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Antifungal and Anticancerous activity of 5-methyl-phenazine-1-carboxylic acid: *Candida albicans CA ATCC10231 - Pseudomonas aeruginosa PA01* interaction bioactive metabolite

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Abstract:

The significance of microbial interactions goes beyond simple antibiosis which is commonly studied about them. A most suitable, efficient and effective method of extracting adequate amounts a phenazine derivative, 5-methyl-phenazine-1-carboxylic acid (5MPCA)resulting from molecular interaction of Candida albicans CA ATCC10231 and Pseudomonas aeruginosa PA01 in yeast potato dextrose agar (YPDA) media, was devised. The red pigment formed on the surface of the YPDA media in which C. albicans and P. aeruginosa were co-cultured, was scooped and dissolved in water and syringe-filtered. The extracted metabolite was purified through repeated lyophilization and dialysis and then analyzed individually before subsequently combining it with bacterial cellulose (BC) to form a hybrid composite. The extracted metabolite and its composite with bacterial cellulose exhibited antifungal activity against C. albicans with zone of inhibition of 18 mm and 14 mm respectively. The pure metabolite was tested for cytotoxicity against prostate cancer-3 (PC-3) cells. The percentage cell viability of PC-3 cells declined significantly

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from 60% on exposure to 200µg/ml of 5MPCA to 25% at 3200µg/ml exposure of the 5MPCA. The PC-3 cells lost their spherical morphology as they shrinked to appear like rods. The results indicate that 5MPCA induced apoptosis on PC-3 cell lines following mitochondrial intrinsic pathway through the activation of caspase-3 and Bcl-2 down regulation. Antifungal studies of the composite were also performed and other characterizations by SEM, FTIR, TGA, XRD and WHC, were conducted. The FTIR result indicated additional characteristic peaks unique to 5MPCA at wavelengths of 1306.73 cm^{-1} and 1236.95 cm^{-1} which represent C-O and C-N bonds respectively, within the 5MPCA – BC hybrid. In TGA, 10% weight loss occurred in BC composited with 0.200 w/v 5MPCA and weight loss of about 7% in lower 5MPCA (0.100w/v) concentration within the temperature range of 90-100°C. The 5MPCA thermogram alone was about 20% within the same temperature range. The observed weight loss in the composite was due to loss of water molecules adherent to the BC fiber surfaces and molecules trapped between BC layers. Findings of this study revealed that 5MPCA is a promising potent antifungal and anticancerous product. Its combination with BC allows for its application in addressing biomedical challenges like wound healing.

Key words: Molecular interaction; metabolite, 5MPCA, Candida albicans, Pseudomonas aeruginosa, prostate cancer-3 cells.

INTRODUCTION

More than just their natural coexistence, fungi and bacteria tend to interact within various ecological niches (Lindsay & Hogan, 2014). These types of interactive phenomena often take place in ecological consortia called biofilms. The biofilm assemblages can either be derived from single species or multiple species. Directly or indirectly, the characteristic behaviour of the interacting species differ from the mono species in various aspects such as microbial behaviour, their distribution, chances of survival in the environment and their

response to antimicrobial agents (Lindsay & Hogan, 2014). Microorganisms within biofilms exhibit more chances of survival compared to sessile planktonic ones hence their characteristic resistance to antimicrobial substances (Tshikantwa, Gashe, & Mpuchane, 2017).

Manipulating microbial communities promises to be useful as it can yield essential outcomes that are applicable in various fields such as waste water treatment (Werner et al., 2011), food production (Mounier, 2008), and for the prevention and treatment of diseases such as caries (Marsh, 2003), inflammatory bowel disease (Maloy & Powrie, 2011) and obesity (Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh, Bäckhed, Fulton, & Gordon, 2008). For inflammatory bowel disease and obesity, the gut ecosystem is a prime target for modelling. However, few attempts have been made so far to develop models for the gut microbiota (Faith, McNulty, Rey, & Gordon, 2011). On the other hand the most outstanding challenge is to devise means through which the safety of food is ensured and guaranteed through reduction or total elimination of contaminants from surfaces where food is handled(Tshikantwa et al., 2017).

Pseudomonas aeruginosa, a common gram-negative soil bacterium and an opportunistic human pathogen, is well known for its ability to produce a blue phenazine, called pyocyanin, which is toxic to numerous bacteria and fungi and damages mammalian cells(Kerr et al., 1999). *P. aeruginosa* culture supernatants also contain phenazine carboxylic acid (PCA), 1-hydroxyphenazine, and phenazine-1-carboxamide. In addition, *P. aeruginosa* can produce two red pigments, aeruginosins A and B (5-methyl-7-amino-1-carboxymethylphenaziniumbetaine and 5-methyl-7-amino-1-carboxy-3-sulfo-methylphenazinium). In a study by Gibson, *et al*, an interplay of biochemical, genetic and microscopic experiments revealed that a pyocyanin precursor, 5MPCA, was previously found to be responsible for

the formation of the red pigmentation(Gibson, Sood, & Hogan, 2009). Further characterization showed that the red pigment accumulated within fungal cells, where it remained redox active, and that its formation correlated with decreased fungal viability(Gibson et al., 2009).

Presently, there is minimal information available about the various types of interactions of several organisms that colonize the humans despite their effect on them. This field however, needs to be cautiously considered because of the complex nature of mixed type infections. A clearer comprehension of mixed species infections (e.g. bacteria-fungi infections) in humans is also crucial in that the consequences of such infections can vary from those caused by individual mono-species (Peleg, Hogan, & Mylonakis, 2010). As a result, the alternative methods of treatment have to be applied. Opportunistic pathogens are often involved in multispecies infections of this nature. Consequently, immunocompromised individuals are more susceptible to opportunistic infections. The bacteria-fungi interactions during an infection, can either be classified as neutral (interaction with no impact on the outcome of a disease), synergistic (acting together of bacterial and fungal pathogens against the human host), or antagonistic (inter-inhibition of microorganisms on each other)(Scherlach, Graupner, & Hertweck, 2013).

Investigations of these diverse bacteria-fungi interactions occurring in humans demonstrated that the outcome of an interspecies infection ranges from virulence enhancement to antagonistic interaction. Treatment for mixed-species infection, therefore, should be carefully measured, as an increasing growth of *C. albicans*, for example, was observed after antibacterial therapy of a mixed infection of *P. aeruginosa* and the yeast (Burns et al., 1999). However, an additional antibacterial treatment may be required in infections where bacteria contribute to the virulence of a fungus. Additionally,

further research on molecular antagonistic interactions might serve new treatment options or hitherto unknown points of attack (Scherlach et al., 2013).

Certain genera of bacteria such as*Acetobacter, Rhizobium, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Salmonella, Escherichia*, and *Sarcina* produce BC(Seo et al., 2014; Shoda & Sugano, 2005). BCis potentially useful in a number of applications(Ullah, Ul-Islam, Khan, Kim, & Park, 2016). This is derived from its high degree of purity, superior physico-mechanical and biological properties over the other type of cellulose obtained from plants(Ul-Islam, M., Khan, T., & Park, 2012).The special properties of BC such as high water holding capacity (WHC) and gradual water release rate (WRR), high crystallinity, greater tensile strength, ultrafine fiber network, and its ability to be readily molded into three-dimensional structures, among other things, attest to the fact that BC is very important(Czaja, Krystynowicz, Bielecki, & Brown, 2006; Ul-Islam, M., Khan, T., & Park, 2012; Xian Li, Kim, Lee, Kee, & Oh, 2011). Moreover, it has great potential in being used to form composite material with several biocompatible, bactericidal and fungicidal material; thus, it can be immensely valuable for different applications in the biomedical field(Czaja et al., 2006; Ul-Islam, Shah, Ha, & Park, 2011; Xian Li et al., 2011).

Earlier reports have intimated that the thin microbial cellulose fibrils are interconnected through inter- and intra-molecular hydrogen bonds that assist in providing stability to its reticulate structure (Ul-Islam, Ha, Khan, & Park, 2013). During synthesis, the BC fibrils are arranged loosely with many empty spaces(Ul-Islam, Khan, & Park, 2012)leading to an increased surface area and a densely intertwined matrix network(Dahman, 2009; Meftahi et al., 2010). This network can however be further enhanced through extended BC production achieved by adding extra nutrients with high carbon source or

alternatively extending its availability(Ul-Islam et al., 2012). The physico-mechanical and thermal properties of BChighly affect its structural variations(Shah, Ul-Islam, Khattak, & Park, 2013; Shezad, Khan, Khan, & Park, 2010; Ul-Islam et al., 2011). The strong and stable fibrils confer improved resistance to the force applied and resist changes in the BC structure(Ul-Islam et al., 2013). Similarly, the empty spaces between the pores readily accommodate media components and water molecules that result in the swelling of BC. Actually, it has been shown to accommodate 100–200 times its dry weight in water(Joseph et al., 2009; Schrecker & Gostomski, 2005).Additionally, liquids and small particles can equally be incorporated into the empty spaces, and consequently BC supports the formation of composites with several nano- and biocompatible polymeric materials(Clasen, Sultanova, Wilhelms, Heisig, & Kulicke, 2006). For example, an appropriate moisture content of a dressing material facilitates the wound-healing process and prevents colonization of infectiousorganisms (Kaewnopparat, Sansernluk, & Faroongsarng, 2008). The high porosity of BC favours a highWHC, since water fills the porous matrix and is bound to the fibrils through hydrogen bonding(Gelin et al., 2007). Generally, the uniform fibril arrangement, high surface area per unit mass, and hydrophilic nature of BCgives rise to a high WHC(Dahman, 2009; Schrecker & Gostomski, 2005). Any variations in such features can be attributed to different BC produced under different experimental conditions.

The characteristic features of BC, such as the arrangement of fibrils and the conformation of the porous matrix, are directly dependent on such factors as the activity of producing organisms, culture media composition, variation in culture time and conditions, amount of inoculum, and the source of carbon(Kaewnopparat et al., 2008; Tang, Jia, Jia, & Yang, 2009).Based on its structure and the associated

mechanical properties, microbial cellulose can be composited with some naturally synthesized bioactive metabolites and applied in crucial medical interventions. The composite will be possessing the characteristic traits of the microbial cellulose and the metabolite as a hybrid. The expectation is that the metabolite will be embedded in the microfibrils of the cellulose hence be applied to elicit the required biomedical response/activity.

Lately, the focus of researchers has been on devising methods that can overcome the clinical challenge of biofilm formation. On the other hand, less attention was paid on the possible potential usefulness of the interactions. In some rare cases, useful products have been discovered through microbial interactions. The current study is one typical examples of those seldom identified cases where 5MPCA as a metabolite resulting from interaction between *C. albicans* and *P. aeruginosa* is extracted, characterized and ultimately composited with BC for biomedical applications.

In this study, the physiochemical properties of the resultant metabolite of *C. albicans* and *P. aeruginosa* interaction, 5MPCA and its composite with BC produced by *Acetobacter xylinum*, were investigated. The physiochemical analysis is a method used to determine the characteristic, physical and chemical properties of a material. This includes Fourier transform infrared (FTIR) analysis, field emission scanning electron microscopy (FESEM), thermal stability using thermogravimetric analysis (TGA), water holding capacity (WHC) and water release rate (WRR). Additionally, the antifungal properties of BC-5MPCA as well as the cytotoxicity of the extracted 5MPCA against prostate cancer cells have been investigated.

MATERIALS AND METHODS

Materials

The chemical reagents that include yeast extract, glucose, peptone, disodiumphosphate and citric acid were purchased from Sigma-Aldrich (St Loius, MO, USA). All reagents in the research were utilized without any additional processing.

Microorganisms and cell culture

Yeast Potato Dextrose Agar (YPDA) was prepared using the individual components in weight ratio as 2%peptone, 1% yeast extract, 2% glucose and solidified with 2% agar (Gibson et al., 2009). The individual components were done by taking 4g of peptone; 4g of yeast extract; 4 g of glucose and 4 g of agar and dissolving them in 5ml of distilled water. The mixture was heated slightly to dissolve the agar and the final volume was made up to 200ml. The preparation was then sterilized in the autoclave at 121 ^{o}C for 15 minutes. The YPDA was then allowed to cool to about 60 ^{o}C and then poured on agar plates to await culturing.

Candida albicans was cultured in the Yeast Peptone Dextrose Agar (YPDA) for 48 hours at 30 ^{o}C A lawn of *C. albicans* was produced and 10µl of a 24 hour-old LB culture of *Pseudomonas aeruginosa* (*P. aeruginosa*) was inoculated into the cultured lawn of CA and incubated for 48 hours at 30 ^{o}C

P. aeruginosa: The organism is motile, gram- negative, catalyase- positive, oxidase-positive or negative(Gibson et al., 2009)and slightly straight or curved rods that measure 0.5 - 0.8µm by 1.5 – 3.0 µm with almost all strains being motile by means of a single polar flagellum; its optimum temperature is 37^{o}C but it can grow in temperatures as high as 42^{o}C (Society for General Microbiology., 1999). It is aerobic (Gibson et al., 2009). This bacterium is a versatile organism that grows in soil, marshes and coastal marine habitats as well as on plant and

animal tissues. People with cystic fibrosis, burn victims, cancer patients and individuals that require extensive stays in intensive care units are mostly at high risk of diseases resulting from *P. aeruginosa* infections(de Lima Brossi, Jiménez, Cortes-Tolalpa, & van Elsas, 2016; Society for General Microbiology., 1999).

P. aeruginosa is notorious for its resistance to antibiotics, therefore its particularly dangerous and one of the highly dreaded pathogen. It is naturally resistant to many antibiotics due to the permeability of barrier afforded by its outer membrane Lipopolysaccharride (LPS). Moreover, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics. Since its natural habitat is soil, living in association with *Bacilli*, *Actinomycetes* and *Moulds*, it has developed resistance to a wide array of their naturally-occurring antibiotics. *Pseudomonas* on the other hand, maintains antibiotic resistance plasmids, both R-factors and RTFs, and it's able to transfer these genes by means of processes of transduction and conjugation(Todar, 2006). However, only a few antibiotics are active against some strains of *Pseudomonas*; these drugs include fluoroquinolones, gentamycin and imipenem (Society for General Microbiology., 1999).

C. albicans is a polymorphic fungus with yeast, hyphal and pseudohyphal forms in its life cycle. It is a commensal organism in healthy individuals and an opportunistic pathogen inimmunocompromised patients (Douglas, 2003). *C. albicans*is found as part of the normal flora ofthe oral cavity, gastrointestinal tract and female genital tract of humans (Mason et al., 2012).Immunosuppressive therapy, antibiotic therapy, use of indwelling devices, HIV infection, diabetes and old age are some of the predisposing factors for C. albicans infection(Ramage, 2002).

Production and purification of 5MPCA

An inoculating loop was used to scoop some part of the red pigment on the surface of the YPDA media in which the *P. albican*s – *C. albicans* co-cultures was made. This scoopful was inoculated into 100µl of sterile water, then shaken and let to stand for five minutes.A 1 ml-capacity syringe and a filter sterilizing instrument was used to filter the water-dissolved 5MPCA. A semi-opaque liquid was obtained in a petri dish. The filtered solution of 5MPCA in the petri-dish was placed in the oven set at 60^{o}C to evaporate the water and retain the crystals overnight. The filter-sterilized solution of 5MPCA (50ml) was then frozen at -20^{o}C overnight and then freeze-dried. This resulted in whitish crystals which were collected in a tube to await further steps in the experiment.

Preparation of 5MPCA -BC composite

Sterile BC samples were placed in various dilutions of 5MPCA stirred on magnetic stirrer for 4 days. Subsequently the samples were removed and excess liquid removed then frozen at -20^{o}C for 24 hours. The sample was then freeze dried for further 24 hours before being used for further tests.

Cytotoxicity of 5MPCA on prostate cancer-3(PC-3) cells

The antitumor activity of 5MPCA on prostate cancer-3(PC-3) cells was determined using CCK-8 assays. The PC3 tumor cells cultured to the logarithmic growth stage, were digested and regulated to the cell density of 2*10^{5} /ml, and seeded to 96 cell culture plates (200ml/hole), after incubation in 5% CO_2, 37 ° C for 24 h, the F12 medium containing different concentrations of drugs replaced the original medium. The drug-free group was used as a negative control group. 5-MPCA dissolved in F12 medium, and filtered with 0.22µm membrane filter, and then it was diluted to 200, 400, 800, 1600, 3200µg/ml, respectively. After 72h of culture, the original medium was aspirated, CCK-8

solution (CCK-8: F12 = 1: 10) added to each hole. After incubated at 37° C for 1h, aspired 100ul CCK-8/medium to a new 96-hole plate and determined the OD value at 450nm. Each concentration of the drug was made in 6 parallel samples and repeated three times. The cytotoxicity is calculated as follows:

Cell viability(%)=(OD_{sample}-OD_{blank})/(OD_{sample}-OD_{blank})×100%.

Characterizations

The bio composite films were evaluated by SEM and FT-IR techniques, TGA and XRD, Antimicrobial assay, and Biocompatibility.

Field-emission scanning electron microscopy (FE-SEM)

The surface morphology of freeze-dried samples BC andBC-5MPCA composite was observed using FESEM. The samples were fixed on a brass holder and coated with gold on a Cu SEM disk and analyzed through a Nova NanoSEM450 FE-SEM (Nova NanoSEM450, FEI, Holand)

Fourier Transform Infrared (FT-IR) spectroscopy

Infrared spectroscopy is one of the most powerful analytical tools, which provides the possibility of chemical identification. FTIR spectra of freeze-dried samples of 5MPCA and BC-5MPCA composite were recorded using a VertexFTIR spectrophotometer (FTIR,VERTEX 70, Germany; spectral range 4000-400cm^{-1}; beam splitter: Ge-coated on KBr; detector: DTGS; resolution: 0. 25 cm^{-1} (step selectable)] to determine the chemical structure of the composite films and possible interactions between their components. The obtained IR data was analyzed through a statistical program, Origin 8 to acquire the spectrum of the constituents of the composite.

X-Ray Diffraction study (XRD)

The XRD patterns of the BC, 5MPCA and BC-5MPCA composite samples were recorded using an X-ray diffractometer with an X-ray generator (3 kW) and anode (LFF Cu). The radiation was CuK-α at 1.54 A˚, the X-ray generator tension and current was 40 kV and 30 mA, respectively, and the angle of scanning varied from 10 to 55°.

Thermal Gravimetric Analysis (TGA)

Thermal gravimetric analysis (TGA) was performed using a thermal gravimetric analyzer (Shimadzu, TGA-50)on about 5 mg samples. The samples were heated in open alumina pans over 25ºC -450ºC at a heating rate of10ºC/min under nitrogen flow.

Water holding capacity analysis

The WHC of BC and BC-5MPCA composite were measured by thesieve-shake method as described previously (Ul-Islam et al., 2012).Both types of cellulose sheets were dipped in distilled water for a sufficient amount of time until completely swollen. The sheets were then taken out using tweezers, placed on a sieve and shaken twice to remove the surface water, and weighed. The samples were then dried at room temperature for 24 h and their weights were measured at different time intervals. Thereafter, both samples were oven-dried for 24 h at 60 ◦C for complete water removal. Finally, WHC was calculated as the mass of the water removed during drying per the dry weight of cellulose using the following formula:

$$\text{Water holding capacity} = \frac{\text{Mass of water removed during drying in (g)}}{\text{Dry weight of the Composite (BC} - \text{5MPCA)(g)}}$$

Similarly, for determination of WRR, the wet weights of both cellulose samples produced in static conditions were measured,

followed by continuously weighing the samples stored at ambient conditions at different time intervals until a constant dry weight was achieved. The weights of both cellulose samples at different time intervals were plotted against time, as reported previously(Shezad et al., 2010).

RESULTS AND DISCUSSION

Red pigment formation in *P. aeruginosa*-fungal cocultures.

The study proposed that the intracellular accumulation of a 5MPCA-derived product within target cells was likely to be representing an important aspect of phenazine-mediated antagonism between *P. aeruginosa* and other species, including fungi.When *P. aeruginosa* strain PA14 was point inoculated onto established lawns of *C. albicans* strain SC5314 yeast and further incubated at 30°C, a red-pigmented zone was observed to have developed around the inoculation point within 12 h and grew to develop 48 h (Gibson et al., 2009) (Figure 1). The red pigmentation also developed when *P. aeruginosa* was inoculated onto established lawns of filamentous *C. albicans* cells, as demonstrated using the constitutively filamentous *C. albicans tup1/tup1* mutant (Brehm-Stecher & A Johnson, 2004). The results further indicated that the Inoculation of *P. aeruginosa* onto a lawn of the filamentous *C. albicans* strain led to a larger zone of pigmentation than that formed on*C. albicans yeast*. It was further proved that when*P. aeruginosa* and*C. albicans* were grown individually under similar conditions, there was no red pigmentation produced hence a clear evidence that the pigmentation came as a result of the interaction between the filamentous *C. albicans* and *P. aeruginosa*.

In another study, the red pigmentation was observed regardless of whether the co-cultures were grown on rich or minimal agar plates, i.e., YPD or M63 medium with 0.2%

glucose, respectively(Gibson et al., 2009), there was no obvious red pigmentation observed when *P. aeruginosa* and *C. albicans* were grown together in liquid cultures of the same medium composition (Gibson *et al,* 2009). In order to carry out confirmatory tests to determine whether the development of red pigmentation was specific to *P. aeruginosa* and *C. albicans* or not, other strains of *P.aeruginosa*, other *Pseudomonas* species, and other fungal strains were analyzed in coculture assays(Gibson et al., 2009). The result showed a similar, but rather more pale, red pigmentation observed when *P. aeruginosa* strain PA14 was cultured on lawns of *Saccharomyces cerevisiae* BY4742, BY4741, and 1278b and three unidentified environmental yeast-like fungi isolated from soil samples and plant material. Upon co-culture with the *C. albicans tup1*/ *tup1* mutant, *P. aeruginosa* strain PAO1 and 17 of 19 clinical *P.aeruginosa* isolates gave rise to red pigmentation to various degrees. No red pigmentation was observed when *Pseudomonas putida*, *Pseudomonas fluorescens*, or *Pseudomonas chlororaphis* were incubated on lawns of *C. albicans*(Gibson et al., 2009).

The 5MPCA metabolite derived from interaction between *C. albicans* and *P. aeruginosa* is a potential antifungal and anticancer product.

Cytotoxicity of 5MPCA onPC-3cells

The *in vitro* inhibitory activity of 5MPCA was determined by CCK-8 assays in prostate cancer cells (CP-3) for 72 hours. 5MPCA exhibited strong suppressive and dose-dependent cytotoxic inhibitory effect on the PC-3 cells. The percentage cell viability of PC-3 cells declined remarkably from 60% on exposure to 200μg/ml of 5MPCA to 25% at 3200μg/ml exposure of the 5MPCA as indicated in Figure 2. In cells where there was no 5MPCA introduced to the cells (control), there was no cytotoxic effect observed. The same trend is observed in the

microscope images and changes in the cell morphology in Figure 3. The cells introduced to the highest concentration of 5MPCA (3200µg/ml) had greatly declined and their morphology was also no longer spherical but they have greatly shrinked. This suggests an indication of the apoptotic effect of 5MPCA on the PC-3 cells. A similar effect was observed by Kennedy *at al* when another bioactive metabolite, 5-methyl phenazine-1-carboxylic acid betaine (MPCAB), obtained from soil bacterium, exhibited selective cytotoxicity against lung (A549) and breast (MDA MB-231) cancer cell lines(Kennedy et al., 2015). Since the two metabolites, 5MPCA and MPCAB are structurally similar, the current result findings equally affirm that 5MPCA itself had the same effect on PC-3 cell lines as MPCAB on lung (A549) and breast (MDA MB-231) cancer cell lines. The results indicate that 5MPCAinduced apoptosis on PC-3 cell lines following mitochondrial intrinsic pathway through the activation of caspase-3 and Bcl-2 down regulation (Kennedy et al., 2015).

Field-emission scanning electron microscopy (FE-SEM)

The crystalline 5MPCA has incorporated itself on the fibers of BC as observed in Figure 4. The incorporated 5MPCA appears ununiformly distributed across the individual fibers. The image obtained shows the typical BC structure with irregularly arranged fibers. The crystals of 5MPCA are observed embedded of the BC fibrils. The association of 5MPCA and BC provides a unique morphological hybrid of BC (composite). Further analysis of the composite under higher magnification revealed that the BC poses a nanostructure with an irregular size and shape of fibrous network as shown in Figure 4.The nanoparticle provides several advantages and applications in the industries since more surface area can be utilized and as such enhance their overall capability. This include the fact that nanoparticles can be easily manipulated for both passive and active drug

targeting, availability of appropriate matrix that is useful in increasing the efficacy and reducing side effects of drugs, and finally important to control and sustain release of the drug (5MPCA) during the transportation as well as the location of the release(Zahan; et al., 2017).

Fourier Transform Infrared (FT-IR) spectroscopy

Infrared spectroscopy is one of the most powerful analytical tools, which provides the possibility of chemical identification.It is an essential chemical characterization technique used to determine the various constant functional groups as well as the types of chemical bonds within a molecule (Ul-Islam et al., 2011). In Figure 5, the FTIR spectrum of BC indicates one absorption peak at 3699.63 and 339.54 cm^{-1} representing OH and CH_2 grouping. The strongest absorption peak is observed at 1437.42 cm^{-1} which represents the presence of carboxylic acid groups in the cellulose structure and other common parts of the BC structure. The FTIR spectra of the composited BC have some resemblance of that of both BC and 5MPCA. Generally, FTIR spectrum of pure BC has been changed by 5MPCA when composited *ex-situ*. In the BC-5MPCA composite, while the basic BC structure is maintained, the strong absorption peak has been shifted from 1437.42 cm^{-1} to 1054.44 cm^{-1}. This indicates a decline in the bond strength which means a weaker type of bonding.

From the FTIR spectrum results, the 5MPCA has been incorporated into the BC fibers. This is indicated by appearance of additional characteristic peaks unique to 5MPCA at wavelengths of 1306.73 cm^{-1}and1236.95 cm^{-1}whichrepresent C-O and C-N bonds respectively. This is further affirmed by the loss of the characteristic strong peak of 5MPCA around 1625 cm^{-1} in the subsequent increased concentrations. This indicates that a composite has actually occurred and acquired morphological traits of both 5MPCA and the BC. The SEM

characterization affirmed the findings of incorporation of 5MPCA into the BC fibers as depicted in Figure 4. Presently there is no data available on this type of composite as it has not been carried out before. The hybrid composite, however, proves to be a potentially useful material in biomedical applications such as in wound healing.

X-Ray Diffraction study (XRD)

The crystalline structure of BC, 5MPCA and BC-5MPCA composite was determined by XRD and the results reflected in Figure 2. The characteristic XRD pattern for BC is traceable with the peaks around 14.18^{o}, 15.98^{o} and 22.91^{o}(Jasim, 2017). The 5MPCA, on the other hand revealed three peaks; a minor one 27.12^{o}, a highest peak at 32.97^{o} and another one at 45.52^{o}(Figure 6). Comparatively, in the BC-5MPCA composite, the basic characteristic peaks for BC are retained but the ones for 5MPCA are lost. However, a new peak is introduced at 35.02^{o} in a composite with a relatively higher weight/volume concentration of 5MPA (0.200 w/v BC-5MPCA). This results from the crystalline portion of the incorporated 5MPCA attached to the BC fibers. The conclusion that could be drawn from these findings is that 5MPCA is a crystalline structure and has improved the crystallinity of the normal BC. However, increasing the concentration of the 5MPCA in the composite can give a clearer picture to fully asset this conclusion.

Thermal Gravimetric Analysis (TGA)

Thermal gravimetric analysis (TGA) was performed using a thermal gravimetric analyzer (Shimadzu, TGA-50)on about 5 mg samples. The samples were heated in open alumina pans over 25^{o}C -450^{o}C at a heating rate of10^{o}C/min under air flow.The degree of stability of BC under thermal conditions directly influences its potential for commercial applicability adding to other physio-mechanical and biological properties

(Lin & Burggraaf, 1991). Based on this background, the thermal stability of BC, 5MPCA and BC-5MPCA composite were determined through TGA technique and their thermograms reflected in Figure 6. The basic processes involved in the thermal degradation of the BC and the composite are degradation, depolymerization and decomposition of glycosylic fragments and subsequently the charred residue synthesis (George, Ramana, Bawa, & Siddaramaiah, 2011). Two major loss zones were depicted in both BC alone and the composited one (Ullah et al., 2016). A loss of about 10% was observed in the BC composited with 0.200 w/v 5MPCA while a weight loss of about 7% was observed in the composite with a lower 5MPCA (0.100w/v) concentration within the temperature range of 90-100^{o}C. By contrast, the thermogram for 5MPCA alone was about 20% within the same temperature range. The account for the observed weight loss in the composite is the loss of water molecules adherent to the BC fiber surfaces coupled with those molecules trapped between the layers of BC(Khan, T., & Park, 2008; Margarita Darder, Montserrat Colilla, & Ruiz-Hitzky*, 2003). The higher weight loss observed in the 5MPCA is attributed to its solubility in water and subsequent high release rate (Figure 7&8). The subsequent phase of the curves indicated a rapid weight loss for the composite primarily due to the high WHC (Table 1) of the BC (Margarita Darder et al., 2003; Ul-Islam et al., 2012) which forms the major part of the composite. The 5MPCA on the other hand showed a reduced weight loss due to its chemical bonding with water molecules.

Antimicrobial activity tests

The extracted 5MPCA has inhibitory effect on the growth of *Candida albicans* as depicted in Figure 9 (a). Additionally, on the BC-5MPCA composite (Figure 9b), the antifungal activity of 5PMCA seem to have reduced slightly possibly because of the possible interactions between BC and 5MPCA. The account for

the inhibition is as a result of the accumulation of the 5MPCA in fungal cells to reach toxic levels (K Morales et al., 2010). The 5MPCA is therefore viewed as having great potential to be used as a potent antifungal agent (K Morales et al., 2010) which can be harnessed naturally through microbial interactions. The mechanisms of action for the antifungal activity of 5MPCA is described by (K Morales et al., 2010).

CONCLUSION

In this study, almost suitable, efficient and effective method of extracting adequate amounts a phenazine derivative, 5MPCAresulting from molecular interaction of *C. albicans* CA ATCC10231 and *P. aeruginosa* PA01 in YPDA media, was established. The extract exhibited antifungal activities against the *C. albicans*. It also showed remarkable cytotoxic effects on prostate cancer cells (PC-3). This places the resultant metabolite from the microbial interaction of the two organisms at a good place for use in some biomedical applications such as antifungal agent and an antitumor agent. In the subsequent experiments, a nobel hybrid composite of 5MPCA and bacterial cellulose (BC) was made.

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Figure1: Red pigment formed from *C. albicans* and *P. aeruginosa* co-cultured in YPDA.

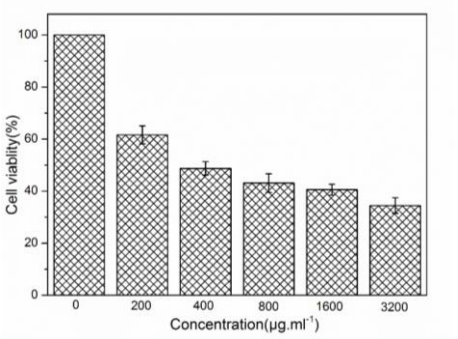

Figure 2: Cytotoxicity of 5MPCA onprostate cancer-3(PC-3) cells. The metabolite showed significant cytotoxic effects on the prostate cancer-3(PC-3) cells across all the dilutions.

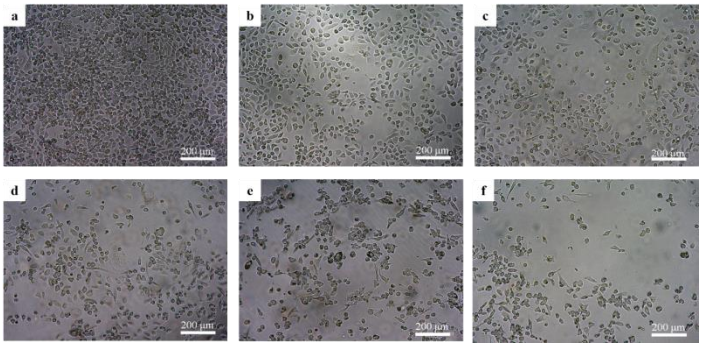

Figure 3: Cytotoxicity of 5MPCA on prostate cancer-3(PC-3) cells. (a) 0µg/ml (control) (b) 200µg/ml, (c) 400µg/ml, (d) 800µg/ml, (e) 1600µg/ml, (f) 3200µg/ml. The metabolite showed significant cytotoxic effects on the prostate cancer-3(PC-3) cells all the dilutions.

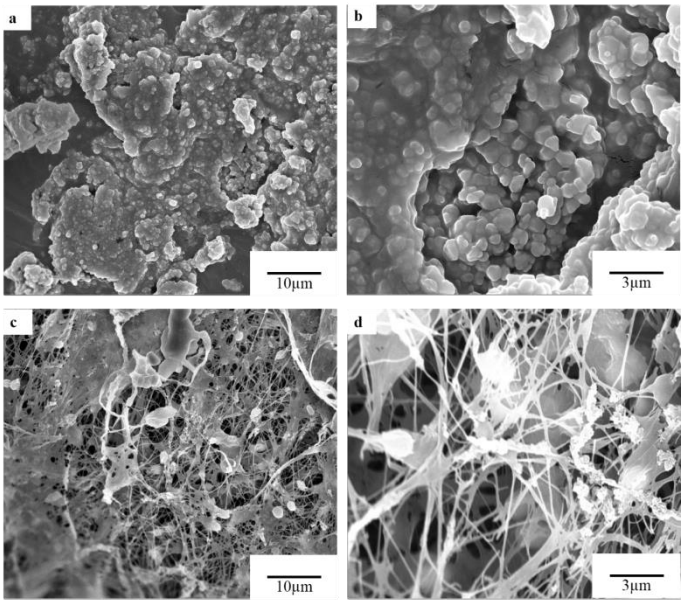

Figure 4.FESEM picture of 5MPCA and BC-5MPCA composite at two different magnifications(a) 5MPCA at lower magnification; (b) 5MPCA at higher magnification; (c) BC - 5MPCA composite at lower magnification and (d) BC - 5MPCA composite at higher magnification.

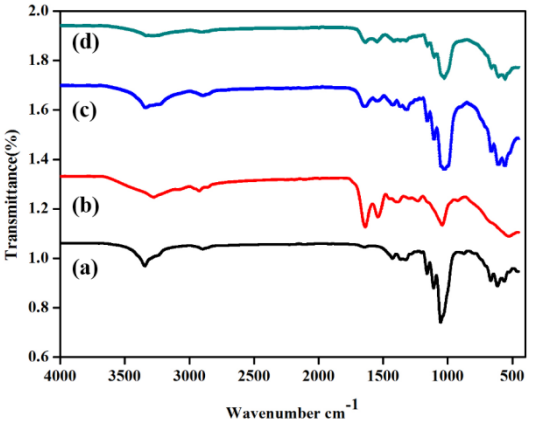

Figure 5: FTIR Spectrum of BC, 5MPCA, and the composite. (a) 5MPCA; (b) BC; (c) BC + 0.100 5MPCA; (d) BC + 0.200 5MPCA

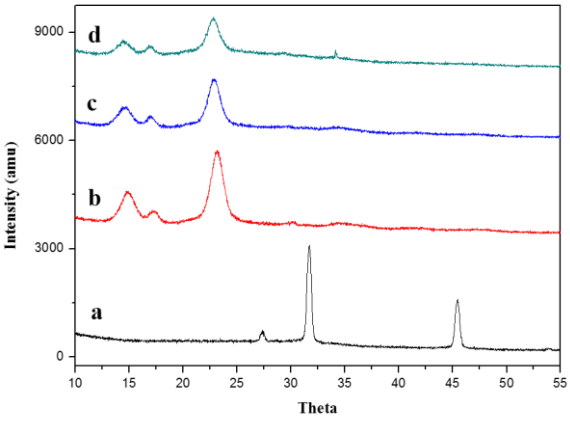

Figure 6: XRD spectrum of BC, 5MPCA, and the composite. (a) 5MPCA; (b) BC; (c) BC + 0.100 5MPCA; (c) BC + 0.200 5MPCA

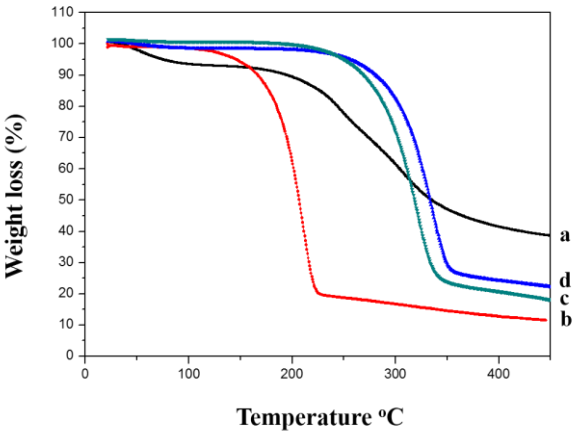

Figure 7: TGA curves for5MPCA, and the composites. (a) 5MPCA; (b) BC; (c) BC + 0.100 5MPCA; (d) BC + 0.200 5MPCA

Water holding capacity (WHC)

Table 1: WHC values for BC, 5MPCA, and the composite.

Sample	Dry weight (g)	Weight (g) after 24 hours	WHC (%)
BC	0.0012	0.0190	14.83
BC-5MPCA	0.0023	0.0598	25

5MPCA release rate (5MPCA RR)

Table 2: The rate of release of 5MPCA from the BC-5MPCA composite at room temperature (21°C)

	Weight (g)	
Time (hours)	BC	BC-5MPCA Composite
0	0.0583	0.1866
2	0.0193	0.1424
4	0.0101	0.1022
8	0.0096	0.0425
12	0.0094	0.0062
24	0.0093	0.0056

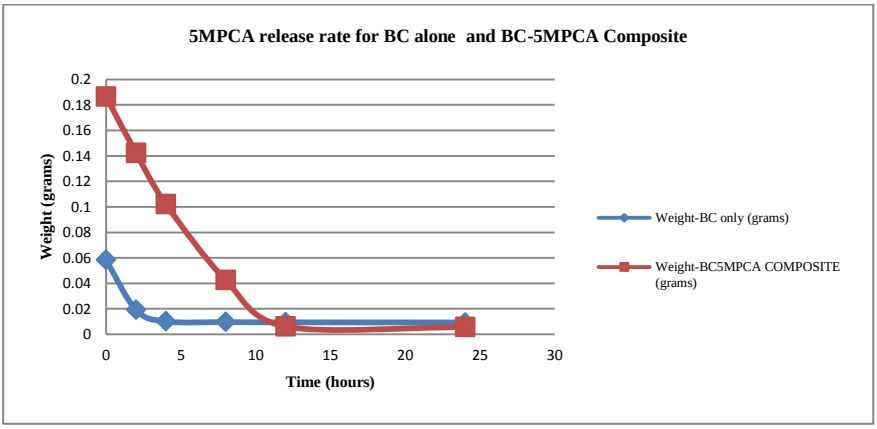

Figure 8: 5MPCA release from BC alone and BC-5MPCA composite

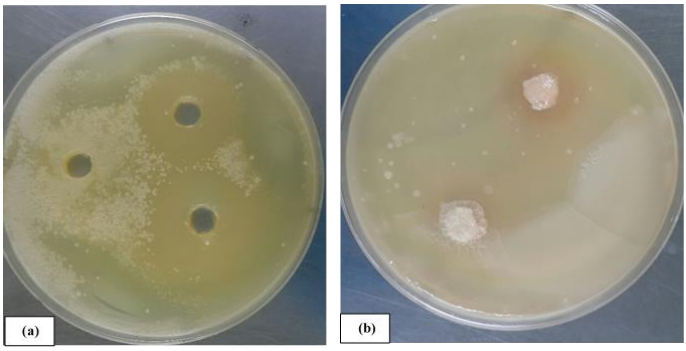

Figure 9: (a) Zone of inhibition reached 18mm after 48 hours of incubation at 30 °C in a culture of *C. albicans* for a liquid 5MPCA extract. (b) Zone of inhibition reached 14mm after 48 hours of incubation at 30 °C in a culture of *C. albicans* for BC-5MPCA composite.