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A Diffusion Study of Quercetin and Trans-Resveratrol Using Bacterial Cellulose as Matrix

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Abstract
Bacterial cellulose (BC), characterized by its high purity, strength, moldability and water holding capacity, has attracted several industries in using BC in biotechnology and medical applications. In this study, the diffusion behavior of two polyphenols, namely quercetin and trans-resveratrol (tRSV), in the bacterial cellulose matrix was investigated. The experiments were divided into three set-ups to monitor the diffusion which are polyphenol incorporated bacterial cellulose (BC-tRSV or BC-Quercetin) with solution in the upper chamber (Set-up 1), pure BC with polyphenol in the upper chamber (Set-up 2) and polyphenol incorporated bacterial cellulose in a solution (Set-up 3). Results show significant diffusion in tRSV in all three setups while negligible diffusion was observed in quercetin. Quercetin has more hydroxyl groups than tRSV, which can form extensive intramolecular hydrogen bonding with the microfibrils of BC, causing it to be trapped in its fibril network. On the other hand, tRSV has lower degree of hydroxyl groups and therefore form lesser hydrogen bonding with the BC fibrils. It was concluded that the degree of hydrogen bonding contributed to the diffusion observed. Furthermore, diffusion through the BC (setup 2) and diffusion from the BC (setup 3) both contributed to the diffusion of tRSV, which was quantified using HPLC. The diffusion of tRSV was further confirmed using LCMS. Thus, BC can be utilized as natural, affordable, and effective delivery system of therapeutically relevant drugs.

Keywords: *Bacterial cellulose, polyphenol, quercetin, trans-resveratrol, diffusion.*

1. Introduction

Cellulose is the most abundant and readily available macromolecule in the world. It can be classified into to two kinds, namely plant cellulose and bacterial cellulose. Plant cellulose is a tough, mesh-like bulk work in which cellulose fibrils are the primary architectural elements. Increasing demand on derivatives of plant cellulose had increased wood consumption as raw material, causing global environmental issues such as deforestation (Esa *et al*, 2014).

Although plant is the major contributor of cellulose, bacteria is capable of producing cellulose as an alternative source. Different types of bacteria belonging to genera *Acetobacter, Rhizobium, Agrobacterium and Sarcina* produce cellulose, which is referred to as bacterial cellulose (Bielecki *et al*, 2004). BC has different properties from plant cellulose and is characterized by high purity, strength, moldability and water holding capacity (Jonas *et al*, 1997). Since its discovery, BC has shown tremendous potential as an effective biopolymer in various fields. The structural features of BC are more superior plant cellulose, which impart it with better properties (Esa *et al*, 2014).

BC is the final product of carbon metabolism which involves either the pentose phosphate cycle or Krebs cycle, coupled with gluconeogenesis. BC is produced in regulated process which involves production of uridine diphosphoglucose, the substrate for polymerization of glucose to form the polymer of β-1,4 linked glucopyranose residues. Growing chains of BC cluster to form subfibrils with thickness of 1.5nm approximately (Bielecki, Krystynowicz, & Kalinowska, 2004). These subfibrils are crystallized into fibrils,

bundles and then into ribbons. Ribbons of BC with length varying from 1 to 9µm forms a net like structure and are stabilized by extensive inter-chain and intra-chain hydrogen bonds in cellulose.

The fibril network of BC is highly uniaxially oriented which results in high mechanical strength and cellulose crystallinity. The small size of the fibril results in high surface area, which allows the membrane to absorb large amounts of water (Brown et al., 2007). BC is produced in the form of highly swollen membranes with approximately 90% water content. Depending on the synthetic conditions, BC has a water-holding capacity ranging from 60 to 700 times its dry weight. One reason for this hydrophilicity is that the cellulose ribbons are assembled in an extracellular way in the liquid medium, which causes abundantly formed micelles to trap large quantities of liquid. However, BC has poor rehydration after drying because of its high crystallinity (Chen et al., 2010). Furthermore, BC membrane is very porous which is an important trait for potential transfer of antibiotics or other drugs and its fine network structure allows BC to exhibit a controlled release functionality and a critical skin-barrier function in wound healing (Brown, 2007).

BC has been used in different applications in several industries. BC is traditionally used to make nata de coco, an indigenous dietary fiber in Southeast Asia, as ingredient in a wide variety of food products. Furthermore, BC was investigated for its use in manufacture of artificial blood vessels for microsurgery (Klemm, Schumman, Udhardt & Marsch, 2001), scaffolds for tissue engineering (Backdahl *et al*, 2006) and drug delivery system (Halib & Ahmad, 2012). In addition, BC has the capability to accelerate the process of wound healing in skin injuries due to its ability to combine its protective properties and its ability to absorb exudates with the release of therapeutically relevant drugs (Kwak et al, 2014).

Modern wound dressings are designed to fulfil many different requirements such as to promote a rapid and painless wound healing, maintain a moist environment, optimum pH and temperature and provide protection from potentially irritating wound exudates. The global market currently offers different types of dressings for advanced based materials - including natural or synthetic polymers, as well as their combinations. Implemented in different forms (films, foams, hydrocolloids and hydrogels), these materials may contain growth factors, peptides, bioactive substances or drugs that accelerate recovery (Bergstrom et al., 2005). However, the search for the "ideal" wound dressing material is still ongoing, since most of the modern wound dressings also possess some drawbacks (Lagana et al., 2010). Depending on the material used and its form, important criteria such as non allergenic and sterile composition, high moisture vapour and fluid affinity and mechanical stability cannot be provided by all in one type of dressing.

Bacterial cellulose itself does not have antimicrobial properties to prevent ingrowths and represents just a physical barrier against bacterial infection (Czaja et al., 2006). This may reduce its effectiveness as a treatment for highly contaminated wounds. However, additional modifications can applied to add antimicrobial and anti-inflammatory properties to BC-based wound dressings. The high porosity and surface area of BC allow the potential for introduction and release of antimicrobial agents, medicines and other bio-functional materials. The presence of chemically reactive sites within the structure of BC provides the additional possibility for the introduction and release of specific non-native functionalities. Several approaches can be used to introduce antimicrobial and anti-inflammatory properties into BC material. Impregnation of antimicrobial and anti-inflammatory agents into the BC porous structure provides slow release of the drug into the colonized wound and long-lasting action against microorganism growth (Moritz et al., 2014; Wu et al., 2014).

Previous studies showed the ability of BC membranes to modulate the release of various drugs for percutaneous administration, and hence, they were proposed as supports for topical or transdermal drug delivery (Almeida et al, 2013). One example of this is a silver loaded BC, which possess strong antimicrobial activity against *E.Coli* and *S. aureus* and is used for antibacterial wound dressings (Zhang, Fang & Chen, 2013). Silver sulfadiazine combines the inhibitory action of silver salt along with the antibacterial effect of sulfadiazine making it one of the golden treatments in healing topical burns.

Trovatti *et al* (2011), studied the BC membranes applied in topical and transdermal delivery of lidocaine hydrochloride and ibuprofen drug. In the course of their study, they found that the permeation rate of the lidocaine hydrochloride in BC membranes was lower than that obtained in conventional formulations, which seems to indicate an advantage in the use of the system to address pathologies that require hydrophilic drugs with a complex toxicological profile, requiring more long-term release of the drug. They also discovered that high fluxes were achieved when a lipophilic (lipid and fat soluble) drugs was included in the BC membrane, which could be valuable in delivery systems that provide fast release for the treatment of acute conditions. This technology can be successfully applied to modulate the bioavailability of drugs, which could be particularly advantageous in the design the design of delivery systems that have the ability to absorb exudates and to adhere to irregular skin surfaces.

Silva *et al* (2013), on the other hand, studied BC membranes as transdermal delivery systems for the diclofenac drug. During the course of their study, it was found that the drug loaded membrane samples

were very homogenous, was considerably flexible and showed an increase swelling capacity compared to the pure BC membranes. They also discovered that permeation rate of diclofenac in BC membranes was similar to the observed commercial patches which suggests that this technology can be successfully applied to the transdermal delivery of diclofenac with the advantage of easy application and simplicity of preparation.

Furthermore, Kwak *et al* (2014), investigated the effects of BC membrane application on the healing of burn wounds of the Sprague-Dawley (SD) rats for 15 days. It was discovered that BC membranes promoted improvement of wound skin symptoms, re-epithelization, granulation tissue formation and angiogenesis without toxic effects. Their investigation on the effects of BC membranes on second-degree burn wounds revealed several implications, specifically, BC membranes stimulated detachment of the scab from the burn wound, which is consistent with the results of a previous study in which BC membrane impregnated with SOD (Procel-super) promoted scab detachment in deep dermal burns. Also, they concluded that the BC treatment on the back skin of SD rates stimulated the regeneration of burn tissue. Furthermore, their results provided a rationale for future development of BC membranes with other functional compounds for topical applications to cutaneous wounds.

Most studies used an apparatus called franz diffusion cell to study drug diffusion through biological and synthetic membranes. The franz cell consists of two primary chambers separated by a membrane. The test compound is applied to the membrane via the upper or donor chamber. The bottom or receptor chamber contain fluids from which samples are taken at regular intervals for analysis. The testing determines the amount of active that has permeated the membrane at each point (BMC Publishing, 2010). The most popular application of the franz cell is in the development of transdermal and topical pharmaceuticals. However, the equipment is also used in basic research to study membrane permeability (PermeGear, 2015).

BC has a great potential in the fields of biomedicine and biotechnology due to its unique nanoporous 3-D structure of hydrophilic fibrils. The structure of the BC guarantees that it can greatly serve as an ideal wound dressing, while providing an economical alternative to other moist providing and conventional dressings. Its potential for modifications and composite formation confirms its favorable standing in modern wound care (Sulaeva, 2015).

This study aims to further investigate the diffusion ability of different kinds of polyphenol molecules using bacterial cellulose as matrix. *trans*-resveratrol and quercetin, polyphenols with different degree of hydrogen bonding, were chosen as model drugs for the study. Due to the lack of apparatus in the Philippines, a franz diffusion cell was fabricated using cheap and economically available materials. This study will compare and quantitatively determine the amount of polyphenol diffused in franz diffusion cell using (i) HPLC and confirm the identity of the drug diffused using (ii) LCMS analysis. Furthermore, this study aims to monitor the effect of degree of hydrogen bonding in diffusion and to determine the factors affecting the diffusion using three different set-ups. The bacterial cellulose produced in the study can act as potential drug carrier which can be used to provide controlled release of drugs.

2. Materials and Methods

Bacterial cellose (BC) was produced using *Acetobacter xylinum* in a culture medium purchased from Department of Science and Technology (DOST) – Industrial Technology Development Institute. Quercetin and *trans*-resveratrol (tRSV) was purchased from Sigma Aldrich. Dropper bottles for fabrication of franz diffusion cell (FDC) was purchased from Beabi. HPLC grade ethanol and acetic acid were purchased from Sigma Aldrich.

Preparation of Bacterial Cellulose

Bacterial cellulose was harvested when its thickness was about 0.5-1.0cm and rinsed with distilled water. The BC was treated with 0.5M NaOH at 90°C for 20 minutes and the procedure was done three times. BC was rinsed and placed in distilled water and the pH was adjusted to 7.0. Purified BC was stored in distilled water at 0-4°C until use.

Fabrication of Franz Diffusion Cell

Franz diffusion cell (FDC) was fabricated with a dropper bottle and a plastic cap. The plastic cap was drilled by the engineering department of De La Salle University in order to make a hole for the diffusion area. The plastic cap was used for the diffusion area attached to the lower chamber and also for the upper chamber.

Preparation polyphenol incorporated BC (BC-tRSV and BC-quercetin

The wet mass of the purified BC was measured and 50% of water was taken out from the BC by applying pressure. The polyphenols, tRSV and quercetin, were dissolved in ethanol:water (1:1 v/v) for tRSV and ethanol:water (1:9 v/v) for quercetin to prepare the stock solution (500ppm). The semi-dried BC was cut into the size of the area of diffusion and sonicated in the freshly prepared polyphenol solution for 1 hour and 30 minutes at 30±5°C. The polyphenol solution after the absorption was stored at 0-4°C.

Diffusion of tRSV and quercetin

Three setups were prepared in this experiment. In setup 1, the diffusion from the BC and diffusion through the BC were simultaneously monitored. The polyphenol incorportated BC (BC-tRSV or BC-Quercetin) was placed on the diffusion area of the franz diffusion cell and the upper chamber was filled with the 500ppm polyphenol solution and covered with a glass. The polyphenol solution was frequently replenished. In setup 2, only the diffusion through the BC was monitored. The pure BC was placed on the diffusion area of the franz diffusion cell and the upper chamber was filled with the 500 ppm polyphenol solution and covered with a glass. The polyphenol solution was also frequently replenished. In setup 3, only the diffusion from the BC was monitored. The polyphenol incorportated BC (BC-tRSV or BC-Quercetin) was placed in a beaker with 20ml of the ethanol water solvent that was used for dissolving the polyphenol and the beaker was covered with parafilm. The temperature of the three setups was maintained at 37±1°C. For setup 1 and 2, the medium in the lower chamber of the franz diffusion cell was continuously stirred with a magnetic stirrer at 250 rpm. The diffusion in each setup was monitored for 24 hours. The summary of the three setups are shown in Table 1.

Table 1. Summary of three setups

Setup	BC-polyphenol	Upper Chamber
1	✓	✓
2		✓
3	✓	

Quantitative analysis of tRSV and quercetin

HPLC analysis was performed with Agilent series 1200 Quaternary HPLC with Variable Wavelength UV Detector. Agilent column (4.6 x 150 mm) packed with 5µm sized C18 was used for the separation.

For tRSV, a multi-step gradient method was applied using methanol-water-acetic acid (10:90:1 v/v) mixture as solvent A and methanol-water-acetic acid (90:10:1 v/v) mixture as Solvent B. The gradient profile was 0.0-18.0 min from 0% to 40% B, 18.0-25.0 min from 40% to 100% B and 25.0-27.0 min 100% B (Avar et al., 2007). The tRSV samples were monitored at 306 nm.

For quercetin, a multistep gradient method was applied using methanol and acetic acid (0.5%) as mobile phase. The chromatogram was monitored at 368 nm (Cayman Chemical, 2012).

3. Results and Discussion

Fabrication of franz diffusion cell

The volume of the upper chamber was 4.4ml for all fabricated FDC and the diffusion area and the volume of lower chamber for each FDC was measured, as shown in Table 2. The fabricated FDC is shown in Figure 1.

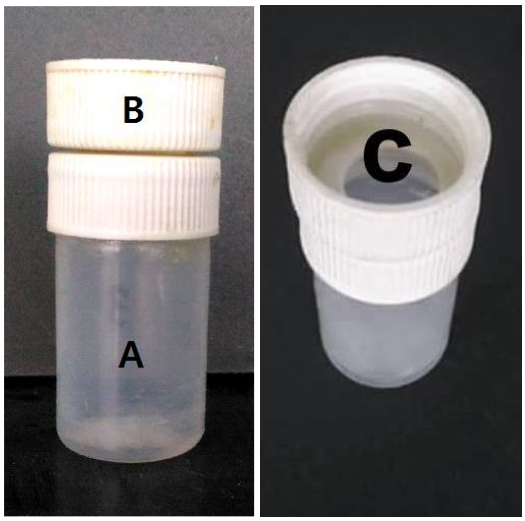

Figure 1. Fabricated FDC; (A) Lower chamber; (B) Upper chamber; (C) Diffusion area

Table 2. Dimension of fabricated FDC

FDC no.	Lower chamber volume (ml)	Diffusion area (cm^2)
1	18.6	1.81
2	18.6	1.52
3	18.8	1.29
4	18.9	1.33

Preparation of bacterial cellulose

The harvested BC was treated three times with 0.5M NaOH which serves as a brightening agent, which encompasses any substance capable of significantly reducing the level of colored impurities in microbially produced cellulose without damaging or substantially altering important properties of the cellulose (Gupta *et al*, 1900). The purified BC was placed distilled water and the pH was adjusted to 7.0 with acetic acid.

Figure 2. Purification of BC

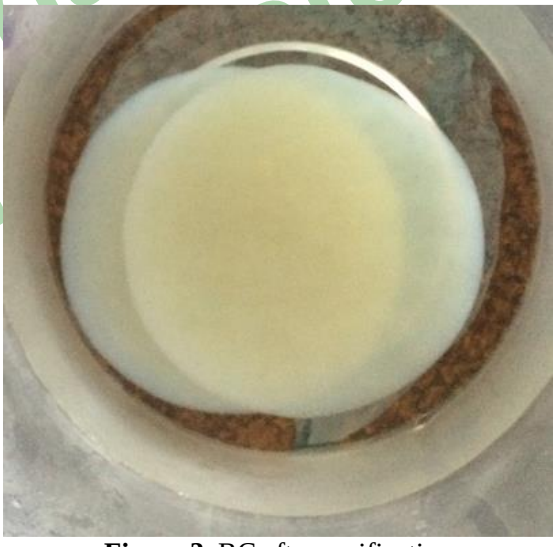

Figure 3. BC after purification

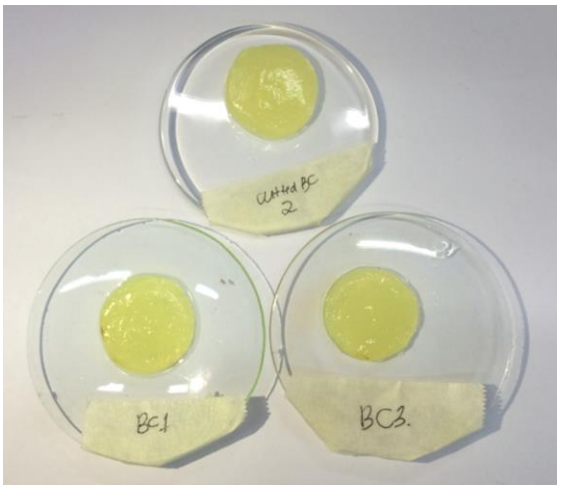

Figure 4. BC-Quercetin. Quercetin incorporated in BC after sonication

Diffusion study of tRSV

After 24 hours, the sample of the diffusion experiment (n=2) was obtained from the lower chamber of the FDC and analyzed by HPLC.

The stock solution with known concentration of 500ppm was first analyzed to determine the retention time of the tRSV, which was found to be 1.79 min.

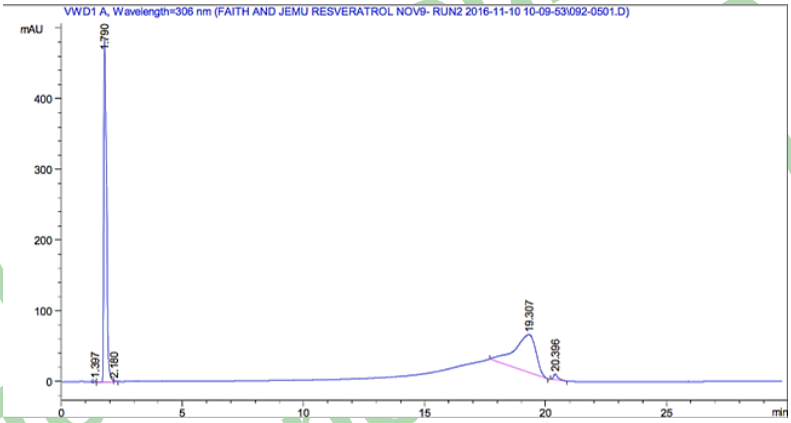

Figure 5. Chromatogram of tRSV stock solution

The concentration of the diffusion was determined using a standard curve (R^2 = 1.00). The three setups resulted to a significant diffusion of tRSV, as shown in table 3.

Table 3. Concentration of diffusion of tRSV from each setup

Setup	Concentration (ppm)
1	42.21±1.53
2	23.56±0.41
3	18.69±0.73

The setup 1 has two factors that directly affect the diffusion, the diffusion from the BC-tRSV and upper chamber. To quantify the diffusion coming from each factor, setup 1 was divided into two parts, setup 1 and 2. In setup 2, there was diffusion through the pure BC with no initiating incorporated tRSV, while in setup 3, it was also observed that there was diffusion from the BC-tRSV.

Furthermore, the sum of concentrations in setup 2 (23.56±0.41 ppm) and in setup 3 (18.69±0.73 ppm) is very close to the concentration of the setup 1 (42.21±1.53 ppm). Thus, the pressure by the concentration gradient between the lower chamber and the upper chamber (set-up 2) and the natural diffusion of the polyphenol (set-up 3) plays a factor in the diffusion of the drug in the franz diffusion cell (set-up 1).

To confirm the diffusion of tRSV, LCMS was used. As shown in Figure 6, The notable mass detected in the chromatogram is 228.87273 m/z, which is close to the known mass of tRSV (228.2 m/z). The other peaks in the chromatogram may be a degradation product of tRSV due to different factors such as light

exposure and temperature. The 182.87623g mass detected may be a degradation product of the tRSV with molecular formula of 2-(3,5-dihydroxyphenyl)-2-hydroxyacetic acid ($C_8H_8O_5$) and molecular weight of 183g/mol (Silva, et al., 2013).

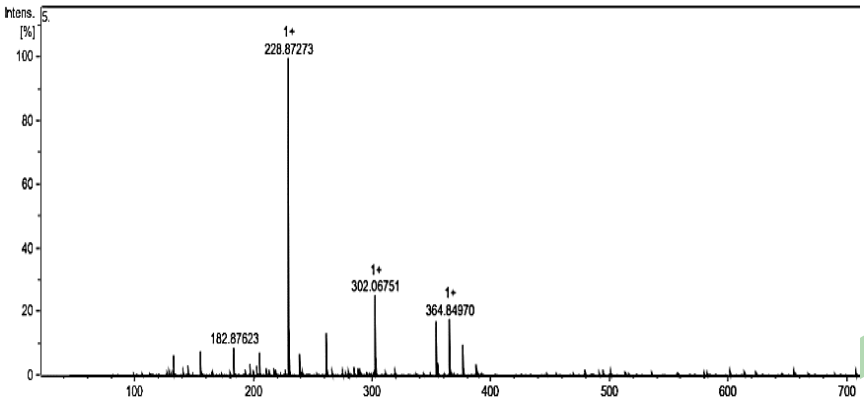

Figure 6. LCMS result of setup 1

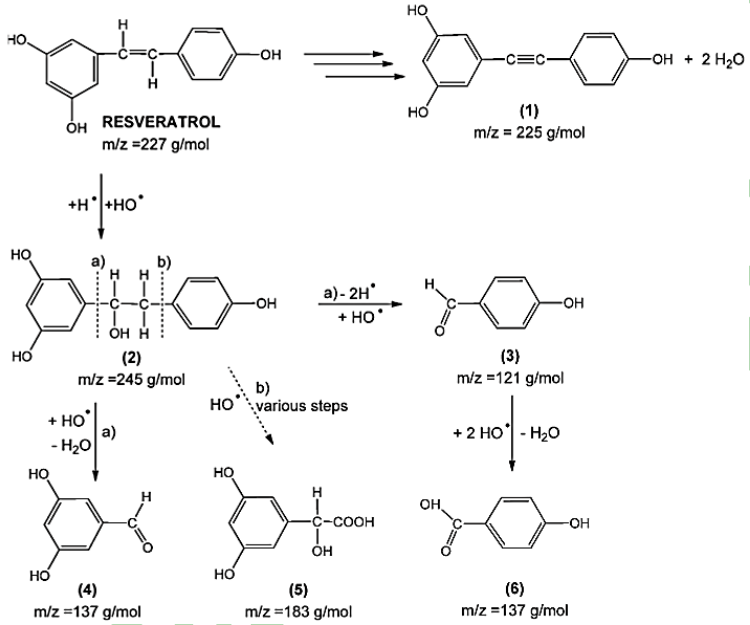

Figure 7. Degradation products of tRSV (Silva et al., 2013).

Diffusion study of quercetin

The stalk solution was analyzed first to determine the retention time of quercetin, which was found to be at 27.71 min, as shown in figure 8. The concentration of each diffusion experiment setup (n=2) was determined using a standard curve (R^2 = 0.961). The three setups resulted in negligible amount of diffusion of quercetin, as shown in table 4.

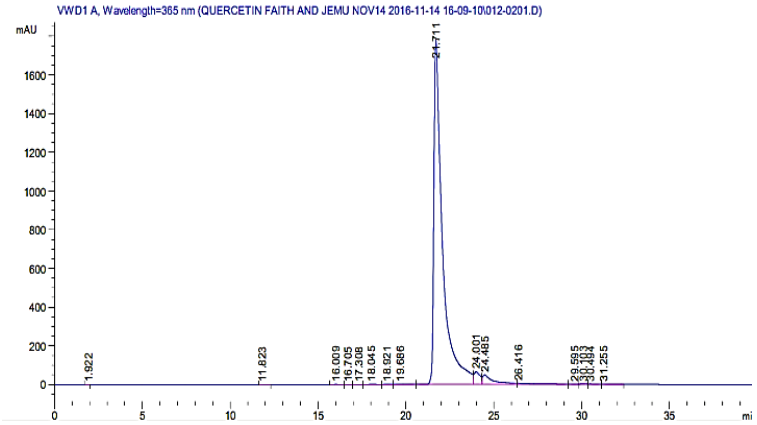

Figure 8. Chromatogram of quercetin stock solution

Table 4. Concentration of diffusion of quercetin from each setup

Setup	Concentration (ppm)
1	2.07±0.014
2	3.56±2.22
3	0

The behavior of diffusion of quercetin in setup 2 may be questionable considering that setup 2 is one of the factors affecting the diffusion of setup 1. A possible reason for this is that the quercetin blocks the diffusion when the BC is saturated with quercetin. Quercetin can form extensive hydrogen bonding with the fibrils of BC and the hydrogen bonds may decrease or stop more quercetin from diffusing through the BC. In setup 1, the BC was already saturated with the 500ppm quercetin solution prior to the diffusion experiment and this means that the quercetin cannot diffuse through the BC anymore since the quercetin molecules are incorporated in the matrix blocking more quercetin to diffuse. On the other hand, pure BC was used in setup 2, and therefore quercetin can diffuse through the BC until the BC is saturated with the quercetin. However, once it is saturated with the quercetin molecules, the diffusion stops, which can explain the negligible amount of diffusion in setup 2.

The setup 3 confirms that there is no diffusion from the BC once it is saturated with the quercetin, since no quercetin was detected in the chromatogram.

Unlike in the diffusion study of tRSV, quercetin exhibited negligible diffusion, and this is due to the hydroxyl groups present in tRSV and quercetin. BC has the net-like fibril structure that is stabilized by extensive hydrogen bonding. Quercetin or tRSV can be trapped in between the fibrils of the BC and its hydroxyl groups can form hydrogen bonds with the fibrils. There are five hydroxyl groups present in quercetin while there are three in resveratrol. Therefore, when quercetin is in the BC as the matrix, it can form more extensive hydrogen bonding with the BC than tRSV.

Thus, the degree of hydrogen bonding of the molecule and the BC significantly affects the diffusion, in such a way that higher degree of hydrogen bonding lowers the diffusion and vice versa.

4. Conclusions

Diffusion of quercetin and trans-resveratrol using bacterial cellulose as matrix was compared and the effect of degree of hydrogen bonding on diffusion was monitored. Three set-ups were used to determine the different factors affecting the diffusion. Significant diffusion was observed in setup 1 of tRSV and the factors affecting the diffusion in setup 1 were separately analyzed in setup 2 and 3. The sum of concentrations of diffusion in setup 2 and 3 were found to be equal to the concentration of the diffusion of setup 1, which means both the concentration gradient and the natural diffusion of the BC played a role in the diffusion of the drug from the membrane. Quercetin exhibited negligible diffusion through BC due to its more extensive hydrogen bonding with the fibrils of the bacterial cellulose compared to tRSV.
In conclusion, the degree of hydrogen bonding of the molecule with the BC significantly affects the diffusion. Higher degree of hydrogen bonding lowers the diffusion and vice versa.

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