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Full Length Research Paper

Antibacterial activity and cellulose acetate electrophoresis in monitoring collagen hydrogels modified with saccharides

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Antimicrobial compounds of plant origin (for example fucoidan, lignans, β -glucans, polyphenols), occurring in roots, leaves, flowers and fruits of plants, have demonstrated antitumor antioxidant, antibacterial or antifungal activities. The samples of collagen hydrogels or saccharide incorporated collagen hydrogels (for example, fucoidan from the bladderwrack or β -glucans from the oatmeal fibre) were examined. They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. In electrophoretic analysis, the samples of oat, oatmeal, furoxin (dietary supplement), cranberry juice, reference β -glucan, baker's yeast, and the dried algae from *Fucus vesiculosus* L. were hydrolysed and monosaccharide derivatives were subjected to electrophoresis on a strip of cellulose acetate membrane. Good bacteriostatic properties were determined for samples of partially hydrolysed fucoidan against the pathogenic bacteria *E. coli* and *S. aureus* as well as *Candida albicans*. It was observed that partially hydrolysed fucoidan incorporated into collagen films can be used as therapeutically active biomaterials that speed up the process of wound healing and may increase the anticancer activity of fucoidan. The microbiological procedure of analysing hydrogels can serve as a kind of monitoring to find their antibacterial properties. Cellulose acetate electrophoresis is a useful method of analysing saccharide hydrolysates, solutions of collagen modifier.

Key words: Antimicrobial resistance, carbohydrates, glucan, collagen.

INTRODUCTION

Several traditional plant extracts have historically been known to have antimicrobial activity, to-date there has been relatively little reports examining the activity against several medically important bacterial and fungal pathogens. Plant-derived phytoalexin, isothiocyanates, alllicins, anthocyanins and essential oils (Alzoreky and

Nakahara, 2003; Smith-Palmer et al., 1998; Dorman and Deans, 2000), tannins, polyphenols (Caanadanovic-Brunet et al., 2008) and terpenoids have demonstrated antibacterial or antifungal activities. They are all useful in treating some forms of cancer and have styptic and antibacterial properties that can assist wound healing

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(Borchardt and Wyse, 2008). The *in vitro* antimicrobial activity of the marine green algae *Ulva lactuca* and brown seaweed *Phaeophyceae* were examined against Gram-positive bacteria, Gram-negative bacteria and a fungus. This result confirms the potential use of seaweed extracts as a source of antibacterial compounds and a health-promoting food (Chotigeat et al., 2004; Kim et al., 2007; Sandsdalen et al., 2003). Cranberries are also distinguished for their antimicrobial properties. They are a particularly rich source of phenolic phytochemicals, including phenolic acids (benzoic, hydroxycinnamic and ellagic acids) and flavonoids (anthocyanins, flavonols and flavan-3-ols) (McKay and Blumberg, 2007). Cranberries are distinguished by a wide spectrum of bioactive substances. American cranberries contain ascorbic acid, phenolic compounds, titratable acids and sugars (Povilaitytė et al., 1998; Úwieczkowska et al., 2004).

Polysaccharides may be a useful material as a skin scaffold with the capability of delivering molecules involved in wound healing; some polysaccharides even improve wound healing (Paulsen, 2002; Yang et al., 2009). It has been found that polysaccharides, crude or purified, are strong scavengers of free radicals as well as inhibitors of liposome peroxidation. Some of them exhibit various biological activities such as anti-inflammatory, anticancer, antiviral, spasmolytic, antibacterial, antioxidant or immunostimulatory (Paulsen, 2002; Yang et al., 2009). A number of studies have concentrated on the anticancer activity of fucoidan polymers (Yang et al., 2009). Until fairly recently, it was believed that only the low molecular weight compounds in plants could be of importance in pharmacy and medicine. Fucoidan and especially, partially hydrolysed fucoidan isolated from different species have been extensively studied on account of their varied biological properties, including anticoagulant and antitumor effects. It was especially thought that the polysaccharides might play a role in wound healing, both internally and externally, and also that they could play a role against inflammation.

Mostly natural polysaccharides have been investigated as biomaterials, such as chitosan, cellulose, collagen and hyaluronic acid. The anticancer properties of the lignans are well documented. Beta-glucan is a major component of the water-soluble dietary fibre of oats and barley. A glucan of cellular origin (Ishurd and Kennedy, 2005) has been isolated from Libyan dates (*Phoenix dactylifera* L). Glucans have been found to exhibit potent antitumor activity that could be correlated to their (1-3)- β -D-glucan linkages (Olafsdottir et al., 1999).

Natural polymers like collagen possess good cytocompatibility which makes them a popular choice for tissue engineering scaffolding applications. Scaffolding materials for cell proliferation and differentiation is one of the key technologies for tissue engineering. Three-dimensional engineered collagen matrixes are fabricated with or without peptide modification and chemical cross-linking

with glutaraldehyde mimicking *in vivo* like conditions (Hosseinkhani et al., 2013a; Hosseinkhani et al., 2013b). A collagen sponge and a 3D hybrid scaffold (Hosseinkhani et al., 2006a) are highly porous with interconnecting openings, good for cell infiltration and for supplying oxygen and nutrients to cells. However, its usage has been limited by poor mechanical properties. To improve these, a novel porous scaffold for bone tissue engineering was prepared with collagen sponge reinforced by polypropylene/polyethylene terephthalate (PP/PET) fibres (Mohajeri et al., 2010).

Incorporation of PP/PET fibres not only improves the mechanical properties of collagen sponge, but also enables mesenchymal stem cells to positively improve their proliferation and differentiation. To improve the mechanical properties of collagen, it is also necessary to fabricate composites of collagen with electrospun poly (glycolic acid) PGA fibres (Hosseinkhani et al., 2010a). PGA/collagen nanofibres fabricated through electrospinning significantly enhance cell adhesion compared with PGA/collagen microfibrils (Hosseinkhani et al., 2010a). The incorporation of nanoparticles into nanofibre sheets is a very promising strategy to genetically engineer mesenchymal stem cells, and it can be used for further applications in regenerative medicine therapy (Hosseinkhani et al., 2012, 2005).

Collagen sponge reinforced by incorporation of poly(glycolic acid) (PGA) fibres was selected as the cell scaffold and was used to evaluate effect of the 3D culture system and cell scaffold type on the transfection efficiency of DNA nanoparticles on mesenchymal stem cells (MSC). Positively charged DNA nanoparticles can interact electrostatically with the cell membrane for internalization (Hosseinkhani et al., 2006b, 2010b). These findings provide an attractive combined strategy of tissue engineering principles with gene therapy for tissue regeneration. With their ability to serve as gene carriers, the nanoparticles are poised to play an important role in the field of regenerative medicine. Collagen also has plenty of applications in the field of wound healing due to its biodegradability and biocompatibility. Implanted collagen is degraded through native enzymatic pathways without any toxic response. There is a growing interest in collagen as a drug delivery vehicle. It seems that the near future may be a renaissance period for collagen, an excellent natural, fully biocompatible and biodegradable drug carrier material. These technological possibilities open a new perspective, especially in creation of product utilizing antibacterial drug for either prophylactic or therapeutic use (Ruszczak and Friess, 2003).

The aim of this research is to find biologically active, exhibiting antibacterial activities, forms of polysaccharides such as fucoidan from the bladderwrack and β -glucans from the oatmeal fibre. Finally, the goal is to obtain hydrogels that can be used as biologically and therapeutically active biomaterials.

MATERIALS AND METHODS

Reagents

The following materials and reagents were used in the examination: reference crude fucoidan from *F. vesiculosus* L (CAS Number 9072-19-9, Sigma–Aldrich, Poland); Flos algae from *F. vesiculosus* L. packed and distributed by Zakład Zielarski, Piotrków Trybunalski, Poland; Furoxin dietary supplement produced by pharmaceutical company LEK-AM, Zakroczyn, Poland; reference β -glucan produced by Walmark, a.s. Oldrichovice, Třinec, Czech Republic; oat, oatmeal and cranberry juice.

Collagen film formation

Fresh carp fish scales were collected, washed thoroughly and stored at -25°C until used. First, they were washed twice in 10 wt% of NaCl solutions to remove unnecessary proteins on the surface by stirring the solution for 24 h. Demineralization was achieved with 0.4 M HCl solution (dry scales: solution = 1:15) for 90 min. The demineralized scales were then washed three times with distilled water for collagen extraction. All the preparative procedures were performed at 4°C . Carp fish scales were extracted with 0.5 M acetic acid for two days, and the extracts were centrifuged for 30 min. The residues were re-extracted with the same solution for one day and these extracts were centrifuged under the same conditions. The supernatants were combined and salted out by adding NaCl to a final concentration of 0.7 M. The precipitated collagens were separated by centrifugation for 30 min and redissolved in 0.5 M acetic acid to precipitate with NaCl again. The samples (0.5 g) of oat, oatmeal, cranberry juice and the dried algae from *F. vesiculosus* L. were extracted with 0.1 M H_2SO_4 and maintained at 80°C or in boiling water with constant mechanical stirring for 30 min. The extract was centrifuged for 30 min and the supernatant was collected. All reagents used were of analytical grade. The samples of collagen hydrogels or saccharide incorporated collagen hydrogels (for example, fucoidan from the bladderwrack and β -glucans from the oatmeal fibre) were examined.

Agar dilution method

They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive *S. aureus* and Gram-negative *E. coli*. Physiological salt (2 cm^3) was poured into two sterile test tubes. Using a sterile (red hot) inoculation loop, *E. coli* sample was taken from its culture on enriched agar (used for growing particularly demanding bacteria strains), inserted into one of the test tubes and diluted in the salt. Using a pipette, 3 drops of the suspension were transferred onto enriched agar; then, using a cooled sterile bacteria spreader, they were spread all over the agar surface. After that, the spreader was sterilized again and 3 drops of collagen hydrogels or saccharide incorporated collagen hydrogels were placed in the centre of the Petri plate, using a dropper. The same procedure was repeated for *S. aureus*, which was placed on mannitol salt agar (containing 7.5% NaCl for inhibiting the growth of other bacteria). The Petri plates were subsequently placed in a tube and then kept in a laboratory heater at 37°C for 24 and 48 h. Collagen hydrogels or saccharide incorporated collagen hydrogels were exposed to the same bacteria: *E. coli* on MacConkey agar (containing salts of bile acids and crystal violet inhibiting the growth of Gram-positive bacteria) and *S. aureus* on mannitol salt agar (with high concentration of NaCl inhibiting the growth of other bacteria). The samples were kept in a laboratory heater at 37°C for 24 h, and then photos were taken (Tables 1 to 3 and Figure 1).

Partially hydrolysed fucoidan acid extracts produced from the common bladder wrack were examined (Figures 2 to 4): collagen hydrogels-pure collagen (PC); partially hydrolysed fucoidan (from algae) incorporated into collagen films; 15 min incubation (H1s), 30 min incubation (H2s). They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive *S. aureus* and Gram-negative *E. coli* and *C. albicans*. Chosen species of microorganisms, *E. coli*, *S. aureus* and *C. albicans*, were being bred on MacConkey, Chapman and Candida agars. Grown cultures were washed out with 1 ml of physiological salt solution, and added to the sterile broth nutrient sample. This was sterilized for 30 min in an autoclave at a temperature of 121°C , and a pressure of 150 kPa. The samples were cultivated in a thermostat at 37°C and tested for antibacterial activity against the sensitive strain of *S. aureus*, *E. coli* and *C. albicans* at one-day intervals up to 1 month (Figures 2 to 4).

Statistical analysis

The results presented here were performed in 10 stages, and arithmetic average and standard deviation were carried out. Standard deviation was determined according to estimator of highest credibility in STATISTICA 6.0.

Electrophoretic analysis

In electrophoretic analysis, the samples (0.2 g) of oat, oatmeal, furoxin dietary supplement, cranberry juice, reference β -glucan, baker's yeast and the dried algae from *F. vesiculosus* L. were hydrolysed into component monosaccharides with 80% H_2SO_4 at 0°C for 24 h, and monosaccharide derivatives were subjected to electrophoresis on a strip of cellulose acetate membrane. The samples (1 μl non-precipitated extract) and references were subjected to electrophoresis on a strip of cellulose acetate membrane (CA-SYS-MINI Cellulose Acetate Systems) in 0.2 M $\text{Ca}(\text{OAc})_2$ (pH 7.5) at 7 mA, max. 240 V for 1.5 h. The strips were stained with 0.5% toluidine blue in 3% HOAc solution and then rinsed in distilled water and air-dried. Semi-quantitative analysis of monosaccharides content in the samples was also conducted using GelScan v.1.45 software (Kucharczyk T.E., Poland) (Figures 5 to 7).

RESULTS

Partially hydrolysed fucoidan incorporated into collagen films can be used as therapeutically active biomaterials that speed up the process of wound healing and may increase the anticancer activity of fucoidan. This thesis, present in many ongoing studies worldwide, was the basis of this investigation.

Three series of semi-quantitative studies of hydrogels placed directly on agars or on paper discs are discussed here (Tables 1 to 3). Antibacterial properties of the modified hydrogels were compared with a reference sample of hydrogels. For collagen gels modified with bladderwrack (*F. vesiculosus* L.) extracts in 0.1 and 0.01 M H_2SO_4 , in distilled water and in 0.1 M NaOH, the inhibition zones are within 6 to 10 mm against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria (Table 1). In the investigated group of gels, the best bactericidal properties, with a 10 mm inhibition zone, can be found in collagen gels

Table 1. Antimicrobial activity caused by flos extract (water or acid fraction) through agar diffusion method.

Parameter	Microorganisms	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Sample 1: Collagen hydrogels in 0.1 m NaOH	-	-
Sample 2: Collagen hydrogels in 0.1 m H ₂ SO ₄	-	+
Sample 3: Flos incorporated collagen hydrogels in 0.1 m H ₂ SO ₄	+	+
Sample 4: Flos incorporated collagen hydrogels in 0.01 m H ₂ SO ₄	+	+
Sample 5: Flos incorporated collagen hydrogels in H ₂ O	+	+
Sample 6: Flos incorporated collagen hydrogels in 0.1 m NaOH	+	+

(+) susceptibility (inhibition zone > 6 mm), (-) absence of susceptibility.

Table 2. Antimicrobial activity caused by plant extracts: Fucoidan and Furoxin (water or acid fraction) through agar diffusion method.

Parameter	Microorganism	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Sample 1: Collagen hydrogels in 0.1m H ₂ SO ₄	-	-
Sample 2: Fucoidan incorporated collagen hydrogels in H ₂ O	-	+
Sample 3: Fucoidan incorporated collagen hydrogels in 0.1m H ₂ SO ₄	+	+
Sample 4: Fucoidan incorporated collagen hydrogels in 0.01m NaOH	+	+
Sample 5: Furoxin incorporated collagen hydrogels in 0.1m H ₂ SO ₄	+	+

(+) susceptibility (inhibition zone > 3 mm), (-) absence of susceptibility.

Table 3. Antimicrobial activity caused by plant extracts: β -glucan and cranberry juice (water or acid fraction) through agar diffusion method.

Parameter	Microorganism	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Sample 1: Collagen hydrogels in H ₂ O	+	+
Sample 2: Reference β -glucan incorporated collagen hydrogels in H ₂ O	-	+
Sample 3: β -glucan (from oat) incorporated collagen hydrogels in H ₂ O	+	+
Sample 4: β -glucan (from oat) incorporated collagen hydrogels in 0.1 m H ₂ SO ₄	+	+
Sample 5: Cranberry juice incorporated collagen hydrogels in H ₂ O	-	+
Sample 6: β -glucan (from oatmeal) incorporated collagen hydrogels in 0.1 m H ₂ SO ₄	+	+

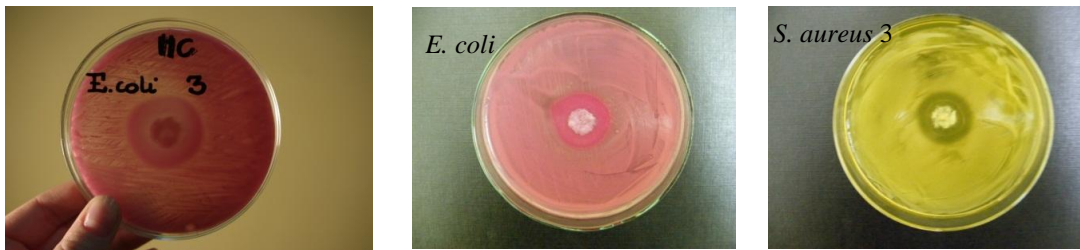
(+) susceptibility (inhibition zone > 2mm), (-) absence of susceptibility

modified with bladderwrack extracts in 0.1 and 0.01 m sulfuric acid (VI).

Do the antibacterial properties of the hydrogels obtained depend on the type of modifier? To answer this question, the properties of gels obtained using *F. vesiculosus* L. extracts (Table 1) were compared with those obtained using reference fucoidan extracts (Table 2). When analysing the results presented in Tables 1 and 2, it should be said that fucoidan solutions as the modifier of collagen hydrogels are responsible for the bactericidal properties of these gels. Examples of increasing inhibition zones are shown in Figure 1, while Tables 1 and 2 provide complete information on the antimicrobial properties

of the modified hydrogels against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria.

Do the antibacterial properties of the hydrogels obtained depend on the type of modifier? To verify this, the properties of gels obtained using extracts of oats and oatmeal as the source of β -glucan were compared with those obtained using β -glucan extracts from a dietary supplement (Table 3) as well as using cranberry extracts from Furoxin dietary supplement (Table 2) and cranberry juice (Table 3). For collagen gels modified with β -glucan extracts in 0.1 m H₂SO₄ and in distilled water, the inhibition zones are within 1 to 3 mm against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria (Table 1).



Sample 3. Flos incorporated collagen hydrogels in 0.1m H₂SO₄

Sample 3. Fucoidan incorporated collagen hydrogels in 0.1m H₂SO₄

Figure 1. Antimicrobial activity caused by flos and fucoidan extracts (acid fraction) through agar diffusion method.

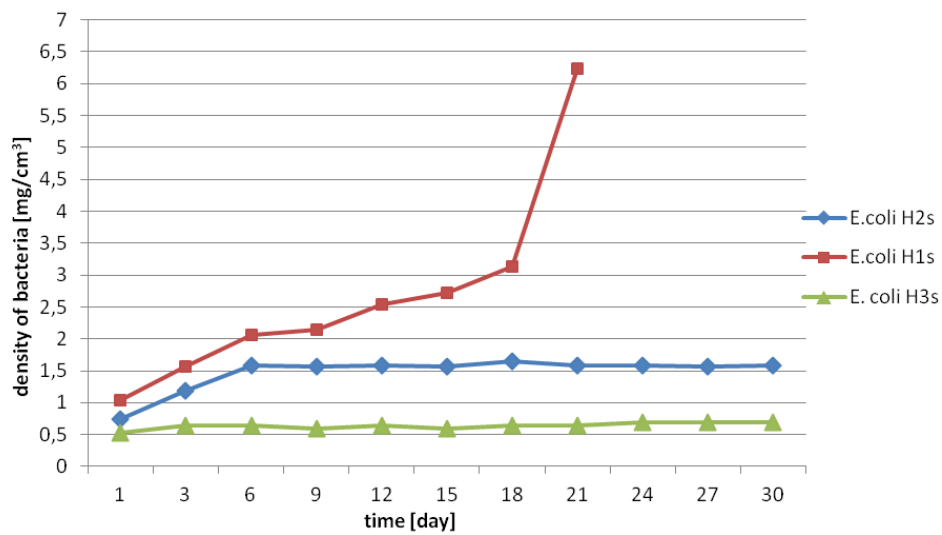


Figure 2. Change of density *E. coli* biomass in time.

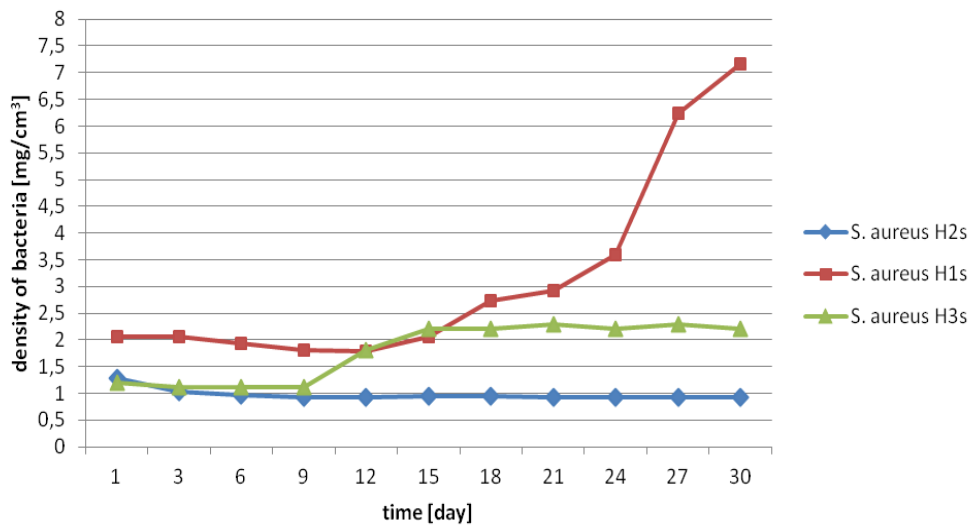


Figure 3. Change of density *Staphylococcus aureus* biomass in time.

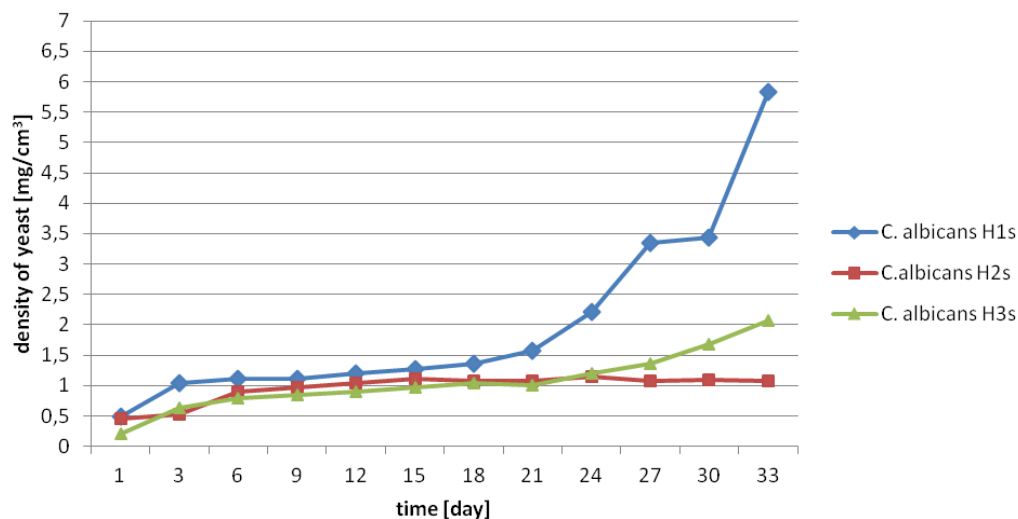


Figure 4. Change of density *Candida albicans* in time.

It should be noted that in this study of β -glucan extracts the method of placing diluted hydrogels on paper discs was adopted. Also in this case, the gels reveal good antibacterial properties (Table 3).

A variable sensitivity of *S. aureus*, *Enterococcus faecalis* and *Micrococcus luteus* to proanthocyanidin-rich fractions was also observed (Leitão et al., 2005). It was found that the extracts from common Finnish berries (blueberry, raspberry, lingonberry, blackcurrant, cloudberry, cranberry, sea buckthorn berry and strawberry) inhibited the growth of Gram-negative bacteria, while Gram-positive bacteria were quite resistant (Puupponen-Pimiä et al., 2001). However, other researchers reported that there was no correlation between Gram-positive or Gram-negative bacterial status and their sensitivity to the raspberry, blackberry, cranberry and black currant fruits (Cavanagh et al., 2003). For collagen gels modified with cranberry extracts in 0.1 m H_2SO_4 and in distilled water, the inhibition zone is 5 mm against Gram-negative (*E. coli*) bacteria (Table 2; Furoxin) while for collagen gels modified with cranberry juice extracts, the inhibition zone is 3 mm against Gram-positive (*S. aureus*) bacteria (Table 3; cranberry juice; on paper discs). Generally, the antibacterial activity of all samples is better against *S. aureus* than against *E. coli*.

As stated earlier, the object of this study was partially hydrolysed fucoidan incorporated into collagen films that can be used as biologically active biomaterials. Partially hydrolysed fucoidan incorporated into collagen films was investigated in wound dressings against the pathogenic bacteria *E. coli* and *S. aureus* and also *Candida albicans*. Figures 2 to 4 show partially hydrolysed fucoidan acting

against tested bacteria and yeast. This antibacterial and fungicidal activity depends on the conditions of the hydrolysis. Microbiological analysis revealed, in particular, density changes of the suspensions of *E. coli*, *S. aureus* and *C. albicans* (Figures 2 to 4) in contact with partially hydrolysed fucoidan (H2s and H3s) obtained by 30- or 60 min hydrolysis of 0.1 m H_2SO_4 . H2s and H3s samples were demonstrated to exhibit better bacteriostatic properties compared with the partially hydrolysed fucoidan (H1s) obtained by 15 min hydrolysis of 0.1 m H_2SO_4 . For H1s samples, increased density of bacteria and yeast was found, starting on the 18th day of incubation, while samples H2s and H3s essentially retained constant density of *E. coli* and *S. aureus*.

The microbiological procedure of analysing hydrogels, as discussed above, can serve as a kind of monitoring to find their antibacterial properties. Key to this study is also selecting a therapeutic concentration of the modifier solution, promoting antibacterial properties of hydrogels. In this investigation, cellulose acetate electrophoresis is a useful method of analysing saccharide hydrolysates, solutions of collagen modifier. In electrophoretic analysis, a polysaccharide is hydrolysed into component monosaccharides with 80% H_2SO_4 at 0°C for 24 h, and monosaccharide derivatives are subjected to electrophoresis on a strip of cellulose acetate membrane. Representative examples of this analysis are presented in Figures 5 and 6.

Based on the analysis of these electrophoregrams, it can be said that CAE is a convenient analytic medium for monitoring the presence of saccharides in samples of herbs, algae, food products and dietary supplements. In

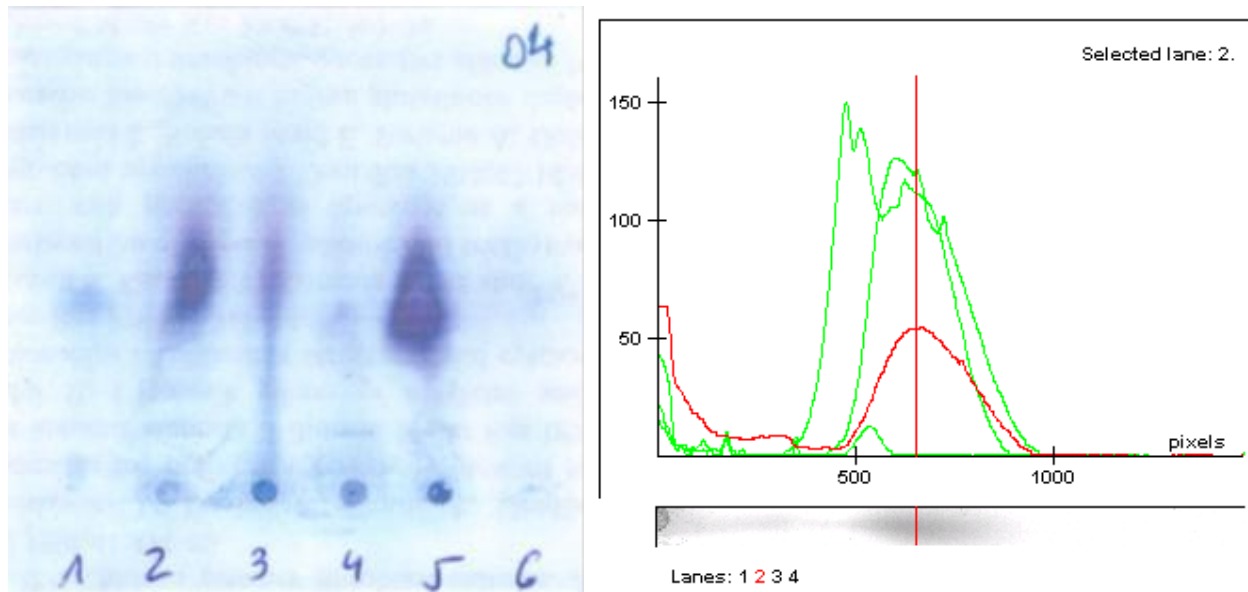


Figure 5. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of hydrolysates (0.2 g of substance, 1 ml 80% H_2SO_4): from left to right: oatmeal, oat, baker's yeast, reference β - glucan.

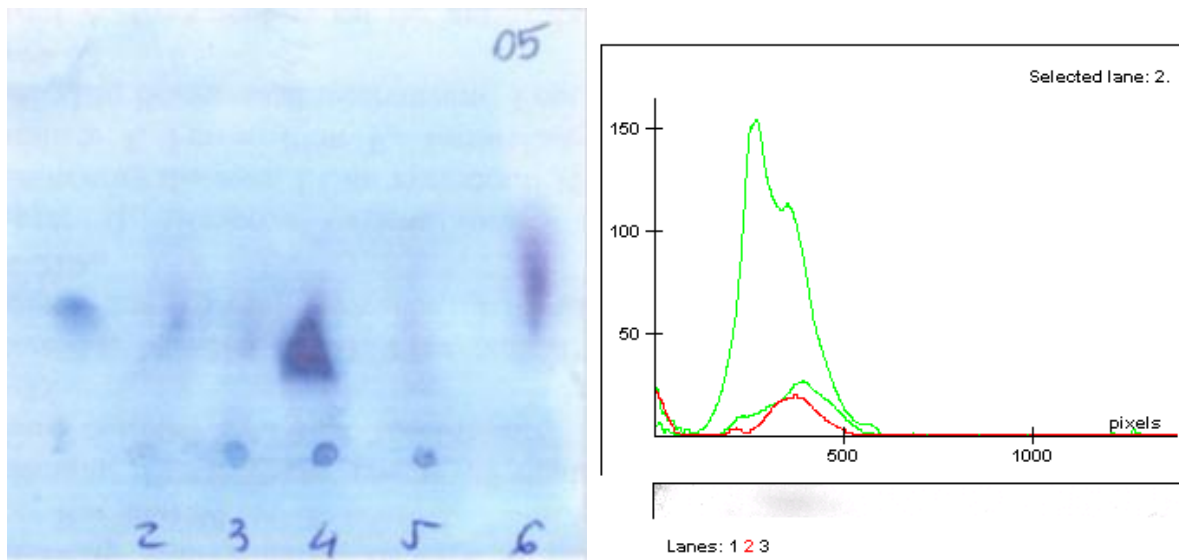


Figure 6. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of hydrolysates (0.2 g of substance, 1 ml 80% H_2SO_4): from left to right: oatmeal, oat, reference β - glucan, baker's yeast.

the electrophoretic tests shown in Figure 7, the same concentrations of modifiers were selected as for the microbiological tests (that is, extraction at 80°C for 30 min in an aqueous medium or in 0.1 m H_2SO_4). CAE was performed on hydrolysate solutions in 80% H_2SO_4 . Two distinct bands were observed: from an extract of *F. vesiculosus* L. (fucoidan) and from an extract of Furoxin,

a dietary supplement (source of phenolic phytochemicals). No electrophoretic bands were found for the extracts of β -glucan, cranberry juice and oatmeal (Figure 7). The identification of electrophoretic bands for fucoidan and Furoxin can be explained by a weaker binding of sugars with the cell wall in herbs and algae, while the extraction and release of β -glucans is more

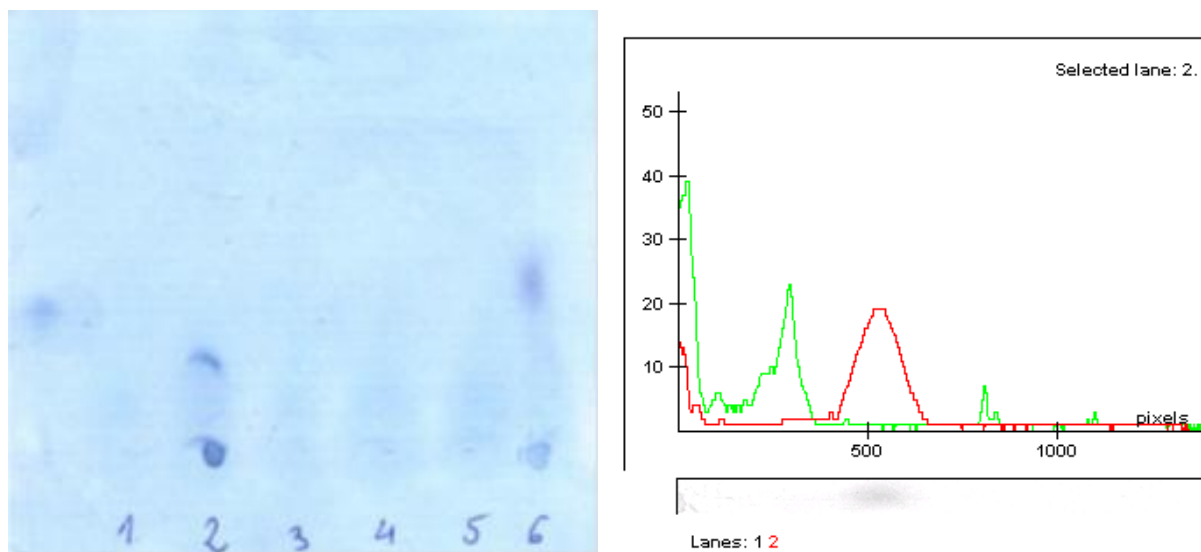


Figure 7. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of hydrolysates : from left to right: reference β – glucan, furoxin, cranberry juice, oatmeal, *F. vesiculosus* L.

complex and requires a more aggressive environment.

DISCUSSION

The aim of this research is to find biologically active and therapeutically desirable chemical forms of polysaccharides such as fucoidan from the bladderwrack and β -glucans from the oatmeal fibre. The aim is also to develop a series of solutions and gels exhibiting antibacterial and potentially anticancer activities. Finally, the goal is to obtain hydrogels that can be used as biologically and therapeutically active biomaterials. For marking fucoidan in the bladderwrack and monosaccharides herbs and the fibre from oat, CAE electrophoresis has been selected with a system of computer image analysis. Research will be complemented by microbiological analyses to prove the influence of hydrolysates and gels on pathogenic Gram-negative bacteria *E. coli* and Gram-positive *S. aureus*.

As stated earlier, this study concentrated on partially hydrolysed fucoidan incorporated into collagen films that can be used as biologically active biomaterials. The reason for this choice is as follows: Fucoidans isolated from different species have been extensively studied because of their varied biological properties, including anticoagulant and antitumor effects. Their anticancer activity can be significantly enhanced by lowering their molecular weight only when they are depolymerized under mild conditions. Many modern wound dressings have a variety of properties that are designed to create an environment to encourage conditions that support

wound healing.

Since bacteria are often present in high numbers in wound fluid, it is also important that dressings with high fluid retention levels be able to absorb and retain bacteria. Once the skin barrier is broken, there is a much greater risk for infection as the majority of wounds provide a favourable environment for both aerobic and anaerobic bacteria. In a bacteria-free wound, infection obviously cannot occur. The prevention of wound infection and a reduction in cross-infection of wound pathogens are primary concerns in infection control. It is crucial to investigate the possibility of producing antimicrobial films or biomaterials for modern wound dressings by incorporation of AgNO_3 and H_4SiO_4 or fucoidan and partially hydrolysed fucoidan (Pielesz et al., 2011b).

Do the antibacterial properties of the hydrogels obtained depend on the type of modifier? Modifying collagen with polysaccharide solutions was meant to obtain not only an antibacterial dressing, but one that would also reveal antitumor activity. Until fairly recently, it was believed that only the low molecular weight compounds in plants (Yang et al., 2008) could be of importance in pharmacy and medicine. Fucoidan and especially, partially hydrolysed fucoidan isolated from different species have been extensively studied on account of their varied biological properties, including anticoagulant and antitumor effects. The objective was to analyse, as reported in the literature, the anticancer activity of partially hydrolysed fucoidan polymers (Koyanagi et al., 2003; Matou et al., 2002; Yang et al., 2008; Zemaniet al., 2005) and to investigate the effects of molecular weights, also known from the literature (Matou

et al., 2002; Zemani et al., 2005) and different hydrolysis conditions on potential inhibition of cancer-cell growth. This study continues earlier analyses of structural changes in fucoidan from *F. vesiculosus* L., examined by means of cellulose acetate membrane electrophoresis and Fourier transform infrared spectroscopy spectroscopy (Pielesz et al., 2011a).

In the investigated group of gels, the best bactericidal properties, with a 10 mm inhibition zone, can be found in collagen gels modified with bladderwrack extracts in 0.1 and 0.01 M sulfuric acid (VI). Without any doubt, methodology based on mild acid hydrolysis can be used as an efficient tool for studying the relationship of biological activities of partially hydrolysed fucoidan. In an earlier study (Pielesz, 2011a), it was determined that the formation of well-defined molecular-size oligosaccharides is notably dependent on the particular structure of the sulfated polysaccharides. Therefore, the specific cleavage of the polysaccharides by mild acid hydrolysis was influenced specifically by their pattern of sulfation. The band shifts observed in electrophoretograms and Raman wavenumber shifts (Pielesz et al., 2011a) may indicate increased anticancer activity of fucoidan, which remains to be determined in subsequent investigations. This study confirms the suggestions made in (Pielesz et al., 2011a).

Generally, the antibacterial activity of all the samples is better against *S. aureus* than against *E. coli*. This contrasting response towards the Gram-positive and Gram-negative bacteria could be attributed to the morphological differences between these bacteria. Indeed, the structure of the cell wall of the Gram-negative strain (*E. coli*) is much more complicated than that of the Gram-positive strain (*S. aureus*) because there is another layer outside the peptidoglycan layer, called “the outer membrane,” which is mainly constructed from tightly packed lipopolysaccharide molecules, resulting in the effective resistive barrier against foreign compounds attack (Liu et al., 2006).

Conclusions

The microbiological procedure of analysing hydrogels can serve as a kind of monitoring to find their antibacterial properties. There is no doubt that methodology based on acid hydrolysis can be used as an efficient tool for studying monosaccharides and their biological activities. The advantage of this study was that a simple, repeatable analytical procedure was developed, using modern but inexpensive apparatus such as cellulose acetate membrane electrophoresis.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

A new multivariate similarity factor for *in vitro* therapeutic equivalence assessment

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Pharmaceutical equivalence is an important issue in the regulatory approval of generic and similar drug products, particularly for those that will not be tested for bioequivalence. However, there is no scientific approach that provides us an objective measure of quality and similarity of the results obtained for testing (generic or similar) and branding drug products simultaneously. This paper describes a new multivariate similarity factor for the assessment of *in vitro* therapeutic equivalence between two medicines by using pharmaceutical equivalence study. We performed pharmaceutical equivalence studies for acyclovir cream, metronidazole injection, meropenem for injection and atropine sulfate injection. All tests and assays results were standardized using an appropriate desirability function. Multivariate similarity factors for pharmaceutical studies were calculated based on individual acceptance factors and similarity deviations for brand, generic, and similar drugs. We found a perfect correlation among multivariate similarity factor and regulatory requirements. The multivariate similarity factor is a useful tool for *in vitro* therapeutic equivalence assessment, and may be used for regulatory approval of generic and similar drugs.

Key words: Therapeutic equivalency, *in vitro*, multivariate analysis.

INTRODUCTION

Drug access provision is a worldwide concern, and generic drug products play an important role in this issue. Generic drug products increase market competition, provide a more effective price control, promote national industrial development and allow physicians and patients to choose among different manufacturers (Dias and Romano-Lieber, 2006; Rumel et al., 2006). In Brazil, the generic drug products are part of the drug national policy, which obligates the government to provide drugs for

Brazilian citizens (Rumel et al., 2006; Brasil, 1999). Since the introduction of generic drug products in pharmaceutical market, Brazilian's regulatory agency (Agência Nacional de Vigilância Sanitária – ANVISA) approved about 3,495 new generic drug products (Rumel et al., 2006; Brasil, 1999, 2007a). Also, similar drug products (non-generic copies of reference drug products) are important for Brazilian's drug national policy (Brasil, 2007b). Currently, ANVISA classifies drug products as: brand, generic, si-

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milar, biological, phytotherapics, specifics and new drugs. However, about 60% of drug products marketed in Brazil are generic and similar drug products.

Even though, there are some concerns about the efficacy, safety and quality of generic and similar drug products (Agudelo and Vesga, 2012; Endrenyi and Tothfalusi, 2010; Gauzit and Lakdhari, 2012; Tschabischer et al., 2008; Bialer, 2007; Kesselheim et al., 2008; Borgherini, 2003; Davit et al., 2009; Meredith, 2003; Durden and Hughes, 2010; van Wijk et al., 2006). According to ANVISA requirements, both generic and similar drug products have to confirm inter-changeability by pharmaceutical equivalence, and when appropriate, bioequivalence (Brasil, 2007a;b). About 36% of generic drug products (for example, injectable drug products, dermatological drug products, ophthalmic drug products, etc.) were tested only for pharmaceutical equivalence. On the other hand, oral drugs (for example, tables, capsules, oral suspensions, etc) were tested for pharmaceutical equivalence and bio-equivalence. Due to the biopharmaceutical classification of active pharmaceutical ingredients, some oral drug products are exempted from bioequivalence (Brasil, 2007a;b). In other words, these drug products are tested only for pharmaceutical equivalence.

Pharmaceutical equivalents drug products contain the same amount of the same active ingredient (salt or basis), are of the same dosage form, route of administration, indications and uses. Conversely, in the cases mentioned earlier, pharmaceutical equivalence may be considered as an *in vitro* therapeutic equivalence test. As a consequence, pharmaceutical equivalence assessment is an important issue on regulatory approval of generic and similar drugs. According to the requirements of most regulatory agencies, two drugs are pharmaceutically equivalent if both test (generic or similar) and brand comply with all specifications of tests and assays included in the study. However, even if both test and brand drug products were pharmaceutical equivalents, there may be a significant difference among their attributes of quality, efficacy and safety (Vesga et al., 2010; Fujimura et al., 2011; Zuluaga et al., 2010; Warren, 2012). Pharmaceutical equivalence studies need to ensure the identity, strength, quality and purity of the test (generic or similar) and brand of drug products (Brasil, 2007a;b).

To guarantee the *in vitro* therapeutic equivalence assessment by using pharmaceutical equivalence study, it is important to have a measure of the quality and similarity of both test (generic or similar) and brand drug products results. Several methodologies are available for comparison of dissolution profiles (for example; difference and similarity factors – f_1 and f_2 , analysis of variance, two one-sided equivalence test - TOST, multivariate methods, etc) (O'Hara et al., 1998; Shah et al., 1998). Conversely, there are a few methodologies applied to *in vitro* therapeutic equivalence of drug products tested only for pharmaceutical equivalence.

Most of these methodologies are employed only to active pharmaceutical ingredient content comparison (for example; t-student tests, two one-sided equivalence tests - TOST, compliance decision based on measurement uncertainty) (Zuluaga et al., 2009; Lourenço and Pinto, 2012; Okamoto et al., 2013). In addition, all these methodologies cannot be employed for simultaneous comparison of all tests and assays.

An objective measure of *in vitro* therapeutic equivalence based on compliance of all tests and assays results is a promising concept for regulatory agencies. In this paper, we described a new multivariate similarity factor for *in vitro* therapeutic equivalence assessment for drug products not tested for bioequivalence. The multivariate similarity factor will also be used for *in vitro* therapeutic equivalence assessment of acyclovir cream, metronidazole injection, meropenem for injection and atropine sulfate injection, since they are tested only for pharmaceutical equivalence.

MATERIALS AND METHODS

Instruments

A high performance liquid chromatograph (Thermo, Accela) equipped with a photo-diode array detector (PDA) was used for assays and identification. A UV-visible spectrophotometer (Thermo, Evolution 201) was used for assays and identifications. An analytical balance (Shimadzu, AUY220) was used for weighting of reference standards and samples. A pH meter (Gehaka, PG 1800) was used for pH determination. A biological safety cabinet (Veco, Biosafe Class II B2) was used for sterility tests.

Chemical reference standards and drug samples

Acyclovir and meropenem reference standards were supplied by United States Pharmacopeia. Metronidazole and atropine sulfate reference standards were supplied by Brazilian pharmacopeia. Commercial samples of reference, generic and similar drugs were acquired in Brazilian market. All reagents and solvents were supplied by Carlo Erba, Merck, J.T Baker, Oxoid and Difco.

Pharmaceutical equivalence of drug products

Pharmaceutical equivalence studies of acyclovir cream drugs included identification (UV spectrophotometry (UV)), limit of guanine (thin-layer chromatography (TLC)), minimum fill, microbiological enumeration (bacterial and fungal counts), microbiological tests for specified microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* sp.), and acyclovir content assay (UV) (United States Pharmacopeia, 2012; Farmacopeia Brasileira, 2010). Identification (infrared spectroscopy (IR) and high performance liquid chromatography (HPLC)), pH determination, volume, sterility test, bacterial endotoxin, and metronidazole content assay (UV and HPLC) were performed for pharmaceutical equivalence studies of metronidazole injection pharmaceutical equivalence studies (United States Pharmacopeia, 2012; Farmacopeia Brasileira, 2010). Pharmaceutical equivalence of meropenem for injection drug included identification (HPLC), loss

Table 1. Results of tests and assays performed in pharmaceutical equivalence studies among acyclovir cream brand, generic and similar drug products.

Test and assay	Specification	Brand drug	Generic drug	Similar drug
UV identification	Positive	Positive	Positive	Positive
Limit of guanine	NMT 1%	Pass	Pass	Pass
Minimum fill (g/unit)	NLT 10	10.3	10.6	10.4
Bacterial count (CFU/g)	NMT 1000	<10	<10	<10
Fungal count (CFU/g)	NMT 100	<10	<10	<10
<i>E. coli</i>	Absence in 1 g	Pass	Pass	Pass
<i>S. aureus</i>	Absence in 1 g	Pass	Pass	Pass
<i>P. aeruginosa</i>	Absence in 1 g	Pass	Pass	Pass
<i>Salmonella</i> sp.	Absence in 1 g	Pass	Pass	Pass
UV assay (%)	90.0 -110.0	102.1±2.7*	104.8±2.7*	98.3±2.7*

NMT = not more than. NLT = not less than. *95% confidence interval obtained from 3 independent determinations.

Table 2. Results of tests and assays performed in pharmaceutical equivalence studies among metronidazole injection brand, generic and similar drug products.

Test and assay	Specification	Brand drug	Generic drug	Similar drug
IR identification	Positive	Positive	Positive	Positive
HPLC identification	Positive	Positive	Positive	Positive
pH determination	4.5-7.0	4.8	5.5	4.8
Volume (ml/unit)	NLT 100	110	102	102
Sterility test	Sterile	Pass	Pass	Pass
Bacterial endotoxin	NMT 0.35 EU/mg	Pass	Pass	Pass
HPLC assay (%)	90.0 -110.0	101.0±3.5*	101.3±3.5*	101.9±3.5*
UV assay (%)	90.0 -110.0	98.5±1.6*	90.6±1.6*	96.1±1.6

NMT = not more than. NLT = not less than. *95% confidence interval obtained from 3 independent determinations.

on drying, uniformity content, sodium content (atomic absorption spectrophotometry (AA)), sterility test, bacterial endotoxin, and meropenem content assay (HPLC) (United States Pharmacopeia, 2012; Farmacopeia Brasileira, 2010; Lourenço and Pinto, 2012). Identification (IR and TLC), pH determination, volume, sterility test, bacterial endotoxin, and atropine content assay (HPLC) were performed for pharmaceutical equivalence studies of atropine sulfate injection drugs (United States Pharmacopeia, 2012; Farmacopeia Brasileira, 2010). All tests and assays were performed according to Brazilian and United States pharmacopeia (United States Pharmacopeia, 2012; Farmacopeia Brasileira, 2010).

Multivariate similarity factor for *in vitro* therapeutic equivalence assessment

All results were standardized using an appropriate desirability function. The desirability functions were chosen based on the specification limits of each test or assay. In other words, we chose an isosceles triangle function for active pharmaceutical ingredient content. On the other hand, we chose a “0 or 1” function for sterility tests and bacterial endotoxin tests. Moreover, all desirability results were combined in individual acceptance factors. When any result is out-of-specification the individual acceptance factor is equal to 0, since it is the geometric mean of desirability functions results. Therefore, individual acceptance factor is a measure of general quality of drugs. Finally, we calculated a combined multivariate

similarity factor for *in vitro* therapeutic equivalence assessment of acyclovir cream, metronidazole injection, meropenem for injection and atropine sulfate injection. These multivariate similarity factors were compared the other approaches of *in vitro* therapeutic equivalence assessment.

Statistical analysis

The two one-sided t-student tests (TOST) were employed as equivalence testing to compare the results of active pharmaceutical ingredient (API) content in brand-name, similar and generic drug products. To test equivalence, 90% confidence intervals (90% CI) were determined, using as basis the standard deviations obtained from the results of API content in drug products. In these TOST we select $\alpha = 0.05$. We assume that two drug products are pharmaceutical equivalents if the 90% CI for the difference of API content is completely contained in the equivalence range ($\pm 10\%$). We considered that an appropriate range to equivalence testing should be defined based on the regulatory (or pharmacopeial) specifications for the content of API in drug products.

RESULTS

The current regulatory criteria for pharmaceutical equivalence defined that all tests and assays should

Table 3. Results of tests and assays performed in pharmaceutical equivalence studies among meropenem for injection brand, generic and similar drug products.

Test and assay	Specification	Brand drug	Generic drug	Similar drug
HPLC identification	Positive	Positive	Positive	Positive
pH determination	7.3–8.3	7.9	8.0	7.6
Loss on drying (%)	9.0–12.0	10.1	9.9	10.8
Uniformity content (%)	85.0–115.0 (RSD ≤ 6.0)	94.0–98.1 (RSD = 1.3)	91.1–102.7 (RSD = 3.4)	94.7–100.5 (RSD = 2.2)
Sodium content (%)	80.0% - 120.0	102.5	100.7	129.9
Sterility test	Sterile	Pass	Pass	Pass
Bacterial endotoxin	NMT 0.125 EU/mg	Pass	Pass	Pass
HPLC assay (%)	90.0-110.0	96.1±3.7*	99.6±3.7*	97.5±3.7*

NMT = not more than. NLT = not less than. *95% confidence interval obtained from 3 independent determinations.

Table 4. Results of tests and assays performed in pharmaceutical equivalence studies among atropine sulfate injection brand, generic and similar drug products.

Test and assay	Specification	Brand drug	Generic drug	Similar drug
IR identification	Positive	Positive	Positive	Positive
TLC identification	Positive	Positive	Positive	Positive
pH determination	3.0 – 6.5	3.7	5.8	5.3
Volume (ml/unit)	NLT 1.1	1.1	1.1	1.1
Sterility test	Sterile	Pass	Pass	Pass
Bacterial endotoxin	NMT 55.6 EU/mg	Pass	Pass	Pass
HPLC assay (%)	90.0-110.0	102.7±4.5*	112.7±4.5*	106.9±4.5*

NMT = not more than. NLT = not less than. *95% confidence interval obtained from 3 independent determinations.

comply with the specifications for both test (generic or similar) and brand drug products. All tests and assays performed for acyclovir cream and metronidazole injection drug products comply with the specifications (Tables 1 and 3). On the other hand, out-of specification results were found for atropine injection and meropenem for injection drug products (Tables 2 and 4). Therefore, atropine generic drug and meropenem similar drug were not pharmaceutical equivalents to their brand drug products.

Although most of the tests and assays comply with the specifications, it is important to consider how close these results are to the specification target. The quality of a drug may be evaluated by the distance of their parameters to the specification target. The closer a result is from the specification target the higher is the drug products quality. In contrast, the closer a result is from the specification limit the lower is the drug products' quality. In addition, the comparison of results from different tests and assays is difficult, once they correspond to different properties of the drugs.

Therefore, each result should be converted to a standardize value using a suitable desirability function. The desirability function should be chosen according to the specification limits and specification target of the test or

assay. An isosceles triangle function (Figure 1A) may be used for tests and assays that have a central target specification (for example, content of active pharmaceutical ingredient, content of sodium, minimum fill, etc). A rectangular triangle function (Figure 1B and C) may be used for tests and assays that have a lower or higher limit specification (for example, volume, loss on drying, etc). Other combined desirability function (Figures 1D and E) may also be used for these tests and assays. For 'pass/fail' tests and assays a '0 or 1' function (Figure 1F) is appropriated (for example, limit of guanine, sterility test, bacterial endotoxin, etc). The standardize results will range from 0 (no compliance or worst fit compliance) to 1 (best fit compliance).

When we standardize the results, all tests and assays will have the same weight in the evaluation of *in vitro* therapeutic equivalence. However, some tests and assays are more relevant than others. An exponential weight factor (w) may be used to attribute a weighted relevance to each test or assay. An exponential weight factor greater than 1 will fine tune the shape of the desirability function (Figure 1G), and may be used to high relevant tests and assays. On the other hand, an exponential weight factor less than 1 will expand the shape of the desirability function (Figure 1H), and may be used to low

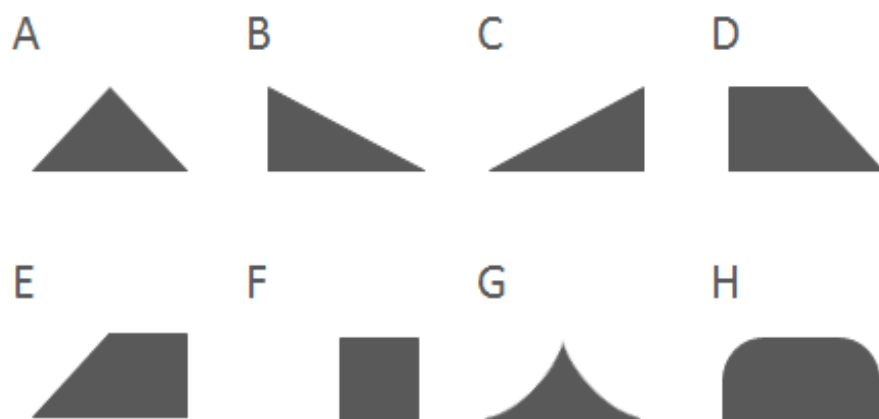


Figure 1. Desirability functions for several types of tests and assays. (A) Isosceles triangle function; (B) and (C) rectangular triangle function; (D) and (E) combined function, (F) 'pass/fail' function, and (G) and (H) exponential weighted function.

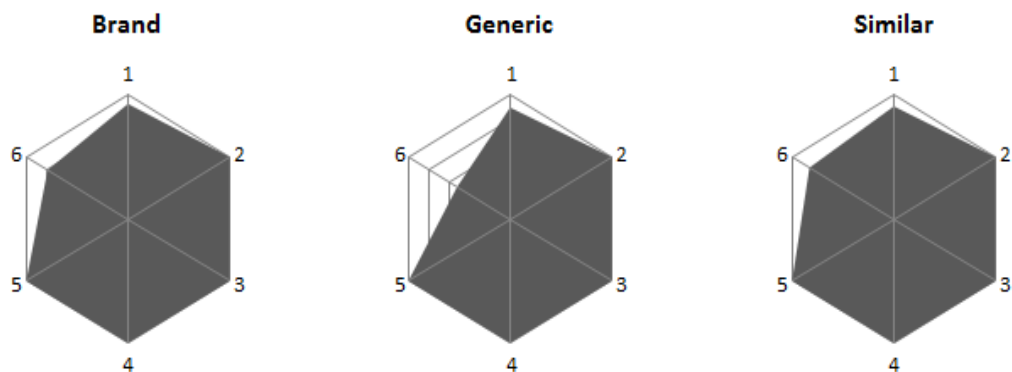


Figure 2. Star graphics for brand, generic and similar acyclovir cream drug products. (1) weight, (2) guanine limit, (3) UV identification, (4) microbiological enumeration, (5) microbiological tests for specified microorganisms, (6) UV assay.

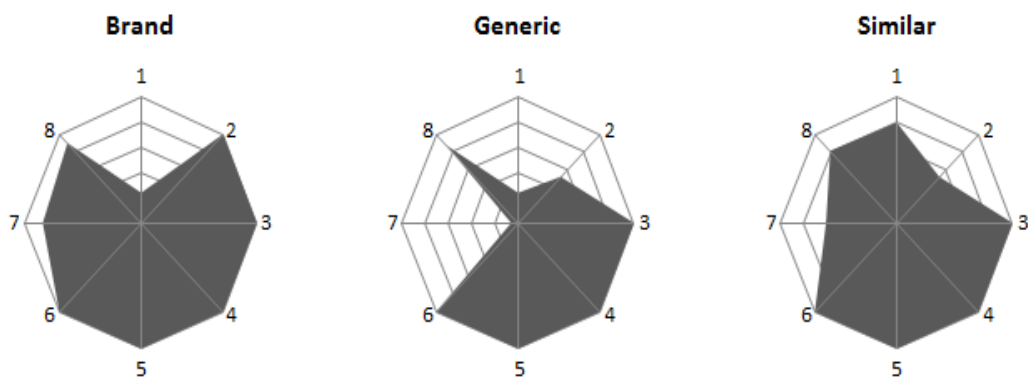


Figure 3. Star graphics for brand, generic and similar metronidazole injection drug products. (1) pH, (2) volume, (3) IR identification, (4) HPLC identification, (5) sterility test, (6) bacterial endotoxin, (7) UV assay, and (8) HPLC assay.

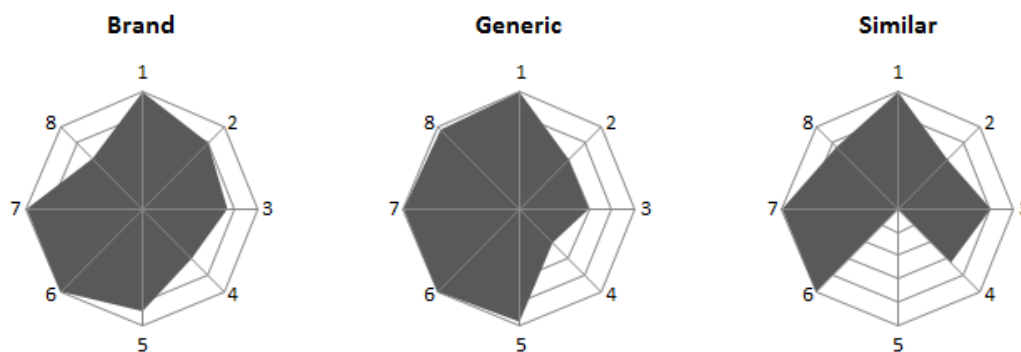


Figure 4. Star graphics for brand, generic and similar meropenem for injection drug products. (1) HPLC identification, (2) pH, (3) loss on drying, (4) uniformity content, (5) sodium content, (6) sterility test, (7) bacterial endotoxin, and (8) HPLC assay.

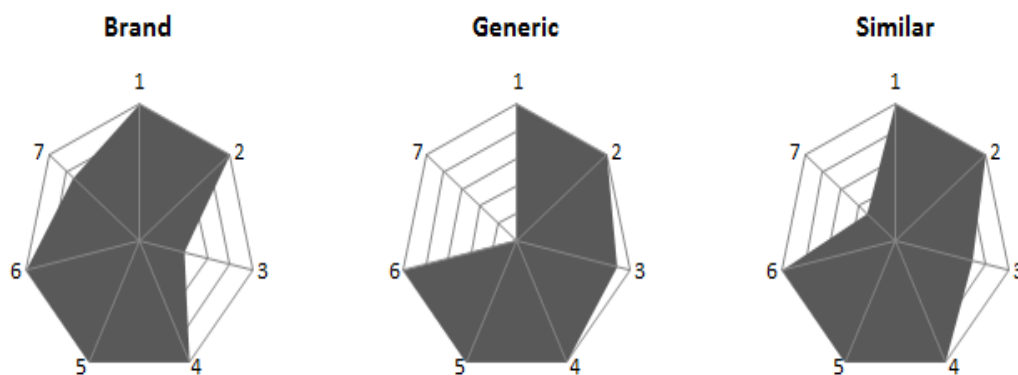


Figure 5. Star graphics for brand, generic and similar atropine sulfate injection drug products. (1) IR identification, (2) TLC identification, (3) pH, (4) volume, (5) sterility test, (6) bacterial endotoxin, and (7) HPLC assay.

relevant tests and assays. In this work, we adopted an exponential weight factor of 1 for all tests and assays. The desirability function allows us to standardize the results, as a consequence it is possible to compare the results among different tests and assays. The standardized results of brand, generic and similar drugs are presented as ‘star’ graphic (Figures 2 to 5). We can evaluate similarities and differences among the drugs, since the shape of ‘star’ graphic changes as the desirability results change. However, these comparisons are subjective. An individual acceptance factor for each drug was calculated as the geometric mean (Equation 1) of the desirability function results of all tests and assays.

$$a = (\prod_{i=1}^n (p_i^w))^{1/n} \quad \text{Equation 1}$$

Where, a is the individual acceptance factor, p is the desirability function results for each i test or assay, w is the exponential weight factor, and n is the number of tests and assays performed.

A multivariate similarity factor is an objective way to assess *in vitro* therapeutic equivalence. We calculated the multivariate similarity factors for *in vitro* therapeutic equivalence according to Equation 2. Multivariate similarity factor includes two terms: (1) Quantification of combined multivariate acceptance factors, and (2) quantification of deviations between test (generic or similar) and brand drug products results. The first term indicates how tests and assays results are closed to the specifications targets. The second term is a measure of similarity between test (generic or similar) and brand drug products results.

$$f_{ms} = (\prod_{i=1}^n (\sqrt{|p_{T^w}| \times |p_{R^w}| \times \left(\frac{2-|p_{T^w} - p_{R^w}|}{2}\right)}))^{1/2n} \quad \text{Equation 2}$$

Where, f_{ms} is the multivariate similarity factor, p_T desirability function results (for each i test or assay) of generic or similar drug product, p_R desirability function results (for each i test or assay) of brand drug product,

Table 5. Individual acceptance factors for brand, generic and similar drug products.

Drug	Brand drug product	Generic drug product	Similar drug product
Acyclovir ^{*1}	0.95	0.88	0.95
Metronidazole ^{*2}	0.81	0.53	0.82
Meropenem ^{*3}	0.81	0.78	0.00
Atropine sulfate ^{*4}	0.84	0.00	0.80

^{*1}Individual acceptance factor, including: IR identification, HPLC identification, pH, volume, sterility test, bacterial endotoxin, UV assay, and HPLC assay.

^{*2}Individual acceptance factor, including: UV identification, weight, guanine limit, microbiological enumeration, microbiological tests for specified microorganisms, and UV assay.

^{*3}Individual acceptance factor, including: HPLC identification, pH, loss on drying, uniformity content, sodium content, sterility test, bacterial endotoxin, and HPLC assay.

^{*4}Individual acceptance factor, including: IR identification, TLC identification, pH, volume, sterility test, bacterial endotoxin, and HPLC assay.

Table 6. Summary of conclusions of pharmaceutical equivalence studies and *in vitro* therapeutic equivalence assessment among brand, generic and similar drug products.

Drug		PE ^{*1}	TOST ^{*2}	<i>f_{ms}</i> ^{*5}
Acyclovir	Generic x Brand	Equivalent	Equivalent ^{*3}	0.51
	Similar x Brand			0.72
Metronidazole	Generic x Brand	Equivalent	Equivalent ^{*3}	0.60
	Similar x Brand			0.74
Meropenem	Generic x Brand	Equivalent	Not equivalent ^{*4}	0.75
	Similar x Brand	Not equivalent	Equivalent ^{*3}	0.00
Atropine sulfate	Generic x Brand	Not equivalent	Not equivalent ^{*4}	0.00
	Similar x Brand	Equivalent	Equivalent ^{*3}	0.71

^{*1} Pharmaceutical equivalence among test (generic or similar) and brand drug products.

^{*2} Two one-sided test (TOST), employed for active pharmaceutical ingredient content only

^{*3}equivalent indicates p-value < 0.05 and ^{*4}not equivalent indicates p-value ≥ 0.05.

^{*5} Multivariate similarity factor for *in vitro* therapeutic equivalence assessment.

w is the exponential weight factor, and n is the number of tests and assays performed.

The multivariate similarity factors for acyclovir cream, metronidazole injection, meropenem for injection and atropine sulfate injection are presented in Table 5. Acyclovir cream studies presented higher multivariate similarity factors (close to 1), because most of the tests and assays were found to be close to the specification target (Figures 2 and 3 and Tables 1 and 2). In contrast, multivariate similarity factors for generic and brand of both meropenem and atropine sulfate drugs were found to be 0, due to the out-of specification results (Figures 4 and 5 and Tables 3 and 4). In addition, the multivariate similarity factors showed perfect correlation to the regulatory requirements for pharmaceutical equivalence (Table 6). Besides, the multivariate similarity factor provides a degree of combined quality and similarity for both test (generic or similar) and brand drugs. The multivariate similarity factor also showed a good correlation to two one sided equivalence test (TOST) (Table 6) (Lourenço and Pinto, 2012). However, we found differences in meropenem for injection studies, because TOST is used for evaluation of active pharmaceutical ingredient content only. In contrast, multivariate similarity factor allows us to evaluate all tests and assays results simultaneously.

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DISCUSSION

In this paper, we described a new multivariate similarity factor to assess *in vitro* therapeutic equivalence based on pharmaceutical equivalence studies. Ours results indicate that the higher the multivariate similarity factor the higher the level of similarity between tested drug products. In other words, the multivariate similarity factor is not only an indication of compliance for pharmaceutical equiva-

lence, but it also indicates the level of similarity between test (generic or similar) and brand drug products. The multivariate similarity factor can be used for any kind of research work on pharmaceutical equivalents, since both test (generic or similar) and brand drug product had been submitted to the same assays and tests, including their specifications.

Several drug products, such as injectable, dermatological and ophthalmic dosage forms, have been tested only for pharmaceutical equivalence. Moreover, some oral drug products are exempted from bioequivalence, due to biopharmaceutical classification of active pharmaceutical ingredients. As a consequence, confirmation of *in vitro* therapeutic equivalence is an important issue to regulatory agencies around the world. Despite of its importance, we found in literature a few methodologies applied to *in vitro* therapeutic equivalence of drug products tested. Most of these methodologies are employed only to active pharmaceutical ingredient content comparison (for example; t-Student tests, two one-sided equivalence tests - TOST, compliance decision based on measurement uncertainty) (Zuluaga et al., 2009; Lourenço and Pinto, 2012; Okamoto et al., 2013). In addition, all these methodologies cannot be employed for simultaneous comparison of all tests and assays.

According to ours results, multivariate similarity factor showed perfect correlation to the regulatory requirements for pharmaceutical equivalence and a good correlation to two one sided equivalence test (TOST) (Lourenço and Pinto, 2012). Differences among multivariate similarity factor and two one-sided equivalence test was due to limitations of TOST. TOST does not allow us to compare several tests and assays results simultaneously. On the other hand, multivariate similarity factor provides a simultaneous evaluation of all tests and assays results for both test (generic or similar) and brand drugs.

The multivariate similarity factor will be affected using different exponential weight factors (w) for each test or assay, but it could be useful to give more ($w > 1$) or less ($w < 1$) importance for a single test or assay in an *in vitro* therapeutic equivalency study. This approach could be used to reduce the weight of "pass/fail" tests and assays results in the quantification of deviation term. Alternatively, "pass/fail" tests and assays could not be consider using multivariate similarity factor, which will result in a more rigorous evaluation of *in vitro* therapeutic equivalence. In this case, both test (generic or similar) and brand drug products should comply with all "pass/fail" tests and assays, such as identification, sterility test and others.

The multivariate similarity factor is a measure of the general quality and similarity of both test (generic or similar) and brand drug products. As a consequence, it can be used to assess *in vitro* therapeutic equivalence of drug products that will not be tested for bioequivalence. Also, it can be used as a preliminary analysis for those drugs that will be tested for bioequivalence or bioavailability. In

conclusion, the multivariate similarity factor is a useful tool for *in vitro* therapeutic equivalence assessment, and it can be used for regulatory approval of generic and similar drug products.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

E-Cadherin, beta-catenin and HER2 expression in prostate cancer tissues with perineural invasion and their correlation with Gleason score: A preliminary study

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Prostate cancer (PC) is the most common malignant tumor in men. Early identification of prostate cancer may result in improved cure rates and increased life expectancies. Gleason score and clinical range at the time of diagnosis are important factors to predict prognosis and outcome after therapy but additional accurate and reliable biomarkers are warranted. Few biomarkers of prostate cancer have been successfully implemented and used in clinical practice. In this study, we sought to determine the expression of E-cadherin, beta-catenin and human epidermal receptor (HER2) in biopsy specimens of prostate cancer with perineural invasion, and correlate them with Gleason score in order to verify the relationship between those markers and prostate cancer process. Our study demonstrated abnormal expression of E-cadherin, beta-catenin and HER2. On the other hand, our results showed no correlation between Gleason score and the expression of those markers in invasive prostate cancer tissues. Other different biomarkers remain to be identified, that potentially could improve the evaluation of prognostic of the patient.

Key words: Biomarkers, biopsy specimens, Gleason score, perineural invasion, prostate cancer.

INTRODUCTION

Prostate cancer (PC) is the most common malignant tumor in men and is a major research focus of Pathologists, Urologists and Uro-oncologists. The clinical decision of physicians, whom and how to treat these men, is dependent predominantly on pathological parameters, but still the grid spanned by these is too wide to allow a suitable prognostic of the individual case (Kristiansen, 2012). Early identification of prostate cancer

may result in improved cure rates and increased life expectancies. The most widely accepted indicator for prostate prostate biopsy is a prostate-specific antigen (PSA) value > 4 ng/ml, but patients with a PSA level of ≤ 4 ng/ml, particularly 2.6 to 4 ng/ml, have clinically significant features and are well suited for immediate treatment (Nagao et al., 2011).

Gleason score and clinical range at the time of

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diagnosis are important factors to predict prognosis and outcome after therapy but additional accurate and reliable biomarkers are warranted. Despite extensive research efforts, very few biomarkers of prostate cancer have been successfully implemented and used in clinical practice. Moreover, it is unlikely that a single biomarker will provide all information needed to tell how aggressive is the newly diagnosed prostate cancer (Bjartell et al., 2011).

Epithelial (E)-cadherin is an adhesion molecule, which is expressed at the baso-lateral membrane of epithelial tissues. Intracellular interactions of E-cadherin with beta-catenin, p120, and alpha catenin also support adhesion and stabilization of the adherens junction (Grabowska et al., 2011). E-cadherin is down regulated in most epithelial cancers, and can be correlated to higher mobility and invasiveness of tumor cells (Veveris-Lowe et al., 2005). The human epidermal receptor (HER) kinase family, which includes human epidermal growth factor receptor 2 (HER2), are receptor- and receptor-like transmembrane proteins that are activated in some human tumors. The gene encoding the HER2 protein is amplified in 20 to 25% of breast cancer patients and it is overexpressed in many prostate cancers (Solit and Rosen, 2007).

In this study, we sought to determine the relative expression of E-cadherin, beta-catenin and HER2 in biopsy specimens of prostate cancer patients with perineural invasion and correlate them with Gleason score in order to find the relationship between those markers and prostate cancer process.

MATERIALS AND METHODS

Patient samples

After giving informed consent, 15 prostate cancer patients with perineural invasion were selected from 19 patients diagnosed with prostate cancer. For the purposes of this study, perineural invasion was defined as, "the presence of cancer tracking along or around a nerve" within the prostate. Perineural invasion was available on biopsy reports by two independent pathologists who were blinded to the patients' clinical information. The patient characteristics at diagnosis are listed in Table 1.

Immunohistochemistry and histopathological analysis

Biopsy specimens from those 15 patients were immediately preserved in buffered formalin (phosphate buffer, pH 7.4) with subsequent preparation of paraffin blocks. Representative tumor areas were chosen based on hematoxylin and eosin-stained sections. The corresponding archived paraffin-embedded specimens were sectioned into 4 μ m slices, and immunohistochemical staining was performed, adapted from Kowalski et al. (2003). Immunohistochemical procedures were performed using LSAB Peroxidase® with monoclonal antibodies for E-cadherin (Cell Signaling, 1:150), beta-catenin (Cell Signaling, 1:150) or HER2 (DAKO, 1:350). As negative controls, we replaced primary antibody by non-immune immunoglobulin, phosphate-buffered saline or irrelevant antibodies.

More than to identify the presence or absence of a biomarker, immunohistochemical can be used to quantify its expression. Digital images can be translated into numerical values, and these values

Table 1. Patient characteristics at diagnosis

Patient	Age (years)	Gleason score
1	71	8
2	42	6
3	67	7
4	64	7
5	61	9
6	61	6
7	78	7
8	72	7
9	63	6
10	60	7
11	64	6
12	72	7
13	62	7
14	66	7
15	77	7

are able to describe staining intensity as a numeric variable. Considering numerical data for staining intensity and percentage of labeled cells, a combined digital immunostaining index can be defined (Matos et al., 2006). In our study, the quantification of immunostaining was made by 2 different methods: semiquantitative and computer-assisted digital image analysis. The number of stained cells per 1000 was determined under a microscope in three visual fields, at a magnification of $\times 200$. When the total number of cancer cells observed under microscope was less than 1000, all cells were counted. E-cadherin or beta-catenin expression was interpreted as normal or aberrant (reduced or absent). Aberrant staining was defined as negative staining < 50% of the population of cells examined. Normal staining was defined as $\geq 50\%$ staining of the cancer cells. When over 50% of all cancer cell cytoplasm was stained, the cells were considered HER2 aberrant. This cut-off value (50%) was analyzed by Software ImageLab®.

The histological grade according to Gleason score was assessed on stained sections in accordance with WHO International classification. Histologically, all tumors were adenocarcinomas. The present study was performed retrospectively, but all specimens were evaluated by two independent pathologists who were blind to the patients' clinics characteristics.

Statistical analysis

Differences were analyzed by the Fisher's exact test. The value of significance was taken as $P < 0.05$.

RESULTS

Patients

Table 1 shows patient characteristics at diagnosis. Mean age was 65.5 ± 6.9 . Gleason score was 6 (18.18%), 7 (48.48%), 8 (24.24%) and 9 (9.09%). Gleason score = 6 was considered low grade and ≥ 7 was considered as high grade.

Table 2. Correlation between E-cadherin, beta-catenin and HER2 and Gleason score. Differences were analyzed by the Fisher's exact test.

Parameter	Gleason score ≤ 6		Gleason score ≥ 7		P
	Normal	Aberrant	Normal	Aberrant	
E-cadherin	3	0	7	5	0.505
Beta-catenin	2	1	7	5	1.000
HER2	3	6	5	1	0.250

The value of significance was taken as $P < 0.05$. Number of patients: 15.

Table 3. Correlations between markers and Gleason score. Differences were analyzed by the Fisher's exact test.

Parameter	Gleason score ≤ 6	Gleason score ≥ 7
	P	
E-cadherin vs HER2	0.10	1.00
E-cadherin vs beta-catenin	1.00	1.00
HER2 vs beta-catenin	0.40	1.00

The value of significance was taken as $P < 0.05$. Number of patients: 15.

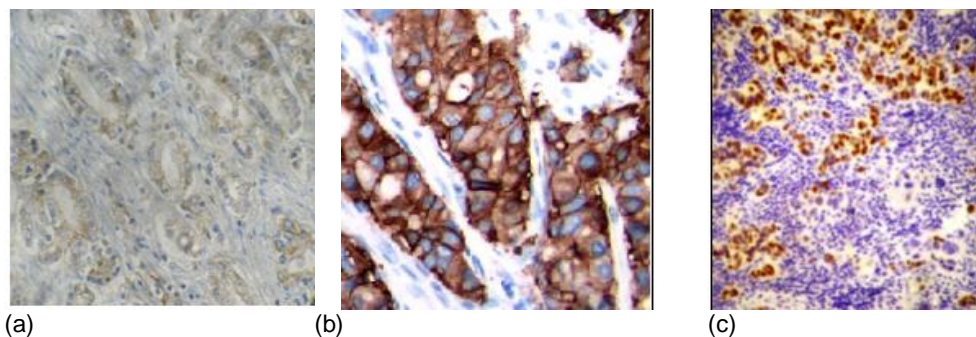


Figure 1. Immunohistochemical expression of (a) E-cadherin ($\times 200$); (b) Beta-catenin ($\times 200$); and (c) HER2 ($\times 200$). Immunohistochemical procedures were performed using LSAB Peroxidase® with monoclonal antibodies for E-cadherin, beta-catenin or HER2. Images were analyzed by Software ImageLab®.

Immunohistochemistry and histopathological results

Relative expression of E-cadherin, beta-catenin and HER2 are shown in Figure 1. This figure is representative of the staining pattern observed for all tissue sections. When E-cadherin, beta-catenin or HER2 were considered with Gleason score (Tables 2 and 3) no correlation was observed.

DISCUSSION

Correlation between immunohistochemistry and histopathological data

There is a need for biomarkers in prostate cancer for

several reasons: (a) to improve cancer detection and staging; (b) to identify subclasses of prostate cancer; (c) to predict outcome after treatment; and (d) to select patients to different treatment opinions (Bjartell et al., 2011). The introduction of the well established biomarker PSA testing has impacted the detection rate of prostate cancer and is responsible for down-staging at diagnosis, with the vast majority of newly diagnosed tumor being localized in prostate. Moreover, Gleason score and clinical stage are important to predict prognosis and outcome after therapy but additional accurate and reliable biomarkers are necessary (Bjartell et al., 2011; Masieri et al., 2012).

The search for diagnostic or prognostic tissue biomarkers in prostate cancer was predominantly based on immunohistochemistry and a large number of tumor

markers with prognostic information were proposed. However, a vast majority of these are not used in clinical practice, probably due to lack of standardized methods to perform and interpret immunohistochemistry. In fact, the only prostate cancer biomarker routinely used in prediction models is PSA in blood (Bjartell et al., 2011). In this work, we focused on the expression of three tissue biomarkers in prostate cancer: E-cadherin, beta-catenin and HER2. Our results demonstrated abnormal expression of those markers. These discrepant results may be explained by other mechanisms, which, in turn may be important steps in the progression of cancer (Delgado et al., 2013).

E-cadherin plays critical roles in epithelial cell maintenance, and its loss from the cell surface during prostate cancer progression has been well documented (Grabowska et al., 2011). Mechanism by which a tumor cell invades the surrounding structure is poorly understood. Among numerous factors, cadherins and catenins are thought to be key molecules involved in the maintenance of the integrity of the epithelium and are likely to be involved in the earlier steps of the metastatic process. Indeed, a disruption in cadherin/catenin expression could lead to both haematogenous and/or lymphatic spread of cancer cells (Loric et al., 2001).

Mutational inactivation of alpha-catenin can be the cause of the impaired E-cadherin function, loss of catenin expression could be one of the mechanisms responsible for the loss of E-cadherin mediated cell-cell adhesion in human prostate cancer and might in some cases provide prognostic information. According to Buhmeida et al. (2006), the study should evaluate E-cadherin as a potential biomarker of disease progression. Furthermore, there is evidence that the loss of E-cadherin adhesion results in a spontaneous increase in PSA secretion to the environment. The effects of PSA secretion may alter growth regulation and behavior of the prostate cells (Kril et al., 2001).

Our group (Serpa Neto et al., 2010) investigated the prognostic impact of HER2 over expression in patients with prostate cancer and its correlation with other pathological and clinical variables. We found a consistent association of HER2 over expression with death and recurrence. Histological grading is a very important factor for the assessment of PC prognosis. Although the reproducibility is not perfect, still the Gleason grading system is the most used prognostic factor, and highly significantly associated to survival and/or progression. When E-cadherin, beta-catenin or HER2 were considered with Gleason score (Tables 2 and 3) no correlation was observed.

Slater et al. (2003) had demonstrated that E-cadherin was unsuitable to be marker of early neoplastic transformation. In addition, our findings indicated that E-cadherin had no correlation with Gleason score. Our results differ from those observed by Nagao et al. (2011). They found a significant correlation between the low positive rate for E-cadherin and a high Gleason score.

Further studies should be conducted in order to determine the real role of E-cadherin in prostate adenocarcinoma. Although markers of neoplasia are needed to improve the accuracy of diagnosis of prostate cancer, this present study adds to previous work that expression of E-cadherin, beta-catenin and HER2 could not be related to Gleason score.

Conclusion

Our results showed no correlation between Gleason score and the expression level of E-cadherin, beta-catenin and HER2 in prostate cancer tissues with perineural invasion. Other different biomarkers remain to be identified that potentially could improve the evaluation of prognostic of the patient.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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