

Original Article

Evaluation of Potential Anti-Pathogenic and Anti-Retroviral Effects of a Proprietary Bioactive Silicate

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Summary: BioActive Silicate a modified sodium silicate dietary supplement manufactured by Cisne Enterprises Inc. (Odessa, TX) was evaluated for its in vitro anti-retroviral and anti-pathogenic effects. Effects on nitric oxide (NO) dependent antiviral activities were measured in neutrophils using standard assays. Assays for inhibition of HIV-II reverse transcriptase (RT), HIV-II protease (PR) and glucosyltransferase [glucuronidase (GH-1) and glucosidase (GH-2)] important for viral replication, coat assembly and virulence respectively, were performed using standard kits. Higher NO (~ 2 fold) was detected in neutrophil medium indicating an increase NO mediated antipathogenic activity. Results suggest that the product significantly decreased HIV-RT activity in a dose dependent manner (ED₅₀= 20.4 mM). HIV-PR activity decreased (IC₅₀=14.6 mM) with increasing product concentration. The product also decreased the HIV-II virulence by inhibiting the GH-1 (IC₅₀= 34.29 mM) and GH-2 (IC₅₀=14.6 mM) activity which decreased protein glucosylation and glucuronylation. Changes in surface EPS carbohydrates assayed in *Pseudomonas aeruginosa* suggested a modulatory effect on various carbohydrates and therefore the composition of EPS.

Industrial relevance: Pathologies caused by retroviral agents and microorganisms are prevalent both in developed and developing countries. Indiscriminate use of single target medical drugs has resulted in the development of resistance in these pathogens. Development of novel therapeutic agents that can effect multiple intrinsic and extrinsic targets in host and the pathogen may prove to be more effective and are less likely to promote drug induced selection. The effectiveness of BioActive Silicate in mitigating various virulence and survival pathways in pathogens is promising. Additionally, it was also effective in inducing a NO mediated immune response.

Keywords: Sodium silicate; anti-pathogenic, anti-retroviral, immune response.

Introduction

Microbial pathogenic diseases caused by bacteria, viruses and parasites are prevalent across all countries globally. Development of pharmaceutical drugs and agent specific vaccines in last 20th century have led to control and in some cases eradication of many infectious diseases and saved millions of lives (Mainous and Pomeroy 2010; Mayers 2010). This is however true only for mortalities associated with bacterial infections. Little success has been achieved worldwide in controlling viral and parasitic infections either due to lack of any significant drug

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development breakthroughs or due to geopolitical disturbances resulting in poor implementation of policy and prophylactic measures imperative for effectiveness of these treatments (Feasey et al., 2010). Among viral diseases retroviral diseases have been especially challenging to control and treat, because of their error prone reproductive cycle which facilitates mutations which sometimes make them resistant to drugs (Mainous and Pomeroy 2010). Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV), is an immunosuppressive disease that results in life-threatening opportunistic infections and malignancies is especially of concern. Despite continuous advances made in anti-retroviral therapy, AIDS has become the leading cause of death in Africa and fourth worldwide. Several therapeutic drugs have been developed to control the onset of AIDS. Common drugs, such as reverse transcriptase inhibitors (ZDV and AZT) and protease inhibitors that suppress HIV replication have several limitations which effect compliance and effectiveness. These limitations include high cost and serious side effects such as inhibition of hematopoietic function and development of resistant strains of HIV (McKeegan 2002; WHO 2006; Mee et al., 2008). Moreover, indiscriminant use of drugs and have substantially contributed to resurgence of many bacterial and parasitic infections due to evolutionary selection of drug resistant strains. Tuberculosis, malaria, influenza, cystic fibrosis are some examples of diseases which have become public health concerns across many countries. *Pseudomonas aeruginosa* often found in cystic fibrosis patients is transmitted nosocomially, many strains of this pathogen have already become resistant to several antibiotics and because of its large genome, it is eventually expected to become resistant to almost all drugs (Hancock, 1998; Lewis, 2001; Hentzer and Givskov, 2003; Harbarth and Samore, 2005). Obviously, there is an urgent need for development of new category of economical complimentary therapeutic strategies that can contribute to effective management of infectious diseases and reduce the risk for development of drug resistance (Spoering and Lewis 2001; Mainous and Pomeroy 2010; Mayers 2010).

BioActive Silicate is a modified value-added silicon-based compound developed by Cisne Enterprises Inc. Recent research with this compound has shown that it has very potent antipathogenic functionality on different virulent bacteria, yeast and mold. In numerous privately collected testimonials, the compound has been suggested to have anti-viral effects. It has been noted in these claims that this compound can alleviate symptoms associated with several bacterial and viral diseases such as skin infections, ulcers, respiratory infections, common cold and HIV-AIDS. Additionally, unconfirmed reports have suggested that it may aid in drug therapy by increasing effectiveness of drugs, reducing their effective dosage associated side effects. We have previously shown that the product has antioxidant and redox modulating properties in *in vitro* systems (Townsend et al., 2010). In this study, our objective here was to evaluate the anti-retroviral and antipathogenic effects of BIOACTIVE SILICATE in model systems. Additionally, we also evaluated the effectiveness of the product in modulating nitric oxide mediated host innate immune response.

Materials and methods

Study Compound: BioActive Silicate manufactured using a proprietary patent pending process was supplied by Cisne Enterprises Inc.(Odessa, TX)

Growth and culture of *Lumbricus terrestris*: Standard growth and culture conditions as described previously (Hutton, 2009; Townsend 2010) were used for culture, maintenance and treatment of *L. terrestris*. Briefly, sexually mature *L. terrestris* with a fully developed clitellum were primed on petri dishes containing 15 ml of Lumbricus growth medium (LGM; 1.25% agar, 0.31% Gerber oatmeal, single grain) for 48 hrs at 10 °C. They were then transferred to treatment plates containing LGM with different concentrations of test compound (1.14 mM, 0.114 mM, 0.0045 mM and 0.0023 mM). Seminal vesicles were harvested and homogenized in calcium free –Lumbricus balanced salt solution (LBSS; LBSS (1.5 mM NaCl, 4.8 mM KCl, 1.1 mM MgSO₄, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 4.2 mM NaHCO₃, and adjusted to pH 7.3 with HCl) and used for further analysis. The homogenate is centrifuged at 1100 rpm for 2 minutes at 4°C. The supernatant was then transferred to a clean microcentrifuge tube and stored -80°C for future analyses. The remaining pellet is used for DNA fragmentation analysis.

Nitric Oxide Production: Nitric oxide production was measured using a modified Griess assay for the detection of total nitrites (Miranda et al., 2001). Briefly, 100 µl of whole cell extract was transferred to a microplate followed by addition of 100 µl of vanadium chloride (0.08 g/10 mL 0.1 M HCl) and 100 µl Griess reagent. The microplate incubated for 30 minutes at 37°C and absorbance was measured at 540 nm using the Bioteck EL 808 (Houston, TX). The concentration of nitric oxide was determined by calculating the % change based on a linear standard curve equation: [Conc (umol/L) = (A540-0.0344)/0.0057].

Reverse transcriptase (RT) assay: The effect of different concentrations of BIOACTIVE SILICATE on reverse transcription was tested using a non-radioactive HIV-RT colorimetric ELISA kit from Roche Diagnostics, Germany. The protocol outlined in the kit was followed using 2 ng of enzyme in a well and incubating the reaction for 2 h at 37°C.

Glycohydrolase enzyme assays: The method described by Collins et al., (1997) was followed with modifications, to measure the inhibition of the glycohydrolase enzymes using a p-nitrophenol substrate hydrolysis microplate assay. A 200 μ l reaction mixture containing 2 mM substrate, 0.25 μ g enzyme, and different dilutions of BIOACTIVE SILICATE was transferred to a microplate and incubated for 15 min at 25 °C. Reaction was terminated by the addition of 60 μ l 2 M glycine–NaOH, pH 10. The release of p-nitrophenol was measured at 412 nm using a Biotek EL 808 (Houston, TX) microplate reader. β -glucuronidase (MP Biomedicals, Solon, OH) and its substrate p-nitrophenyl- β -d- glucuronide (Sigma, MO, USA) were dissolved in 50 mM MES–NaOH buffer at pH 6.5. β -glucosidase (MP Biomedicals, Solon, OH) and its substrates p-nitrophenyl- β -d-glucopyranose were dissolved in 50 mM sodium acetate buffer at pH 5.6.

Protease (PR) assay: Effect of different concentrations of BIOACTIVE SILICATE. On IV-1 protease activity was determined spectrofluorometrically using a SensoLyte® 490 HIV Protease Assay Kit (Anaspec, San Jose, CA) using manufacturer's protocol.

EPS Isolation and purification: 100 μ l of growth phase *Pseudomonas aeruginosa* (PA-O1) a kind gift from Prof. Robert McLean (Texas State University) was inoculated in 10 ml of LB broth and grown overnight at 32°C on a orbital shaker (250 rpm) to induce EPS production. Once the A_{600} of the overnight culture reached 1 unit, the broth was centrifuged (8000 \times g, 20 min; 4°C) to remove bacterial cells. 22 ml volumes of chilled (-20 °C) absolute ethanol was added to the supernatant to precipitate the EPS. After incubation for 1 h at -20°C, the precipitate was collected by centrifugation (8000 \times g, 20 min; 4°C) and air dried in a laminar flow hood for 6 hr. Dried EPS was re-suspended in and deionized water and used for analysis. For treatments, PA-O1 was cultured in LB containing various concentrations of BIOACTIVE SILICATE and EPS was collected and purified like above (modified from Korakli et al., 2001).

EPS analysis: Total carbohydrates in EPS were determined by the phenol sulfuric acid method modified for a microplate format (Masuko et al., 2005). Glucose based carbohydrates were assayed using the anthrone reagent by modifying an assay described by Laurentin et al., (2003). Pentose sugars in the EPS were assayed using the ferric-orcinol method (Pramod and Venkatesh, 2006). Sialic acid residues in the EPS were determined by modifying a method described by Jourdian et al.,(1971). Uronic acid residues were measured as glucuronic acid equivalents using a microplate method as described by van den Hoogen (1998). The Heptose concentration in the EPS isolate was assayed using thiobarbutyric acid reagent by modifying a method described by Ghosh et al., (1999). Protein content in EPS was measured using the Biorad assay (Biorad, 1976) all concentrations were expressed per mg of protein to normalize all samples.

Statistical analysis: All experiments were conducted at-least in triplicates. Data is expressed as mean with standard error. Two tailed students 't' test was used to determine significance ($p < 0.05$).

Results

Effect of BIOACTIVE SILICATE on nitric oxide (NOS) levels: Nitric oxide levels in the neutrophils in response to treatment with BIOACTIVE SILICATE increased at all the concentrations tested over a duration of 6 days (Figure 1). Nitric oxides were measured indirectly as total nitrates, and a fold change of 1.79, 2.37 and 1.22 in nitric oxide levels was observed at concentrations of 0.029 mM, 0.29 mM and 2.9 mM respectively compared to controls (Figure 1)

Effect of BIOACTIVE SILICATE on Glycohydrolase enzymes: BIOACTIVE SILICATE at various serially diluted concentrations was assayed for its ability to inhibit different glycohydrolase enzymes. The effect of BIOACTIVE SILICATE on inhibition of β -glucosidase increased in a dose dependent manner. At a concentration of 94 mM there was a 96% decrease in the activity of the enzyme compared to the control. The IC_{50} or concentration at which 50% of the enzyme activity was inhibited was determined to be 14.56 mM (Figure 2). The effect of BIOACTIVE SILICATE on glucuronylation was determined by adding BIOACTIVE SILICATE at different concentrations to a β -glucuronidase reaction mixture. The enzyme activity was inhibited even at the lowest concentration of BIOACTIVE SILICATE (0.0094 mM), subsequent increases in BIOACTIVE SILICATE concentration increased the inhibition in a linear manner. The highest inhibition was achieved at 98.5 mM, at this concentration of BIOACTIVE SILICATE the activity of the enzyme decreased by 98.5 % compared to control. From the dose response curve the IC_{50} , of BIOACTIVE SILICATE was calculated to be 11.03 mM. with 94 mM. (IC_{50} =14.6 mM) activity which decreased protein glucosylation and glucuronylation (Figure 3).

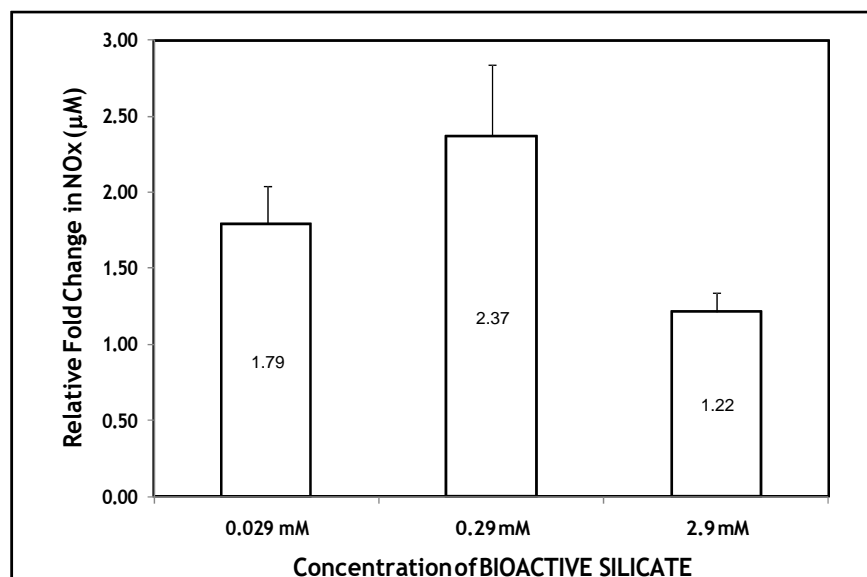


Figure 1. Effect of BIOACTIVE SILICATE silicates on nitric oxide levels as measured by total nitrates.

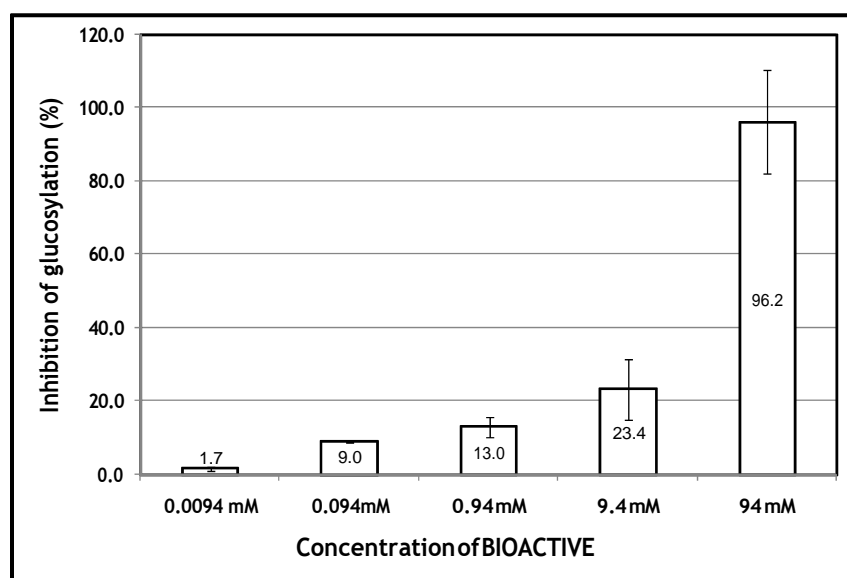


Figure 2: Effect of various concentrations BIOACTIVE SILICATE on HIV envelop protein glycosylation

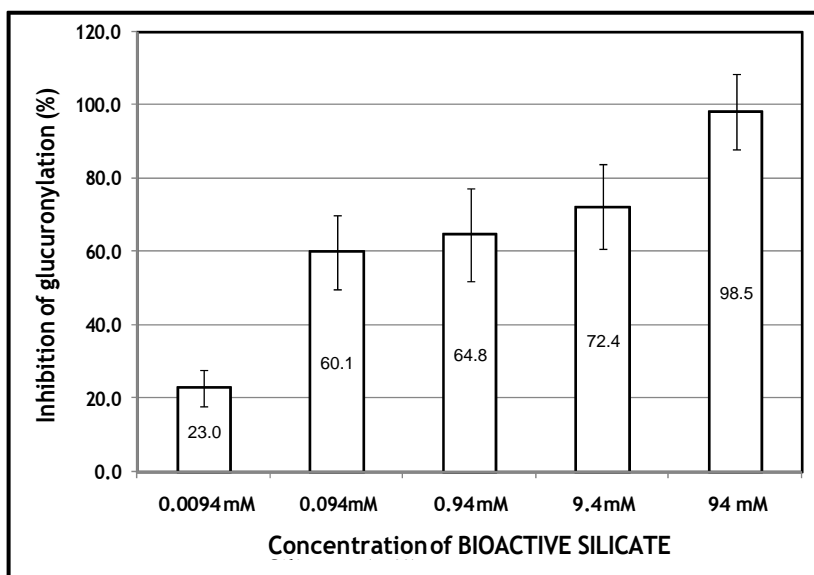


Figure 3: Effect of various concentrations BIOACTIVE SILICATE silicates on HIV envelop protein glucuronylation

Effect of BIOACTIVE SILICATE on inhibition of HIV-1 Reverse Transcriptase activity: Activity of the HIV-1 reverse transcriptase (HIV-1 RT) decreased with increasing concentration of BIOACTIVE SILICATE. At the lowest concentration of 0.0126 mM the enzyme activity decreased by 5.2 % followed by 13.2 % at 0.126 mM, 16.2 % at 1.26 mM and 18% at 12.6 mM. At the highest dosage (126 mM) the HIV-1 RT activity was completely inhibited (Figure 4). From the dose response curve (Figure 4) the IC₅₀ was calculated to be 20.5 mM.

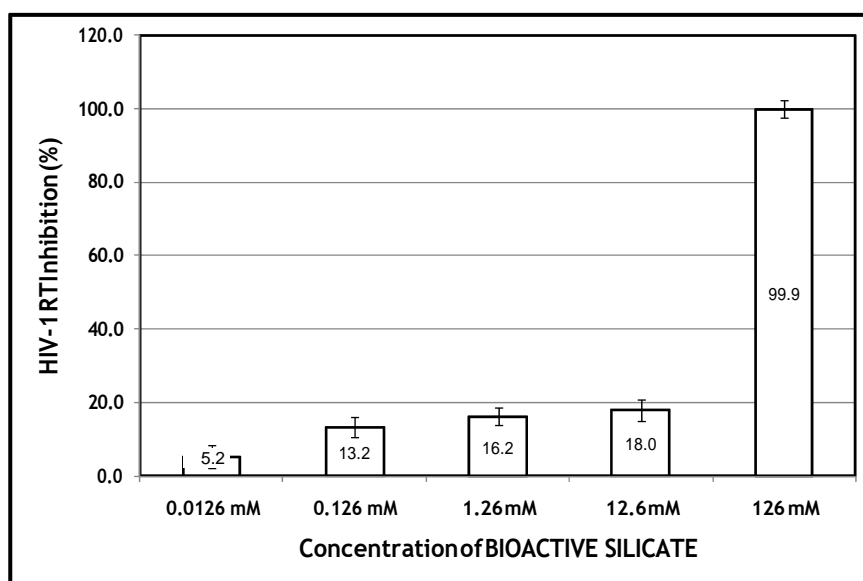


Figure 4: Effect of various concentrations BIOACTIVE SILICATE silicates on HIV-1 reverse transcriptase activity

Effect of BIOACTIVE SILICATE on inhibition of HIV-1 Protease activity: A dose dependent inhibition of the HIV-1 protease (HIV-1 PR) was observed with increasing concentrations of BIOACTIVE SILICATE. At the highest concentration tested (75 mM) the enzyme activity was inhibited by 65 % compared to the control. At a concentration of 7.5 mM, 0.75 mM, 0.075 mM and 0.0075 mM of BIOACTIVE SILICATE, the protease activity decreased by 35.5%, 22.6%, 18.7% and 5.1 % respectively (Figure 5). From this dose response curve the IC₅₀ was calculated to be 14.69 mM.

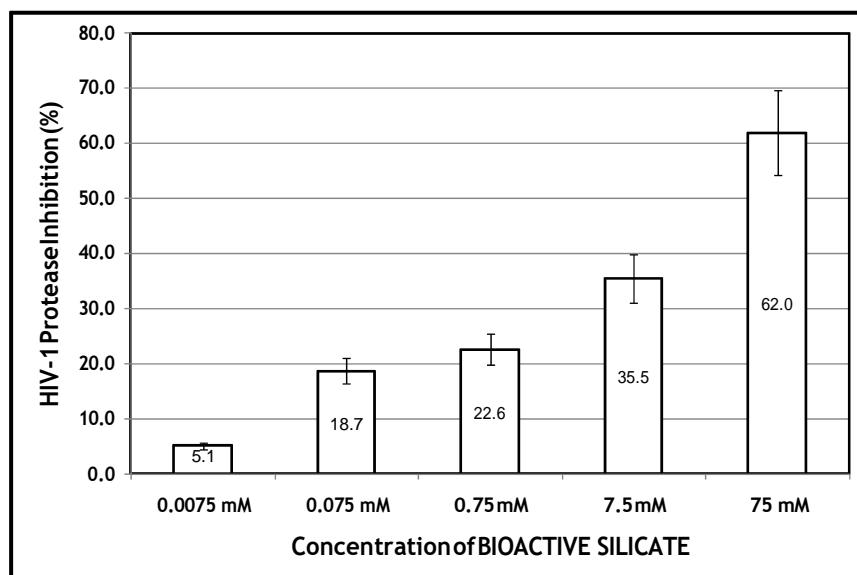


Figure 5: Effect of various concentrations BIOACTIVE SILICATE on HIV- protease activity

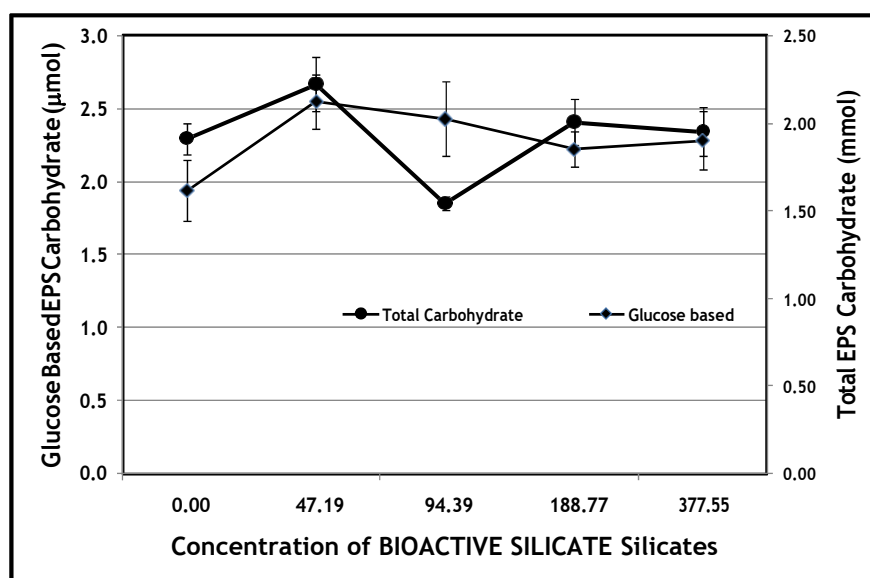


Figure 6: Effect of various concentrations of BIOACTIVE SILICATE silicates on total carbohydrate and glucose based carbohydrate composition of PA-O1 EPS.

Effect of BIOACTIVE SILICATE on PA-O1 EPS composition: We evaluated the effect of BIOACTIVE SILICATE on the carbohydrate composition of EPS secretion induced in pathogen PA-O1. In the presence of BIOACTIVE SILICATE, the total carbohydrate composition of the EPS did not show any dose dependent effect. It was observed that at a concentration of 47.1 μ M the total carbohydrate content of EPS increased compared to EPS in the control medium (Figure 6). However, at 94.39 μ M of BIOACTIVE SILICATE the carbohydrate content decreased to a value below control (Figure 6). At higher concentrations of BIOACTIVE SILICATE there was no significant difference between the BIOACTIVE SILICATE formed in the control and the BIOACTIVE SILICATE supplemented LB broth (Figure 6). The total glucose based carbohydrates did in the EPS overall increased with addition of BIOACTIVE SILICATE to the PA-O1 growth medium. Similar to the total carbohydrate content, the concentration of glucose based carbohydrates in the EPS increased significantly at a concentration of 47.19 μ M BIOACTIVE SILICATE compared to control (Figure 6).

Further increase in BIOACTIVE SILICATE in the LB medium, resulted in a gradual decrease in glucose based carbohydrates and were always higher than in control (Figure 6)

The composition of pentoses (measured as ribose) in the PA-O1 EPS did not differ significantly from the EPS in control medium at a concentration of 47.19 μM and 94.39 μM . At 188.77 μM of BIOACTIVE SILICATE silicates, the concentration of riboses in EPS decreased by 34% (Figure 7) which decreased by 53 % compared to control at a concentration of

377.5 μM in the medium. Compared to the control, the composition of uronic acid residues in EPS measured as glucuronic acid equivalents decreased by 10.3 % when the concentration of BIOACTIVE SILICATE silicates was 47.19 μM in LB medium (Figure 7). The concentration of uronic acid in the EPS gradually increased with increasing concentrations of BIOACTIVE SILICATE, and increased to 26% more than the control at 188.77 μM (Figure 7). Sialic acid residues in EPS gradually decreased compared to the control with an increase in BIOACTIVE SILICATE silicate concentration in medium. At the highest BIOACTIVE SILICATE silicate concentration of 377.55 μM the concentration of sialic acid residues in the EPS were 13% lower than in control EPS. The concentration of heptoses in the EPS did not change with concentration of BIOACTIVE SILICATE silicates in the medium (Figure 7).

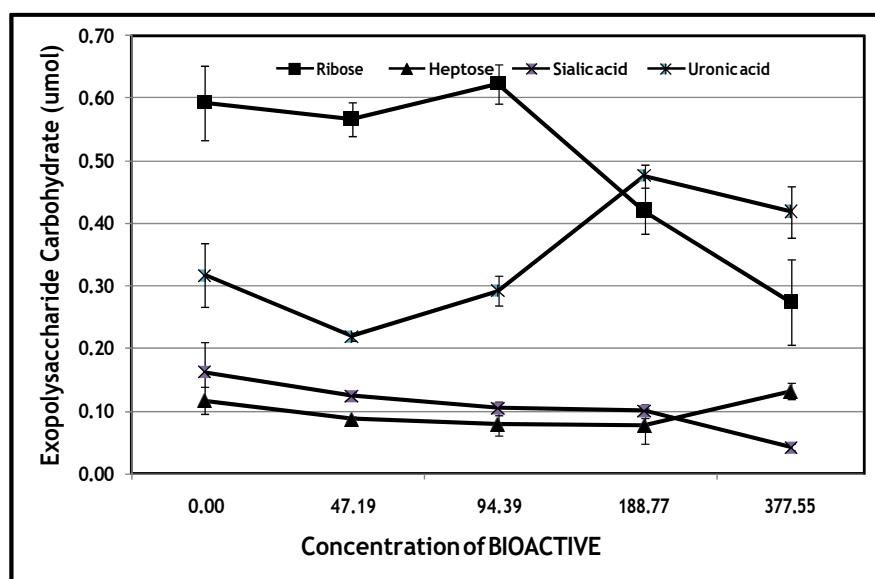


Figure 7: Effect of various concentrations of BIOACTIVE SILICATE on ribose, heptose, sialic acid and uronic acid acid composition of PA-O1 EPS.

Discussion

Even though silicon is one of the most abundant minerals (metalloid) in nature, its biological effects are not very well understood. They have been implicated to have important roles in structural development of animals. Manuscripts exploring biological activities of silicon and silicates are very rare. In our previous investigation we have studied in vitro health promoting properties of silicates, especially from BIOACTIVE SILICATE. Structural characterization using FT-IR and NMR analysis suggested that BIOACTIVE SILICATE is a mixture of trimeric sodium silicate and sodium silicate pentahydrate (Townsend et al., 2010). In vitro analysis also suggested a structure dependent anti-mutagenic free radical quenching activity and redox modulatory property of BIOACTIVE SILICATE silicates with potential therapeutic applications in chemoprevention of oxidative stress mediated diseases such as cancer and CVD (Townsend et al., 2010). In this project we evaluated the in vitro anti-infectious properties of BIOACTIVE SILICATE in several model systems, including its ability to stimulate the nitric oxide mediated host immune response in neutrophil like cells. Treatment with BIOACTIVE SILICATE silicates significantly increased the formation of nitric oxide in neutrophil environment (Figure 1). Nitric oxide is a well known biological signaling molecule and plays important role in vascular endothelial function, memory development and in the enteric nervous system (Bogdan 2001; Hopper and Garthwaite 2006; Ignarro 2009). Additionally, research has shown that it also very important in modulating immune function parameters. NO is a signaling molecule which promotes hematopoiesis and myelogenesis and thereby facilitates differentiation of hematopoietic stem cells into leukocytes and lymphocytes. It is also necessary for chemokine induction and leukocyte recruitment in response to a foreign challenge. Additionally, its synthesis is also induced in leukocytes

where it aids in the removal bacterial and viral agents (Shami and Weinberg 1996, Aicher et al., 2003; Thum et al., 2005). Ability of BIOACTIVE SILICATE to increase NO may indicate a possible immunomodulatory role which may be beneficial in strengthening host immune response against bacterial and viral pathogens (Figure 1).

BIOACTIVE SILICATE were also effective in inhibiting several important physiological events important in survival and development of virulence in viral and microbial pathogens. A dose dependent inhibition of glucosyltransferase enzymes was observed in *in vitro* systems (Figure 2 and 3). Glucosyltransferases and glucuronidases are components of important post translational machinery in golgi complex and critical for glycosylation of a number of viral proteins (eg HIV- gp120). Post-translational glycosylation of viral proteins is extremely important in the assembly of the virion, viral maturation, attachment and invasion into the host cells. Additionally, they also facilitate evasion of the viral proteins from immune system. Disruption of these glycosylations is therefore an important target for retroviral drugs (Collins et al., 1997; Robina et al., 2004; Guo et al., 2009; Gloster and Davies 2010). Significant disruption in the glucosyltransferase enzyme activity by BIOACTIVE SILICATE silicates may indicate its application as an anti-retroviral agent. We also investigated the effectiveness of BIOACTIVE SILICATE on inhibiting HIV-1 reverse transcriptase and HIV-1 protease, two enzymes that are imperative in viral replication and survival. Inhibitors of these enzymes are targets for many anti-retroviral drugs and are highly desirable therapeutic agents (Weller and Williams 2001; Gloster and Davies 2010). In our *in vitro* assays, BIOACTIVE SILICATE were very effective in inhibiting both HIV-1 RT (Figure 4) and HIV-1-PR (Figure 5) at the concentrations tested. HIV-1 RT transcribed the viral RNA into DNA and facilitates its integration into the host genome, where it replicates with the host DNA. When conditions are optimal for viral assembly, the viral DNA is transcribed and translated into a single polypeptide chain. Post translational cleavage of this polypeptide into many peptide chains necessary for viral assembly. Inhibition of either HIV-1 RT or HIV-1 PR makes viral replication and assembly improbable and therefore, make BIOACTIVE SILICATE candidates for anti-retroviral therapy.

Exopolysaccharides secreted by many microbial pathogens including *Pseudomonas aeruginosa* are needed in virulence development and eventual infection (Vuong et al., 2004; Begun et al., 2007; Ullrich 2009). They are highly modified glycoproteins important in adhesion, biofilm formation, drug resistance and have anti opsonisation functions. The heteropolysaccharide residues in EPS are specific to a pathogen, its composition and structure is critical for the above mentioned functions. Alteration of EPS composition therefore, can completely disrupt the function of EPS and attenuate virulence development (Vuong et al., 2004; Begun et al., 2007; Ullrich 2009). We noted that in the presence of BIOACTIVE SILICATE silicates the composition of EPS was effected (Figure 6 and 7). There was a change in total carbohydrate composition of the EPS suggesting an alteration in the glycosylation of EPS (Figure 6). Specifically we also found an increase in glucose based (Figure 6) glucuronic acid based carbohydrates (Figure 7) in the EPS. Treatment with BIOACTIVE SILICATE silicates also resulted in lower ribose based pentoses (Figure 7) in the EPS. Sialic acid residues in EPS which decreased upon treatment with BIOACTIVE SILICATE silicates are important in virulence development of many pathogens. Heptoses, an indicator of lipopolysaccharide (LPS) did not change in response to BIOACTIVE SILICATE treatment. LPS, recognized by many cell surface receptors, is an important signal for the rapid activation leukocytes and results in synthesis and secretion of many inflammatory chemokines which contribute to oxidative stress, tissue damage, sepsis. Unaltered levels of heptoses upon treatment with BIOACTIVE SILICATE silicates may potentially suggest a modulatory effect of these compounds on LPS mediated virulence, infection and systemic effects in hosts.

Results of this investigation have indicated a potential *in vitro* antiretroviral and antipathogenic effect of BIOACTIVE SILICATE silicates and merits further investigation using *in vivo* models.

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