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Full Length Research Paper

Antibacterial action of an aqueous grape seed polyphenolic extract

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The potential of a polyphenolic grape seed extract for use as a natural antibacterial agent was evaluated. Pure catechin (CS) and a previously LC-MS characterized grape seed phenolic extract (PE) were evaluated as antibacterial agents against *Escherichia coli* and *Brevibacterium linens* on solid and in liquid culture media. After 48 h incubation on solid medium, PE had a bactericidal effect on the gram positive *B. linens* and a reduction of the microbial growth for *E. coli.* The antibacterial agents tested were effective against *E. coli* for 13 h, after 7 h incubation, but ineffective against *B.linens* in liquid medium. CS and PE both had an antibacterial effect depending on incubation time.

Key words: Catechin, polyphenolic extract, antibacterial activity, *Brevibacterium linens*, *Escherichia coli* B_{41}.

INTRODUCTION

Ensuring food safety and at the same time meeting the demands for retention of nutrition and quality attributes has resulted in increased interest in alternative preservation techniques for inactivating microorganisms and enzymes in foods (Tiwari et al., 2009). Quality attributes of importance include flavor, odor, color, texture and nutritional value. This increasing demand has opened new dimensions for the use of natural preservatives derived from plants. Ahn et al. (2007) investigated the effects of a grape seed extract (ActiVin™), pine bark extract (Pycnogenol®), and oleoresin rosemary (Herbalox®) on microbial growth, colour change and lipid oxidation in cooked ground beef. When compared with the control, 1.0% ActiVin™ effectively reduced the numbers of *Escherichia coli* O157:H7 and *Salmonella typhimurium* and retarded the growth of *Listeria monocytogenes* and *Aeromonas hydrophila*.

Grape, one of the world's largest fruit crops, with more than 60 million tons is cultivated mainly as *Vitis vinifera* for wine production (Amico et al., 2004). It is estimated that around 13% of the total weight of grapes used for the wine making results in grape pomace, which is a by-product in this process (Torres et al., 2002).

Dealing with the problem of this waste disposal the wine producers have to balance two major issues given by this high polyphenolic composition, the properties of

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Abbreviations: CS, Catechin standard; **UV-Vis,** spectroscopy ultraviolet-visible spectroscopy; **PE,** grape seed polyphenolic extract; **LC-UV-DAD,** liquid chromatography with ultraviolet diode array detection; **LC-ESI-MS,** liquid chromatography-electrospray ionisation-mass spectrometry; **PBS,** phosphate buffered saline; **KOH,** potassium hydroxide.

germination's inhibition by this compounds which may have an adverse environmental impact (Morthup et al., 1998) and the beneficial effects on human health of polyphenols (Shi et al., 2003; Torres et al., 2002). If conveniently processed, the grape pomace could provide useful products that may balance out waste treatment costs (Amico et al., 2004). One way of using the potential of the grape pomace is the isolation of seeds and extraction of the polyphenols, since the total extractable phenolics in grape are a maximum 10% in pulp, 60 to 70% in the seeds and 28 to 35% in the skin (Shi et al., 2003). The most abundant phenolics isolated from grape seeds are catechins (catechin and epicatechin) and their polymers, the procyanidins (Prieur et al., 1994). Phenolic compounds from grape seeds, as other phenolic compounds have pharmacological and nutraceutical benefits both antiviral and antimutagenic (Saito et al., 1998) that are closely related to their antioxidant and singlet oxygen quenching ability. Recognition of such health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement (Laparra et al., 1979; Soleas et al., 1997). Besides its antioxidant activity, the grape seed extract proved to act also as antibacterial agent (Ahn et al., 2007; Jayaprakasha et al 2003).

Aiming to determine the beneficial properties for the human health of the flavan-3-ols and procyanidines from grape seeds through food and immediate house medical care and also the exploitation of the potential added-value of this by-product, we have carried out a study on the effect of pure catechin and of an aqueous grape seed extract on the viability of *Brevibacterium linens* and *E. coli* B_{41}. *B. linens* was taken in this study for comparing the activity of the polyphenols on a gram positive bacteria with their action on the gram negative *E. coli* B_{41}. The aqueous extract of polyphenols from the grape seeds was analyzed through LC-UV-DAD and LC-ESI -MS and the quantitative analysis of its components was performed previously (Chedea et al., 2011). The data presented here, along with those from other laboratories might be useful information in searching for safe antibacterial agents added in appropriate amounts to the human diet as nutritive supplements having antibacterial properties.

MATERIALS AND METHODS

Chemicals

Catechin standard ((±)-catechin hydrate) was purchased from Sigma Chemical Co., St. Louis, MO (L-8383) and gentamicine 10 µg/microtablet from Himedia, Cluj-Napoca, Romania. Other chemicals used were all analytical grade.

Microbial strains

Catechin standard (CS) and a grape seed polyphenolic extract (PE) were tested against *E. coli* B_{41} a reference strain and *B. linens*. *E. coli* B_{41} was obtained from the collection of microbial strains of the Department of Microbiology, Faculty of Veterinary Medicine, Cluj-Napoca, Romania. *B. linens* was isolated from the fermented cheese nasal, a Romanian brand of maturated cheese and maintained in the same collection mentioned earlier. *B. linens* decompose some proteins and lipids during cheese maturation, giving the specific flavor to nasal cheese.

Bacterial strains were cultured overnight at 37°C in agar. Pre-culture of test bacteria at 37°C with reciprocal shaking for 24 h with a 250 ml peptone broth (Difco Laboratories) produced a stock preparation containing a log-phase cell density of approximately 10^7 colony forming units (CFU)/ml as evaluated initially by measurements of the optical density at 545 nm.

Standard solution preparation (CS)

Catechin standard was solubilized in pure Milli-Q water and added to the bacterial culture to a final concentration of 310 µM (CS).

Extraction and quantification of polyphenols from grape seeds (PE)

The polyphenols from grape seeds were extracted and characterized as previously described (Chedea et al., 2011). The total polyphenol content of the extract was measured by Folin-Ciocalteu method (Singleton et al., 1999). The grape seed extract contains polyphenols expressed as 3.2 g gallic acid equivalent/kg grape seeds.

Antibacterial assay

The antibacterial tests were carried out by disc diffusion method (Pal et al., 2007), using 100 µl of suspension containing 10^7 CFU/ml of bacteria, spread on nutrient agar. In a volume of 2 ml PBS pH = 7, we added 36 µl CS (to a final concentration of 310 µM) or 36 µl PE (to a final concentration of 35 µg total polyphenols /ml medium). The discs (6 mm in diameter) were impregnated with the 25 µl/disc stoc solution of 310 µM CS (3-on the agar plate), respectively, 35 µg polyphenols/ml medium (4-on the agar plate) of grape seed extract PE and placed on the inoculated agar. Negative controls were prepared using dimethyl sulfoxide (DMSO). Gentamicine (10 µg/disc) was used as positive reference standards to determine the sensitivity of each bacterial species tested. Each experimental variant (CS and PE) was placed on the agar plate 3 times. For *B. linens*, potassium hydroxide (KOH) 6 M was also added were the microorganism was present in order to identify the presence of this bacterium. *B. linens* in the presence of KOH give a reddish-brown colour on the agar plates.

The inoculated plates were incubated at 37°C for 48 h. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms. All inhibitory tests were performed in triplicate. The zone of inhibition was calculated in millimetres and the average of three measurements was calculated.

Growth inhibition assay (turbidometry and spectrophotometry)

Ten microliters (10 µl) of logarithmic-phase bacterial cultures (10^7 CFU/ml) in 200 µl nutrient broth was added in each well. Catechin standard (CS), to a final concentration of 310 µM or 36 µl PE to a final concentration of 35 µg polyphenols/ml medium, were co-cultivated with the microorganisms.The cultures were incubated at 37°C for 26 h and growth inhibition was measured by determination of the absorbance at 545 nm. Absorbance readings (545 nm) were taken periodically. A growth curve was plotted with the obtained readings, according to Farouk et al. (2007) method.

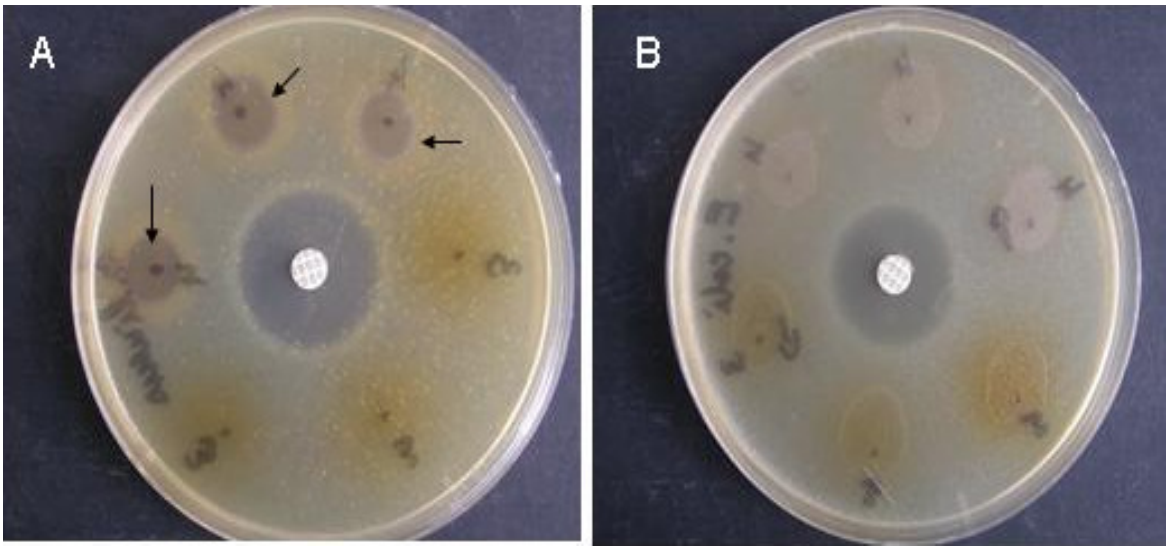

Figure 1. Agar diffusion assay for antibacterial-screening results; (A) against *B. linens* (B) against *E. coli* b_{41}. the arrows indicate the bactericidal effect of PE (discs number 4; discs number 3 represent the CS). 100 µl of suspension containing 10^7 CFU/ml of bacteria were spread on nutrient agar; CS (to a final concentration of 310 µM) and PE (to a final concentration of 35 µg total polyphenols /ml medium) were added. The inoculated plates were incubated at 37°C for 48 h. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms.

RESULTS AND DISCUSSION

E. coli B_{41} and *B. linens* were cultured in liquid and on solid media and CS and PE were added. The composition in polyphenols of the extract tested (PE) was evaluated using the LC-ESI-MS technique and it was found that, epicatechin and catechin are the major compounds in PE, representing together with ECG 60% of total polyphenols, followed by procyanidine dimers (28%) and trimers (12%) (Chedea et al., 2011).

Figure 1a and b, show the effect of CS and PE against *B. linens* and *E. coli* B_{41}. From the figure, we can see that PE (number 4 on the plate) had a bactericidal effect on the gram positive *B. linens* and a reduction of the microbial growth for *E. coli* B_{41} after 48 hours incubation time. The lysis zone in case of PE for *B. linens* was 8.79 ± 0.37 mm.

Measurements of the clear zone around the wells from the antibacterial screening test stage gave a rough estimate of the efficacy of the antibacterial agent and so the growth inhibition was evaluated by spectrometry measuring the turbidity of the liquid cultured bacteria. Absorbance readings obtained from turbidometric analysis helped determine the efficacy of the antibacterial agents over time.

On liquid medium the reading was done up to 26 hours. In this case catechin has a slight better action on *B.linens* after 5 hs, the turbidity of the sample of catechin cocultivated with this bacteria being lower than control, but strongly higher after 7 hours. After 20h of cocultivation with *B. linens* PE has a better action than CS, indicating a bacteriostatic action of this extract at longer incubation time.

The turbidometric analysis showed that the antibacterial agents tested were effective against *E.coli* B_{41} for 13 hs, after 7 hs incubation, but actually stimulating the growth of *B.linens* for the same time period (Fig.2). At 5 hs, test sample absorbance readings for CS in both cases, *B. linens* and *E.coli* B_{41}, were lower than the negative control, suggesting that bacterial cell count at the lag phase was also lower (Fig.2).

Interest in natural antimicrobials has expanded in recent years in response to consumer demand for greener additives. During the last two decades, natural preservatives have been investigated for practical applications.

The applications of natural antimicrobial agents are likely to grow steadily in the future because of greater consumer demands for minimally processed foods and those containing naturally derived preservation ingredients (Tiwari et al., 2009). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Sher, 2009). In a study concerning the protective activity of tea catechins against experimental infection by *Vibrio cholerae* O1, Toda et al., (1992) have shown that epicatechin may disrupt the microbial membrane.

On solid medium screening, after 48 hours incubation time, CS had no bactericidal effect, neither against *B.linens* or *E.coli* B_{41} but reduced the growth rate for both microorganisms tested, with a better action against *B.linens*. In the case of *E.coli* B_{41}. Fig.1B shows that CS

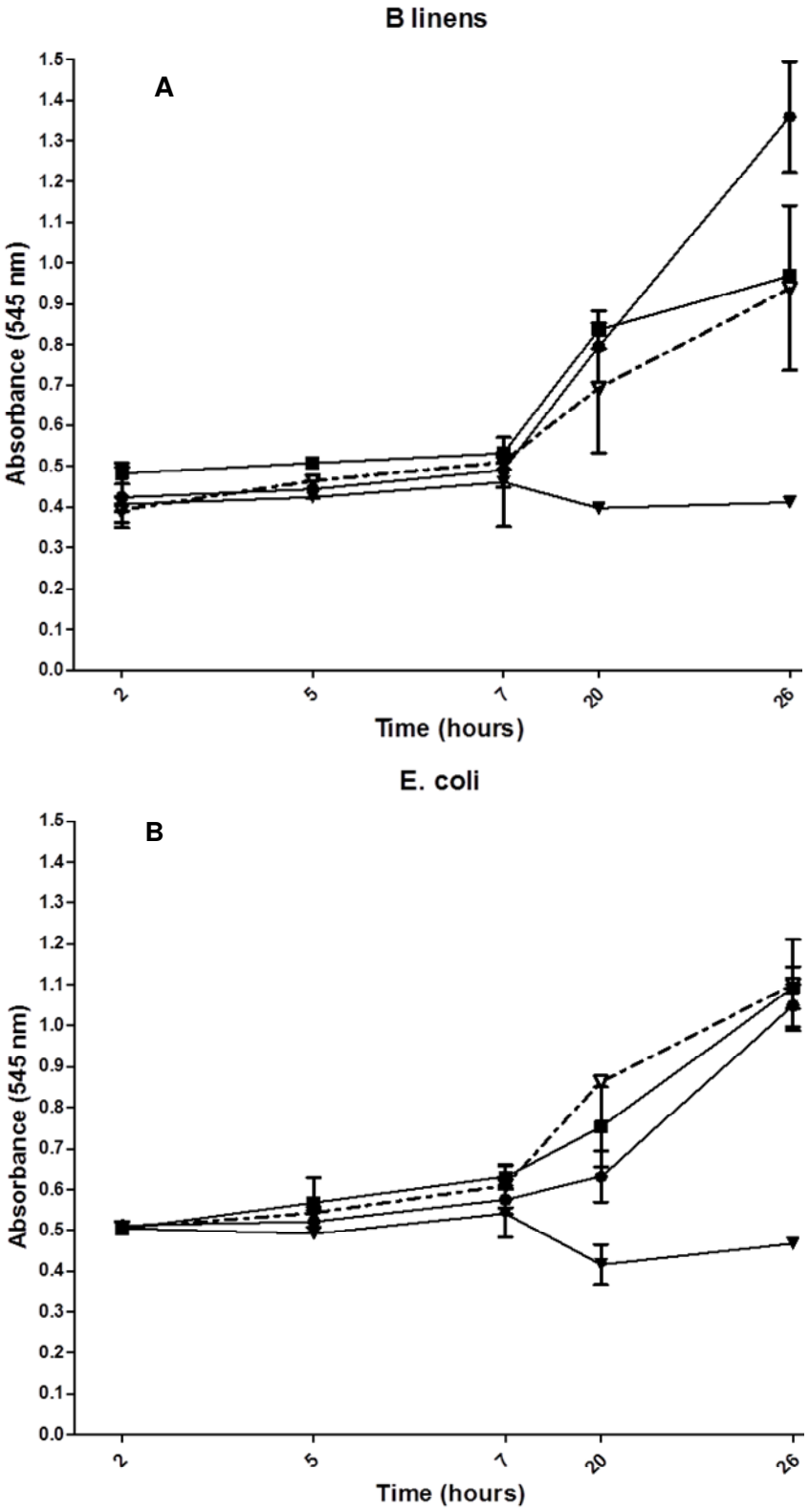

Figure 2. Growth curves of (A), *B. linens* and (B) *E. coli* b_{41} measured by UV-Vis spectroscopy at 545 nm at different time intervals (2, 5, 7, 20 and 26 h), where -●-represents the bacterial co-cultivation with CS (310 µM); -■- the bacterial co-cultivation with PE (35 µg total polyphenols /ml culture medium); -▼- the positive control (antibiotic-gentamicine added on the bacterial culture); -·-▽-·- the negative control (only bacteria).

had a stronger inhibition action than PE on the growth rate of this bacterium. The liquid coculture of studied microorganisms with polyphenols (PE and CS) indicated that the tested antimicrobial agents were mostly ineffective against *B.linens* (Gram-positive bacteria) but having antimicrobial action against *E.coli* B_{41} (Gram-negative bacteria).

Data analysis from both antibacterial screening and turbidometry showed that both CS and PE had antibacterial effect depending on incubation time. Against *B. linens* PE had antibacterial action at longer incubation times. CS had a better antibacterial activity against *E.coli* B_{41} than against *B. linens* and at shorter incubation times.

Our observations confirmed that the grape seed polyphenolic extract, containing beside catechin other components (procyanidins and phenolic acids) may be considered as an alternative natural preservative, either as a food additive, either as simple as a grape seed meal infusion.

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