacillin, and trimethoprim were served as positive controls for antimicrobial activity. The tested EO exhibited highest antimicrobial activity against Gram positive bacteria and yeast, followed by Gram negative bacteria, except lactic acid bacteria were the most resistant. *A. triphylla* EO showed an MIC value of 0.06 µL/ml against *E. coli* and *Sreptococcus* sp., 0.125 µL/ml against *S. aureus*, and 0.07 µL/ml against *C. albicans*. The results justify the use of *A. triphylla* EO in the treatment of genital infections without fear for their impact on survival of probiotic vaginal *Lactobacillus* sp. and *Bifidobacterium* sp.

INTRODUCTION

Aloysia triphylla is belongs to the family Verbenaceae and named (Lemon Verbena); is cultivated in multiple regions of the world and used for the preparation of herbal tea which is known to have antispasmodic, antipyretic, sedative, digestive activity, antimicrobial activity and antioxidant properties. This plant used in traditional medicine practices since ancient to treat asthma, spasms, cold, fever, flatulence, colic, diarrhea, indigestion, insomnia, and anxiety (Rotman et al., 1999; Gomes et al., 2006). Verbenaceae family have medicinal uses closely related to microbial infections, as they have antiseptic properties and can be used for the treatment of wounds, bronchitis, sinusitis, tetanus (Cristina et al., 2018; Megh et al., 2020). Lemon verbena is locally known as Lominat (Beemnet et al., 2013) and is native to South American countries; Argentina, Paraguay, Brazil, Uruguay, Chile, Bolivia and Peru (Sartoratto et al., 2004; Ricciardi et al., 2011; Pina et al., 2012; Cristina et al., 2018). Its cultivation has spread to Europe, especially Portugal, France and Spain. It is also found in Mediterranean countries such as Algeria, Tunisia and Morocco, and parts of Asia (Akroum et al., 2009; Braga et al., 2011; Fentahun et al., 2017). It is a bush with white flowers and fruits, with an intense scent lemon-like, sweet, lightly floral, and herbaceous (Barboza et al., 2001; Gil et al., 2007).

Most Lemon verbena properties are due to the essential oil (EO) composition produced by their secondary metabolism. The composition of EOs is highly variable and determines their physical, chemical, and biological

properties together with their organoleptic characteristics, which therefore determine their commercial use. Many EOs from Verbenaceae were studied by means of *in vitro* tests and demonstrated a high biological activity against bacteria, fungi, and yeasts (Burt, 2004; Hanna et al., 2011; Hasika et al., 2014; Santos et al., 2015b; Dhifi et al., 2016; Bahramsoltani et al., 2018; Djadouni, 2020). Many microorganisms associated with human diseases exhibit antibiotic resistance due to inappropriate or prolonged use **(**Ahmad and Beg, 2001; Klein et al., 2013; Santos et al., 2016; Swamy et al., 2016 ; Cristina et al., 2018; WHO, 2018). Therefore, the search for new antibiotics therapies from natural sources including plant, animal and microorganisms has become an urgent need (Singh, 2005; Singh, 2013; Jyoti et al., 2014; WHO, 2014). This study was carried out with an objective to investigate *in vitro* the antimicrobial potentials of leaves of *A. triphylla* essential oil (EO) and compared it biological efficacy to antibiotics and some medicinal plants EOs used in treatment of vaginitis.

MATERIAL AND METHODS

Plant collection and extraction

Fresh leaves of *A. triphylla* were collected from Mohammedia commune in Mascara Province, north Algeria in March 2017 (Geographical coordinates are 35° 35' 19" North, 0° 40' 7" East). Botanical identification was performed by Dr. Righi Kada, Laboratory of Research on Biologic systems and Geomantic, Department of Agronomy Sciences, Faculty of natural Science, Mascara University, Algeria.

Essential oil extraction and analysis

Fresh leaves (150 g) were submitted to a steam distillation in a Clevenger-type apparatus over 5 h to afford 931 mg of the crude essential oil. The obtained oil was immediately analyzed by testing pH, yield, and organoleptic characteristics (taste, color, flavor, and smell). The oil was diluted in Tween 80 and Dimethyl Sulfoxide (DMSO), filtered by 0.22 µm Micro Filter (Millex-GV, Renner D-67125/GMBH Germany) and stored at - 4°C until biological testing (De Feo et al., 1998; Sartoratto et al., 2004).

Figure 1. Aromatic plant *A. triphylla*: (a) leaves, (b) flowers, (c) dried leaves.

Media and growth conditions

Yeast were cultivated on agar plates containing YPD (1 % yeast extract, 2 % peptone, 2 % dextrose, and 2 % agar) or RPMI1640 (Merck, Germany). Gram-negative bacteria were grown in LB (0.5 % yeast extract, 1% tryptone, 1% NaCl, and 2.0% agar) and Gram-positive bacteria were tested in BHI (Merck, Germany). Lactic acid bacteria were grown in MRS (1% peptone, 1% beef extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80 (also known as Tween 80), 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate, and 1 % agar.

Antimicrobial screening

Test microorganisms

Escherichia coli, *Salmonella* sp., *Proteus* sp., *Eterobacter* sp., *Shigella* sp., *Streptococcus* sp., *Staphylococcus aureus*, *Candida albicans*, *Lactobacillus* sp., and *Bifidobacterium* sp. were used in these studies. All microbial strains were obtained from Deriex Health Center of Mascara (Algeria). Bacterial species were subcultured and maintained in nutrient broth (NB, Merck, Germany) at 4°C. All fungal species were subcultured and maintained in Sabouraud broth at 4°C.

Disk diffusion assay

Antimicrobial activity of essential oil from leaves of *A. triphylla* was evaluated using the disk diffusion method according to the Clinical and Laboratory Standards Institute CLSI (Jorgensen and Turnidge, 2007). Thin agar plates were prepared with 10 ml of YPD (yeast), LB (Gram-negative), MRS (lactic acid bacteria) and BHI (Gram-positive) media. Three milliliters of liquid cultures (10^8 cells/ml) were grown at 30°C with aeration (150 rpm) overnight on culture media already mentioned. A top agar was prepared by mixing 100 µl of each culture with 10 ml of soft agar medium for confluent plates (YPD, LB, MRS or BHI plus 1% agar) and poured on top of the thin agar (2% agar medium). Sterilized 5 mm filter paper disks were then impregnated with 20 µl of crude essential oil diluted in DMSO. The disks were placed on top of agar plates and incubated at 30°C for 24 or 48 h depending on the microbes. Thereafter, the diameters of the inhibition zones were measured in millimeters. Negative control was prepared by impregnating the paper disks with the same amount of DMSO used to dilute the essential oil. All tests were performed in triplicate. For comparative study, antimicrobial activity of plant extracts *Ar. vulgaris*, *Ur. dioica*, *M. vulgare*, and *T. vulgaris* was determined using the disc diffusion method (Santos et al., 2015a). Standard discs of piperacillin 75µg, streptomycin 10µg, cloxacillin 5µg, and trimethoprim 5µg were obtained from SIGMA PROCHIMA and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 µl of distilled water were used as negative controls (Bauer et al., 1959; Bauer et al., 1966).

Minimum inhibitory concentration

Microdilution tests were conducted according to CLSI, OPAS1 M27-A2 for yeasts and OPAS M7-A6 for bacteria. Briefly, minimum inhibitory concentration (MIC) values were determined using microtiter plates (96 wells) with a total volume of 100 µl. Microbial strains were cultured in test tubes filled with 3 ml medium RPMI 1640 for yeast and BHI for bacteria, overnight at 30°C in a rotary shaker (150 rpm). The cultures were diluted and adjusted to 1–2 × 10^2 CFU/ml, which was confirmed by viability counts on YPD and BHI plates (100 µl of diluted cells). Crude essential oil and reference standards were serial diluted (two-fold, 0.125–5 µl/ml) and added to each well. A sterilization control containing medium only and growth control containing cell, DMSO (10 µl), or saline (10 µl), and Tween 80 were included as negative and positive controls, respectively. Depending on the strain the microtiter plates were then incubated at 30°C for 24 or 48 h. Microbial growth was determined by reading the absorbance at 530 nm in a plate reader ELISA (BLA 808, Biotech) and the minimum inhibitory concentration was considered the lowest concentration at which at least 80% of growth was inhibited. All tests were performed in triplicate (Palavani et al., 2013; Santos et al., 2015a).

Statistical analysis

All the measurements were replicated three times for each assay. The mean values ± standard deviations were calculated.

RESULTS AND DISCUSSIONS

Yield of *A. triphylla* essential oil was 0.27%±1.0 and it was fresh yellow-greenish liquid, lemon-like aroma, and acidic pH: 4.0 in characteristics (Table 1). Sartoratto et al. (2004) and Pereira et al. (2007) reported that the yield of Brazilian *A. triphylla* (L´Hér.) was 0.22% compared to *A. triphylla* collected from Rio Primero, La Paz, Las Viñas, Mendoza, San Luis and Paraguay in Argentina was 0.4% (Oliva et al., 2010). The oil yields of *A. citriodora* Palau from Argentina was 0.5-0.7% (Ricciardi et al., 2011). Santos et al. (2013) demonstrated that yields of Brazilian *A. gratissima* was 0.35%. The yield of Brazilian *Lippia alba* was 0.21% (Tomazoni et al., 2016; dos Santos et al., 2016). According to many findings, various factors are responsible for these results such as extrinsic factors related to the extraction method and intrinsic factors related to the plant, interaction with the environment (soil type, geographic location, seasons, and climate, etc.), plant maturity, and harvest time during the day (Oliveira et al., 2012; Ricciardia et al., 2011; Dhifi et al., 2016).

Table 1. Essential oils (EOs) characteristics from Algerian aromatic plant *A. triphylla*

EOs properties	
% Essential oil yield from leaves	0.27±1.0
pH	**4.0**
Taste	**Mobile liquid**
Color	**Yellow/ yellow-greenish liquid**
Smell	**lemon-like aroma**
Flavor	**Fresh**

Antimicrobial potential of essential oil against microbial pathogens is summarized in Figure 2. The results represent the diameter of inhibition zone including diameter of paper disk (5 mm). A broad variation in antimicrobial properties of the analyzed oil was observed in the study. The essential oil of *A. triphylla* showed consistently strong antimicrobial activity against *Streptococcus* sp., *S. aureus*, and *C. albicans*; where the mean hallo diameter was MHD≥14mm. A moderate activity was observed against gram negative bacteria *E. coli*, *Salmonella* sp., *Proteus* sp., *Eterobacter* sp., and *Shigella* sp. HD≤10mm. No antimicrobial activity was observed against *Lactobacillus* sp., and *Bifidobacterium* sp. Previous studies demonstrated that essential oil of *A. tryphila* (L´Hér.) Britton collected from CPQBA/UNICAMP in Brazil was active against Gram positive and Gram negative bacteria, but no activity was observed against *C. albicans* (Sartoratto et al., 2004). However, Oliva et al. (2010) reported that EOs of *A. triphylla* (L`Her.) Britton collected from different regions in Argentina showed the best antimicrobial potentials against yeasts *C. albicans, Rhodotorula* sp. and *Hansenula* sp. followed by the Gram positive bacteria and lastly the Gram negative ones. According to Hossam et al. (2011), *A. triphylla* essential oil obtained from Morocco exhibited an interesting antibacterial activity against Gram positive bacteria. No antibacterial activity was observed against Gram negative bacteria. In contrast, the results showed strong antiyeast activity against pathogenic yeast *C. albicans.* Similar results were obtained by dos Santos et al. (2016), where the yeast are *C. albicans* considerably more sensitive to essential oil from *Lippia alba* than bacterial strains.

Many finding considered that Gram-positive bacteria were more sensitive to essential oils or antibacterial compounds than Gram-negative bacteria, which is in a good agreement with previous reports. This resistance could be ascribed to the structure of the cellular walls of Gram-negative bacteria, mainly with regard to the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict entry of hydrophobic compounds (Russell, 1995; Smith-Palmer et al., 1998; Dorman and Deans, 2000; Burt, 2004; Cox and Markham, 2007). Also, the differences in the biological potentials of *A. triphylla* EOs could be attributed to the quantity and quality of the terpenic composition and the possible associations between them (Oliva et al., 2010). Helander et al. (1998) attributed the thymol antimicrobial action to its phenolic character, which can cause membrane-disturbing activities. According to Ouattara et al. (1997), Tampieri et al. (2005), and Bozin et al. (2006), the variation in antimicrobial activity may be explained by the different composition and percentage content of active constituents in essential oils, which have been found to have an important role in slowing down or stopping the bacterial growth or killing the bacteria. Some factors influencing this variation in composition can be species, subspecies or variety of plants, geographical locations (Sarac and Ugur, 2008; Mechergui et al., 2010), harvesting seasons (Hussain et al., 2008), drying methods (Di Cesare et al., 2003), and also extraction methods (Burt, 2004; Karakaya et al., 2011). Moreover, the methods used to assess the antimicrobial activity could also affect the generated outputs (Hammer et al., 1999; Burt and Reinders, 2003; Burt, 2004). Other factors such as the choice of bacterial strains and their sensitivity, volume of inoculum, incubation time, and temperature should also be related to the variation in the experimental results (Smith-Palmer et al., 1998; Burt, 2004; Bozin et al., 2006).

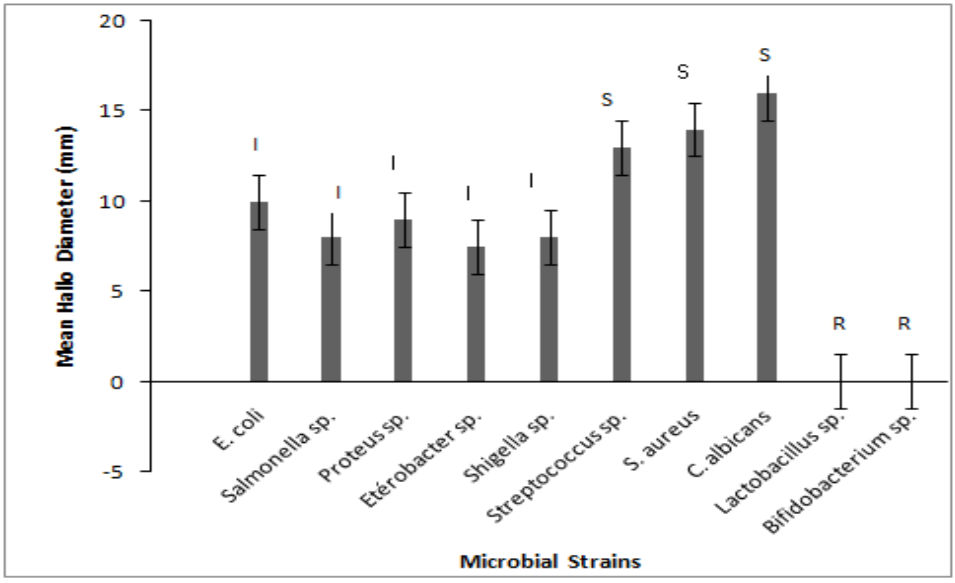

Figure 2. Antimicrobial activity of *A. triphylla* essential oil EO in disc diffusion assays. R: resistant, S: sensitive, I: intermediate.

A. triphylla essential oil showed MIC values 0.06 µl/ml against both *E. coli* and *Sreptococcus* sp., 0.125 µl/ml against *S. aureus* and 0.07 µl/ml against *C. albicans* (Table 2). According to Sartoratto et al. (2004), *A. triphylla* EO MIC values were between 0.05 and >2 mg/ml against *Enterococcus faecium* and *Salmonella cholerasuis.* Duarte et al. (2007) reported that *A. triphylla* inhibited 12 *E. coli* serotypes with MIC values between 400–1000 µg/ml. EOs of *A. gratissima* showed activity against *Pseudomonas aeruginosa* with MIC values 0.8 mg/ml and MIC 0.6 mg/ml against *Streptococcus pneumoniae*. MIC values of *A. triphylla* EO against *P. aeruginosa* was 0.15 mg/ml, 0.025 mg/ml against *S. pneumoniae* and 0.02 mg/ml against *C. albicans* (Santos et al., 2013).

Table 2. Minimum inhibitory concentrations (MIC) for essential oil from *A. triphylla*. Concentrations are given in µl/ml.

Microbial strains	**MIC (µl/ml)**
E. coli	0.06
Salmonella sp.	-
Proteus sp.	-
Etérobacter sp.	-
Shigella sp.	-
Streptococcus sp.	0.06
S. aureus	0.125
C. albicans	0.07
Lactobacillus sp.	-
Bifidobacterium sp.	-

Antibiotic susceptibility of the isolates was done using the **standardized single disk method** and interpreted according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2013). The result shows that *Salmonella* sp., *Shigella* sp., *S. aureus, C. albicans, Lactobacillus* sp., and *Bifidobacterium* sp. were exhibited multiple antibiotic resistances. Other isolates *E. coli*, *Proteus* sp., *Eterobacter* sp., *Streptococcus* sp. were susceptible to all antibiotics (Figure 3). Antibiotic resistance in both Gram negative and Gram positive bacteria is due to an intrinsic difference in the composition of the cytoplasmic membrane (Witte, 1999).

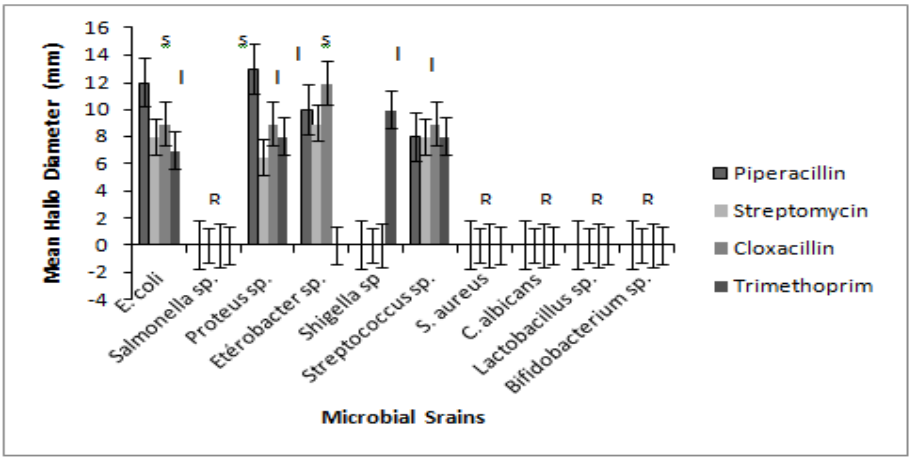

Figure 3. Antibiotic susceptibility profile by a standardized single disk method**.**
R: resistant, S: sensitive, I: intermediate.

The intrinsic resistance of some Gram-negative bacteria to many compounds is due to an inability of these agents to cross the outer membrane (Blair et al., 2015). Lactic acid bacteria are highly adaptable and capable of developing resistance to antibiotics and may have the potential to acquire resistance to other antimicrobials (Mathur and Singh, 2005 ; Humme et al., 2006). According to Gueimonde et al. (2013), bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* present both intrinsic or innate and extrinsic or acquired antibiotic resistance.
Results in figure 4 and 5 show that the *T. vulgaris* essential oil possesses strong antimicrobial properties against *C. albiacns* (MHD≥14mm) and a moderate activity against the rest microbial strains (MHD≤12mm) exsept *S. aureus*, *Lactobacillus* sp. and *Bifidobacterium* sp. were more resistant to all plants Eos. ***Ar. vulgaris*, *Ur. dioica*, and *M. vulgare* showed a moderate activity compared to *T. vulgaris*** (MHD≤10mm)**.** The antifungal and antibacterial potential exhibited by *Thymus* genus essential oil has been demonstrated by several researchers. According to Prasanth et al. (2014), the extract of *T. vulgaris* plant contain high amount of flavonoids that exhibited antioxidant and antibacterial activity against both Gram positive, Gram negative bacteria and fungi. The antimicrobial potentials of EOs depends on their chemical constituents (Mallappa et al., 2016) such as phenolic compounds (thymol) and terpene hydrocarbons (Dorman and Deans, 2000, Rota et al., 2008, Borugă et al., 2014, Swamy et al., 2016).

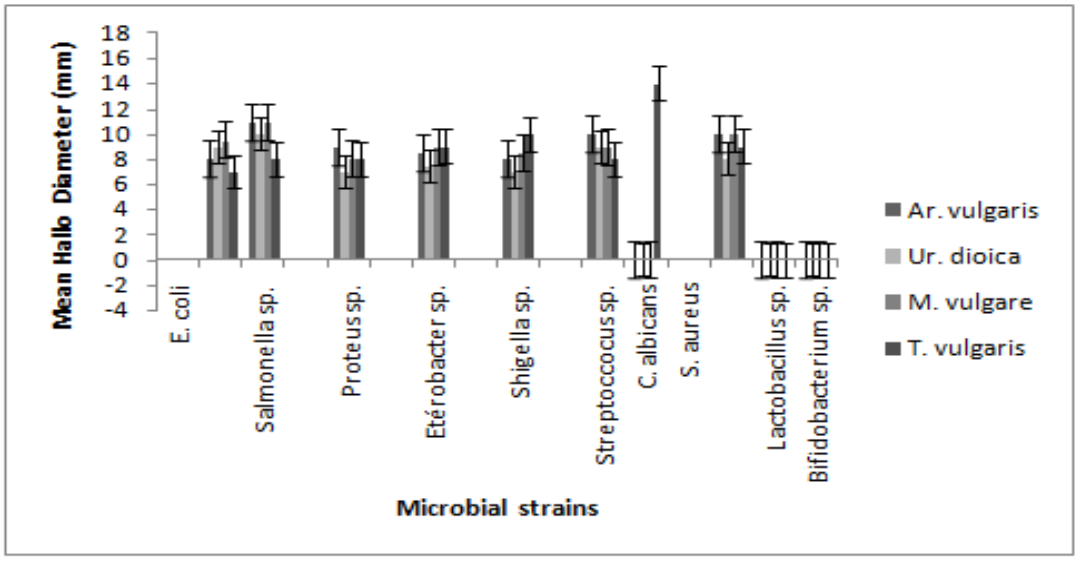

Figure 4. Antimicrobial activity of ***T. vulgaris*, *Ar. vulgaris*, *Ur. dioica*, and *M. vulgare*** in disc diffusion assays.
R: resistant, S: sensitive, I: intermediate.

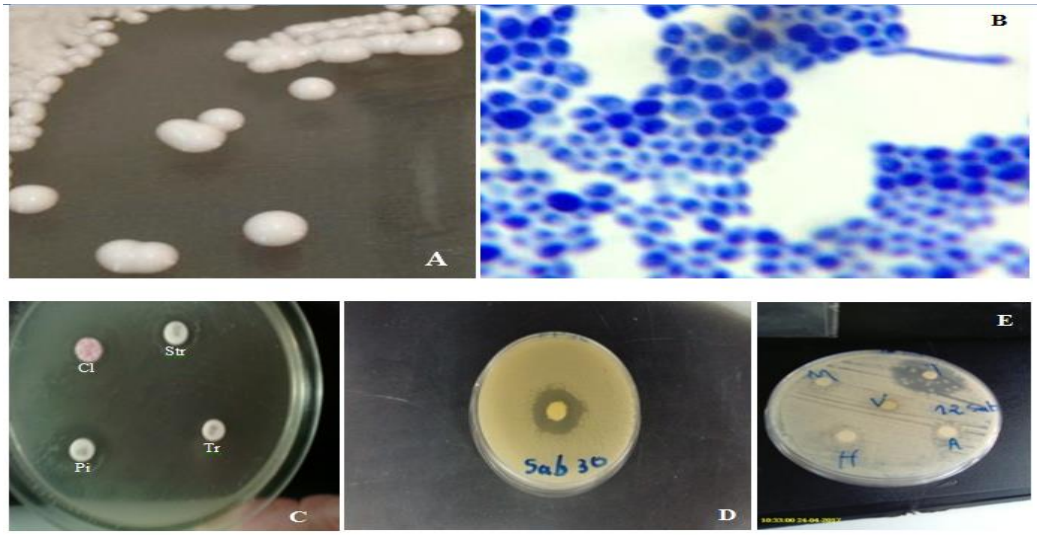

Figure 5. (A, B) Typical colonies of *C. albicans* on YPD. (B) Gram staining of *C. albicans*. (C) **Antibiotic susceptibility profile by a standardized single disk method** against *C. albicans*. (D) Inhibition diameter zones obtained by paper disk diffusion method for **EOs of *A. triphylla* and (E) EOs of *T. vulgaris*, *Ar. vulgaris*, *Ur. dioica*, and *M. vulgare*** against *C. albicans*.

CONCLUSION

Essential oils can be used as an alternative antibacterial and antifungal potentials for pathogens with a problem of resistance to standard antibiotics without therapeutic treatment. These finding justify the use of *A. triphylla* EO in the treatment of genital infections without fear for their impact on survival of probiotic vaginal lactobacilli. It would be interesting to determine the constituent responsible for this biological activity. However, future studies with purified fractions from this oil could uncover more significant biological activities against pathogens. The toxicity study of this plant would also determine the therapeutic index of these oils, so traditional that they could be useful in management of vaginitis.

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