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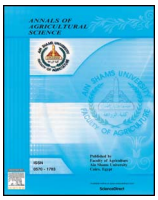

Antibacterial, antibiofilm and antitumor activities of grape and mulberry leaves ethanolic extracts towards bacterial clinical strains

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ABSTRACT

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured at concentrations of 0.01–2.56 mg/mL of grape and mulberry leaves ethanolic extracts. The MIC values were ranged from 0.08 to 0.16 mg/mL against *Ps. aeruginosa* Ps9, and 0.32 mg/mL against each of *S. aureus* St3, *E. coli* Ec3, and *S. typhi* Sa1. Whereas, the MBC values were ranged from 0.32 to 1.28 mg/mL of the tested extracts. The effects of the tested extracts were also studied representing the bactericidal effect of the grape extract with a ratio of 2 against all investigated isolates, except *S. typhi* Sa1. Whereas, the mulberry extract had a bactericidal effect towards *S. aureus* St3 and *E. coli* Ec3 with ratio of 2, and a bacteriostatic effect against *Ps. aeruginosa* Ps9 and *S. typhi* Sa1 with a ratio ≥4. The investigated bacteria found to have a strong ability to form biofilms with densities ranged from 0.67 to 0.80. Both tested extracts inhibited these biofilms with percentages ranged from 48 to 66% at sub-inhibitory concentrations (SICs) ranged from 0.04 to 0.16 mg/mL. In addition, the tested extracts have an excellent cytotoxic activity towards colon cancer cell lines (HCT-16). Five phenolic compounds detected in the tested extracts of grape and mulberry using high performance liquid chromatography (HPLC) after 9.53 min of the retention time. The phenolic compounds of both tested extracts were gallic, coumaric, ferulic, chlorogenic and caffeic with concentrations ranged from 1.28 to 6.56 μg/mL.

Introduction

Despite the tremendous progress in the human medicine, many diseases developed by bacteria are still a major threat to the public health (Mahendiran and Umavathi, 2015). A large numbers of bacterial pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes* are known to be responsible for foodborne infections causing a health risk to human all over the world (Barco et al., 2014; Bloch et al., 2012; Karch et al., 2012; Choi et al., 2014).

Biofilms, or surface-attached communities of cells encapsulated in an extracellular matrix, represent a common lifestyle for many bacteria and also enhanced resistance to antibiotics and other environmental stresses (Sasirekha et al., 2015). The production of these biofilms depends on the component of the growth media, pH, and other biological factors (Sujana et al., 2013). Moreover, biofilm formation occurs in three sequential phases including the specific factor-irreversible attachment to the surface, the production of the extracellular matrix leading to mature biofilms, and the detachment or dispersal (Donlan, 2002). The initial attachment is primarily governed by physicochemical forces such as hydrophobicity and electrostatic forces occurred between the surface of the attachment and attaching microorganism. In addition, bacterial appendages such as flagella and pili, have been shown to be associated with the attachment in the case of *Ps. aeruginosa* (Joo and Otto, 2012).

Phytochemicals are used for treating infection diseases caused by bacterial biofilms (Kubo et al., 2006; Saavedra et al., 2010). These compounds suppress the expression of genes responsible for pathogenesis during interfering with bacterial biofilm formation (Dineshbabu et al., 2015). The inhibition of biofilm formation can be explained by the presence of flavonoids such as quercetin, kaempferol, naringenin and apigenin that have capability of reducing the biofilm synthesis by suppressing the activity of the quorum sensing system functional for cell-to-cell communications (Vikram et al., 2010; Sánchez et al., 2016).

From the dawn of the ancient medicine, chemical compounds derived from plants have been used to treat human diseases. Natural products have received increasing attention over the past 30 years for their potential as novel anticancer agents (Rajendran et al., 2016). Reports on many pharmaceutical plants revealed their importance as an alternative treatment to inhibit cancer cells in many countries around

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the world (Maridass and John de Britto, 2008; Gordaliza, 2009). Currently, an approximate of 60% of drugs used for treating cancer have been isolated from natural products (Gordaliza, 2007). Known recently, most cases of cancer attributes to change in DNA that control the growth, maturation of many cells (Jaikumar and Jasmine, 2016). Such compounds extracted from plants have been investigated for testing their ability to inhibit the growth and initiate apoptosis of cancerous cells (Greenwell and Rahman, 2015). Therefore, there is a demand for alternative treatments naturally derived anticancer agents. The secondary metabolites in some plants such as polyphenols and flavonoids have been investigated for testing their potential effects as anticancer agents. Overall, they have been shown to possess anticancer activities including the antioxidant process, tumor growth inhibition, apoptosis induction and cancer cell cytotoxicity (Gupta et al., 2014; Greenwell and Rahman, 2015).

Beside the well-known pharmaceutical plants (e.g. guava, orange, garlic, onion, lemon, moringa, etc.), other plants such as grape, mulberry, mallow, purslane have been used as antimicrobial and/or antitumor agents (Penecilla and Magno, 2011; Lei et al., 2015; Abdel-Hamid et al., 2017). The extracts of both grape and mulberry were preferred as antibacterial agent than lemon and mallow extracts against *S. aureus*, *Ps. aeruginosa*, *E. coli* and *Salmonella typhi* (Abdel-Hamid et al., 2017).

Here in this study, we identified the bioactive compounds of grape and mulberry extracts using HPLC and evaluated the MIC, MBC and the action mode of grape and mulberry leaf extracts against local human pathogenic bacterial strains. The inhibition of bacterial biofilm and cytotoxic activity by ethanol extracts were investigated.

Materials and methods

Bacterial strains

Four pathogenic bacterial strains (*S. aureus* St3, *Ps. aeruginosa* Ps9, *E. coli* Ec3 and *S. typhi* Sa1) collected from clinical microbiology laboratories, and previously reported by Abdel-Hamid et al. (2017) were selected for this study.

Ethanolic extracts of grape and mulberry leaves

The extracts of grape (*Vitis vinifera*) and mulberry (*Morus alba*) leaves were extracted using 95% ethanol according to a protocol reported by Abdel-Hamid et al. (2017).

Media

Nutrient agar medium (Jacobs and Gerstein, 1960) was used for bacterial culture preservation, while the medium without agar was used for inoculum preparation. Mueller Hinton agar (MHA) medium (Mueller and Hinton, 1941) was used for MBC assay which contained (g/L): meat infusion, 6.0; casein hydrolysate, 7.5; starch, 1.5; agar, 15 and distilled water up to 1000 mL. Mueller Hinton broth (MHB) medium was similar to MHA medium without adding agar, it was used for MIC determination. Luria-Bertani broth (LB) medium was recommended for assessing biofilm formation (Bertani, 1951). This was contained (g/L): peptone, 10; yeast extract, 5; sodium chloride, 5 and distilled water up to 1000 mL. All these media were adjusted to pH 7.2 ± 0.2 and autoclaved for 15 min at 121 °C.

Dulbecco's modified Eagle (DME) medium (Pombinho et al., 2004) was used to maintain the growth of HCT 16 cell line in tissue culture. DME medium was supplemented with (%) 10, fetal bovine serum (SeraLab); 1, antibiotic (penicillin G potassium/streptomycin (Sigma)) and high glucose (Lonza).

Phenolic profile determination by HPLC

The HPLC of the phenolic profiles of the tested extracts of grape and mulberry leaves was measured according to Goupy et al. (1999). In brief, one gram of each ethanol plant extract individually was mixed with 2 mL methanol for 5 min and then centrifuged at 8000*g* for 10 min. The supernatant was filtered using a 0.2 mm Millipore membrane filter and then 1 mL of filtrate was injected into HPLC WinChrome Chromatography (GBC 1100) equipped with an autosampler injector, solvent degasser, GBC ultraviolet (UV) detector set at 280 nm and GBC pump (LC 1110). The column used KROMASIL (150 × 4.6 mm) and incubated at 35 °C. The mobile phase contained methanol: water: tetrahydrofuran: acetic acid with a ratio of 23:75:1:1 at a flow rate of 1 mL/min. The phenolic acid standard (Sigma) was dissolved in a mobile phase, then injected into HPLC. The phenolic compounds concentration was calculated from retention time and peak area. The data were analyzed using WinChrome Chromatography Ver.13 software.

Minimum inhibitory concentration (MIC) determination using resazurin-based microtiter dilution assay (RMDA)

A 96 well microtiter plates (Tarson) were used for RMDA under aseptic conditions. All the wells of the plates were loaded with 100 µL of MHB medium. Twofold serial dilutions were done from stock plant extract. Each well has 100 µL of plant extract in serially descending concentrations, 10 µL of resazurin solution as an indicator and 10 µL of bacterial suspension to obtain a final concentration of 5×10^6 CFU/mL. Each plate was wrapped loosely with cling film to avoid the dehydration of bacterial culture. The microtiter plate included 3 controls as follows: (1) a negative control, without bacterial suspension and presented in columns C1 and C2 and contained the tested plant extracts in a serial dilution + MHB medium + indicator, (2) a positive control in a column C3 without plant extracts which contained bacterial suspension + MHB medium + indicator, and (3) a positive control with antibiotics in a column C4 contained ciprofloxacin in serial dilution + MHB medium + indicator + bacterial suspension (Fig. 1).

After the incubation (37 °C for 24 h), the reactions were visually screened due to changing their colors from purple to pink. MIC value was the lowest concentration (a high dilution) of plant extracts reflecting unchanged color (Gahlaut and Chhillar, 2013).

Determination of minimum bactericidal concentration

The MBC value was detected by sub-culturing the bacteria from broth dilution MIC test (unchanged color) into MHA plates at 37 °C for 24 h. The MBC with the lowest concentration resulted from the inhibition of the growth on the agar plates (Taweechaisupapong et al., 2010).

Evaluating the bactericidal and bacteriostatic effects

The action of ethanol plant extracts as antibacterial activity on selected bacteria can be described by the ratio of MBC/MIC. When the ratio equals to1 or 2, it means bactericidal effect, and when the ratio is ≥4 or 16 it means, bacteriostatic effects (Elshikh et al., 2016).

Evaluation of antibiofilm activity

Detection of biofilm formation

A 96-well microtiter plates (Tarson) were used to assay the ability of bacteria to form biofilms (O'toole and Kolter, 1998) under aseptic conditions. Each well of the microtiter plate containing 50 µL LB broth medium and 50 µL of fresh bacterial suspension (0.5 McFarland). These plates were incubated at 37 °C for 48 h, then the content of each well was carefully removed by tapping the plates and washed with 200 µL of sterile saline for free floating bacteria remove. The biofilms formed by