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FACULDADE DE FARMÁCIA
DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

Citopatogenicidade de *Trichomonas vaginalis* às células epiteliais vaginais humanas: possível envolvimento das atividades de NTPDase e ecto-5'-nucleotidase

Lucia Collares Meirelles

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Trabalho de Conclusão de curso apresentado por **Lucia Collares Meirelles** como requisito parcial para obtenção de GRAU DE FARMACÊUTICO.

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“O importante não é o que se dá, mas o amor com que se dá.” (Madre Teresa de Calcutá)

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Trichomonas vaginalis cytopathogenesis on human vaginal epithelial cells: a possible involvement of NTPDase and ecto-5'-nucleotidase activities

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Abstract

Trichomonas vaginalis is a parasitic protozoan that causes trichomoniasis, a common human urogenital tract infection with relevant health consequences. NTPDase and ecto-5'-nucleotidase activities have already been characterized in *T. vaginalis* trophozoites. Aiming to investigate the role of these enzymes in trichomonads cytopathogenesis, we evaluated the cytolysis, hemolysis and the profile of extracellular nucleotides hydrolysis rates in different isolates. *T. vaginalis* isolates presented hemolytic activity and heterogeneity in cytolysis against human vaginal epithelial cells (HVECs) and in extracellular nucleotide hydrolysis profiles. Although no correlation between the parasite ability to exert cytolysis against HVECs and trichomonads ectonucleotidases activities was found, our data suggest that the formation of adenosine through *T. vaginalis* ecto-5'-nucleotidase may have an important role to decrease the cytolysis injury and consequently, the infection establishment. Levels of cytolysis were correlated with hemolytic activity, suggesting hemolysis and cytolysis as important virulence factors by *T. vaginalis*.

Keywords: *Trichomonas vaginalis;* Trichomoniasis; Cytotoxicity; Purinergic signaling; Ectonucleotidases

1. Introduction

Trichomonas vaginalis is an amitochondriate flagellated protozoan that causes trichomoniasis, the most common non-viral sexually transmitted disease with an incidence of 276 million cases worldwide [1]. This infection is associated with vaginal discharge [2,3], vaginitis [2–5], pregnancy complications [2,4,6], preterm birth [3–6], low birth weight [2–6], infertility [3,4] and pelvic inflammatory disease [2–4]. In men, trichomoniasis is associated with urethritis [2,4,5], prostatitis [3–5], epididymitis [3,4] and also might lead to infertility [3,4]. The infection could predispose to prostatic [6] and cervical cancers [2,4,5] and increases the risk of transmitting and acquiring HIV [2–6]. Although trichomoniasis is a common infection with relevant health consequences [2,4], the pathogenesis mechanisms are not completely established, becoming necessary the investigation of biochemical aspects and the virulence factors of the parasite.

The vaginal epithelium is the primary infection site for *T. vaginalis* [2] and the parasite is able to survive in the vaginal environment establishing a successful parasitism by using multiple complex mechanisms for colonization [4]. To establish parasitism, the cytoadherence is the first and crucial step [5,4]. *T. vaginalis* modifies its typical ellipsoid morphology to an amoeboid shape, increasing the contact surface with the host cells. The cytopathogenic process involves a dependent contact mechanism; nevertheless, contact-independent mechanisms are also involved [5]. Five surface proteins known as adhesins (AP23, AP33, AP51, AP65, AP120) have already been characterized in *T. vaginalis* and are considered cytoadherence mediators [2,4,5]. Lipophosphoglygan (LPG), cysteine proteinases (CPs), and hypothetical proteins are also involved in the cytopathogenic mechanism [4,7].

T. vaginalis parasites can criate a symbiotic relationship with *Mycoplasma hominis*, a intracellular bacteria associated with urogenital and respiratory system infections [8,9], that may act as an opportunist pathogen [8]. Studies demonstrated that the association of both microorganisms might increase the cytopathogenic effect of *T. vaginalis* on epithelial cells [9]. *T. vaginalis* can also be infected with four viruses, known as *T. vaginalis* viruses (TVVs), that are members of *Totiviridae* family [6,10]. Some studies have shown that TVVs might influence the *T. vaginalis* genes expression, increasing the parasite virulence [6,11]. However, the association

between TVVs and *M. homins* in the pathogenicity of the parasite has not been already established.

Extracellular nucleotides like adenosine 5'-triphosphate (ATP) and the nucleoside adenosine, an ATP breakdown product, act as signaling molecules [12]. ATP is involved in many biological effects including cardiac functions, blood flow, secretion, inflammation, and immune reactions, acting as a proinflammatory and an immunostimulatory mediator. In response to extracellular ATP, chemokines, such as IL-1ß, IL-2, IL-8, IL-12, and TNF-α are released by immune cells increasing the recruitment of neutrophils and other immune cells. Therefore, in the microenvironment of damaged cells, ATP might have proinflammatory and cytotoxic activities. In contrast to the ATP pro-inflammatory role, adenosine can act as immunosuppressive modulator, decreasing the activation of macrophages, suppressing lymphocytes proliferation, and stimulating tissue regeneration [13]. Extracellular nucleotides can be hydrolyzed by enzymes known as ectonucleotidases [14]. The ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) family hydrolyzes nucleosides triphosphate and diphosphate [13,15]. Another enzyme, the ecto-5'-nucleotidase, catalyzes the conversion of AMP to adenosine. In *T. vaginalis,* the NTPDase activity has been characterized in intact trophozoites [16], and the ecto-5'-nucleotidase activity has also been identified [17]. These enzymes play a crucial role on the parasite's purine metabolism, since *T. vaginalis* lacks "*de novo"* purine and pyrimidine synthesis. In addition to that, the control of this enzymatic cascade is very important to modulate immune responses [18]. *In vitro* parasite cultures are usually supplemented with 10 % of bovine serum, a source of nutrients as nucleotides and nucleosides. In *T. vaginalis*, adenosine is the main precursor of the purine nucleotide pool through salvation pathways that include the intracellular enzyme purine nucleoside kinase (PNK) [18].

Considering the necessity to investigate the mechanisms involved during the establishment of the infection, the aim of this study was to evaluate the participation of the NTPDase and ecto-5'-nucleotidase activities in *T. vaginalis* cytopathogenesis. For that, the cytolysis, hemolysis and the profile of nucleotide hydrolysis rates in *T. vaginalis* isolates previously classified according to harboring or not *M. hominis* and TVVs were determinated.

2. Materials and Methods

2.1 Parasites and epithelial cell culture

In this study, fresh clinical *T. vaginalis* isolates were used: TV-LACM6, TV-LACM11, TV-LACM15, TV-LACM22, TV-LACM24 (from female patients), TV-LACH4, and TV-LACH6 (from male patients) obtained from urine from the Laboratório de Análises Clínicas, Faculdade de Farmácia, UFRGS, Brazil (UFRGS Ethical Committee approval number 18923); and the isolate ATCC 30236 from the American Type Culture Collection. The criteria used to select the fresh isolates was the previous characterization of the presence or absence of TVVs and *M. hominis* (unpublished work), as shown in Table 1. Parasites were cultivated in trypticase-yeast extract-maltose (TYM), pH 6.0 supplemented with 10 % inactive bovine serum at 37 ºC [19]. Human vaginal epithelial cell line (HMVII) from Public Health England Culture Collection was used to analyze the interaction between human cells and *T. vaginalis*. Cells were cultivated in RPMI 1640 medium supplemented with 10 % inactive fetal bovine serum and 100 μg/mL penicillin-streptomycin at 37 ºC and 5 % CO_2.

2.2 Hemolysis assay

Blood samples obtained from volunteer donors (UFRGS Ethical Committee approval number 616496) were centrifuged (250 *x* g for 5 min), had their plasma discarded, and were washed three times in phosphate buffered saline (PBS) in order to isolate erythrocytes. *T. vaginalis* cultures were washed three times in TYM medium. Hemolysis was performed using 1.0×10^6 trophozoites in 2.5 mL of TYM medium and 50 μL of washed erythrocytes during 24 h at 37 ºC. To eliminate the normal hemolysis of red blood cells, a control was used containing only TYM medium and erythrocytes. After 24 hours, the mixture was centrifuged (6000 g, 5 min) and the supernatant was measured at 540 nm [20]. Hemolysis rates were calculated as percentage of total lyses in comparison to positive control treated with 0.2 % Triton X-100. All experiments were repeated three times with triplicate samples.

2.3 Cytolysis assay

To examine the parasites cytotoxicity, a cytolysis assay was performed by measuring lactate dehydrogenase (LDH) release by cells using the CytoTox-One homogeneous membrane integrity assay (Promega, USA), as instructed by the manufacturer. On

the previous day, 3.0 x 10^4 cells were seeded in a 96-well microplate until monolayers to reach confluency at 37 ºC and 5 % CO_2 [7]. Parasites in the logarithmic phase of growth exhibiting normal morphology and motility were washed three times and were resuspended on a fresh RPMI 1640 medium. An aliquot of 100 μL from a solution containing 5.0 x 10^5 trophozoites/mL was added to confluent HMVII cells and incubated during six hours at 37 ºC and 5 % CO_2. The background was the LDH release by HMVII unexposed cells. Data were expressed as a percentage of total lysis, using as control the LDH release from the cells after 0.18 % Triton X-100 treatment. Knowing that TV-LACM6 is a fresh isolate with high rates of adhesion and cytotoxicity (unpublished results), a time line curve was performed to determine the maximum release of LDH. In order to investigate if adenosine has influence in the cytolysis process, confluent HMVII cells and *T. vaginalis* isolates were exposed to adenosine (500 μM) and adenosine plus EHNA (50 μM) (an adenosine deaminase inhibitor).

2.4 Enzyme assay

The specific activity was measured determining the release of inorganic phosphate (Pi) by a colorimetric test. Trophozoites were washed three times with saline solution and the Coomassie Blue method [21] was used to determine protein quantification. The suspensions were diluted to have a final protein concentration of 0.6 mg/mL. To measure ATP, ADP, and AMP hydrolysis, trophozoites were added to the reaction mixture containing 50 mM Tris buffer pH 7.2 and 5.0 mM $CaCl_2$ (ATP and ADP) and 50 mM Tris buffer pH 7.5 and 3.0 mM $MgCl_2$ (AMP). Before and after the reactions, cellular integrity, viability and motility were assessed using trypan blue dye exclusion method. The reaction initiated by the addition of ATP and ADP (final concentration 1.0 mM), to determine NTPDase activity and AMP (final concentration 3.0 mM) for ecto-5'-nucleotidase assay. The reaction was interrupted after 30 minutes, by adding 10 % of trichloroacetic acid (TCA) [22]. To eliminate the non-enzymatic hydrolysis of the substrate, intact trophozoites were added to a control sample, containing the reaction mixture and 10 % of TCA. Specific activity was expressed by nmol Pi released/h/mg of protein. The experiment was repeated three times with triplicate samples.

2.5 Statistical analysis

Statistical analysis was conducted by Pearson rank order correlation coefficient to measure the correlation between cytolysis and hemolysis, cytolysis and nucleotides hydrolysis as well as between hemolysis and nucleotides hydrolysis [23]. Data from adenosine cytolysis reversion assay were analyzed using Student's t test considering $p < 0.05$ as significant. The Statistical Package for the Social Sciences (SPSS) software was used to perform the analysis.

3. Results

3.1 *T. vaginalis* fresh clinical isolates abilities to lyse erythrocytes

Figure 1 shows, in descending order, the ability of *T. vaginalis* isolates to lyse erythrocytes. All isolates were able to lyse erythrocytes, however, an heterogeneous profile was noted between different parasites. A previous study had classified hemolytic rates as: (i) low hemolytic capacity - less than 20 % of hemolysis and (ii) high hemolytic capacity - between 20 % and 40 % of hemolysis [7]. In our study, isolates which were able to lyse more than 40 % of erythrocytes were considerate with a very high hemolytic capacity. Considering hemolysis rates shown in Figure 1, the isolate TV-LACH6 was considered to display a high hemolytic capacity and the other *T. vaginalis* isolates were considered to express very high hemolytic profile. Further, there was no relationship between *M. hominis* and TVVs infection of *T. vaginalis* isolates and hemolytic activity.

3.2 Heterogeneity on the *T. vaginalis* cytolysis against human vaginal epithelial cells (HVECs)

In order to investigate the cytolysis by *T. vaginalis* against HVECs, the intracellular LDH release was evaluated. The incubation time was previously determined using TV-LACM6 isolate and the maximum LDH released was achieved in six hours (data not shown). Figure 2 shows that all *T. vaginalis* isolates stimulated LDH release from HVECs, indicating that all parasites exert cytolysis at some level. It is important to highlight the high percentage of released LDH by the isolate TV-LACM6 (74 %), comparing with the positive control (0.18 % Triton X-100) and in comparison with all other isolates evaluated. For some isolates, such as ATCC30236, TV-LACH4, and TV-LACH6 LDH levels detected were very low. This data was expected, comparing to previous results in the literature [7]. Interestingly, TV-LACM6 as well as TV-

LACM22 isolate, presented high hemolytic and cytopathogenic effects. On the other hand, TV-LACH6 showed the lowest hemolytic and lactate desidrogenage release rates. Moreover, a strong Pearson rank order correlation coefficient between lyses of erythrocytes and HVECs was found (p = 0.00642). In addition, there was no relationship between *M. hominis* and TVVs infection of *T. vaginalis* isolates and cytolysis.

3.3 NTPDase and ecto-5'-nucleotidase activities profiles among *T. vaginalis* isolates

To evaluate the extracellular nucleotides hydrolysis profile in fresh clinical and ATCC isolates, we assayed the NTPDase and ecto-5'-nucleotidase activities in intact *T. vaginalis* trophozoites. As seen in Figure 3, the NTPDase was higher than the ecto-5´-nucleotidase activity in all isolates. No hydrolysis profile could be established regarding the source of isolates- from female or male patient - or *M. hominis* or TVV infection since all *T. vaginalis* isolates presented heterogeneity in nucleotide hydrolysis. A significant high ATP hydrolysis was observed on TV-LACM15 isolate. On the other hand, TV-LACM22 and TV-LACM6 presented the lowest ability to hydrolyze AMP. Our results confirm that all *T. vaginalis* isolates tested were able to hydrolyze extracellular nucleotides, which is in agreement with previous studies [16,17]. Besides, there was no relationship between *M. hominis* and TVVs infection of *T. vaginalis* isolates and extracellular nucleotide hydrolysis.

3.4 Adenosine's protective role

Finally, in order to investigate a possible immunosuppressive and tissue protective role for adenosine, the most cytotoxic *T. vaginalis* isolate TV-LACM6, was co-incubated with the nucleoside and the cytolysis effect was assessed. As shown in Figure 4, the tested condition leaded to a significant decrease on the released LDH levels in comparison to the parasites from TV-LACM6 isolate, revealing a protective effect by adenosine. As expected, the same reversion effect on cytolysis by adenosine could be observed in presence of EHNA.

4. Discussion

Trichomoniasis presents a wide variation in symptomatology, pathogenesis, complication, sensitivity to treatment, and host susceptibility to acquire other diseases [24]. However, it is still not clear what are the main factors associated with

the parasite's virulence. In this sense, we have investigated some possible virulence factors to better understand the pathogenesis mechanisms.

Erythrocytes might be an important source of nutrients to the parasite [5,25], being hemoglobin a possible iron source. *T. vaginalis* requires up to 300 μM of iron for an optimal metabolism, but it could adapt its need according to iron variation in their host, which is constantly changing specially in women due to menstrual cycle [5]. Because of this, the parasite may regulate its iron necessity by lysing erythrocytes or through endometrial tissue, squamous epithelial debris, white blood cells, and mucus [5,7]. As previously studies demonstrated, *T. vaginalis* isolates are able to lyses erythrocytes [26]. However, the relationship between the hemolytic activity and clinical virulence or cytopathology is conflicting. In our study, the percentage of hemolysis in all isolates tested varied between 26 % and 91 %. Aiming to investigate if there is a correlation with erythrocytes lyses and the virulence of the parasite, we compared hemolysis data with that obtained in LDH release assay with HVECs. A strong correlation between lyses of erythrocytes and HVECs was found ($P < 0.00642$), suggesting that the high ability played by the parasites to perform hemolysis and cytolysis may represent key virulence factors. It has already been reported that hemolytic activity is correlated with parasite virulence [26]. However, in a recent work, a correlation was not found [7]. Nevertheless, the methods used to measure hemolytic activity are different, making the results comparison inappropriate. Our study suggests that the hemolysis process is important to the parasite, which is not surprising, once iron, derived from hemoglobin, is essential to parasite's perfect metabolism. In this way, the erythrocytes may be an important source of iron as well as endometrial tissue, squamous epithelial debris, white blood cells, and mucus.

Regarding the cytolysis assay, *T. vaginalis* isolates used in this study showed the capacity to cause LDH release from HVECs among rates varying between 5 % and 74 %. These data are in agreement with a previous study, that used the same methodology to determine cytolysis (four hours of contact with cells) [7]. Our results demonstrated a large variation on cytolysis effect among different *T. vaginalis* isolates. In some of them, LDH release was even below the background level, which may be related to *T. vaginalis* ability to scavenge and ingest proteins and other metabolites from the media [7]. The interaction between the parasite and host cells

depends on the isolate's virulence. *T. vaginalis* isolates presenting high virulence can rapidly destroy and phagocytize cells debris. In addition to that, parasites are also able to stress the host cells *in vitro*, inducing cytoplasm leaking and death by necrosis [27]. Our data suggest that adenosine, in this context, may act as a protector agent, avoiding the HVECs damage. Interestingly, the isolates which presented the slightest ability to hydrolyze AMP to adenosine - TV-LACM6 and TV-LACM22 - were the isolates with the highest ability to cytolysis. Although no correlation between cytolysis and ectonucleotidases activities profile was found, these data are important to demonstrate that more studies are necessary to investigate the role of nucleotide hydrolysis in the *T. vaginalis* pathogenesis mechanism.

Our experiments showed heterogeneity on nucleotide hydrolysis among different *T. vaginalis* isolates. This lack of hydrolysis profile was expected, being in accordance to a previous study, which also showed heterogeneity of extracellular nucleotides on hydrolysis [28], being ATP the preferred substrate for NTPDase activity when compared to ADP and AMP. The ecto-5'-nucleotidase activity is the last step in the casacade to produce adenosine [28] which acts as an anti-inflammatory molecule, decreasing the nitric oxide generation by *T. vaginalis*-stimulated neutrophils trough A2A receptor [29]. Some isolates, TV-LACM6 and TV-LACM22, presented the lowest ecto-5'-nucleotidase activities. We hypothesize that, *in vivo*, lower AMP hydrolysis rates might be correlated with exacerbated symptoms with the increase in the pro-inflammatory process due to the low amount of adenosine to reverse the cytolytic process with its protective properties.

Our study did not show a major virulence profile in *T. vaginalis* isolates harboring *M. hominis* and TVVs. It is important to emphasize that we analyzed the cytopathogenic effect through LDH release, therefore, it is not appropriate to demonstrate the influence of *M. hominis* and TVVs infection on *T. vaginalis* isolates pathogenesis based in one single methodology. Moreover, only few studies covering this aspect are found in the literature, and a strong relation between the co-infection of *T. vaginalis* by these microorganisms and the increase of virulence is not completely established. Nevertheless, one study clearly demonstrated that the presence of *M. hominis* in *T. vaginalis* isolates impacts on random amplification of polymorphic DNA (RAPD) and suggested variability in *M. hominis* strains infection [30]. Co-infection

with *T. vaginalis* and *M. hominis* consumes greater amounts of free arginine compared to infections containing only *T. vaginalis*, what could reduce the production of nitric oxide by macrophages and interfere in the host defense mechanism [31]. Conrad *et al.* demonstrated a change in the expression of cysteine proteinases and P250 by TVV, providing a phenotype more favorable to the parasite with serious implication for the host [11]. Another study demonstrated that endobiont viruses and viral dsRNA could modify the inflammatory reaction [32]. Although these studies show some possible changes in the virulence of the parasite, more research is needed to verify the contribution of TVVs in virulence, pathogenicity, and symptomatology.

5. Conclusion

In summary, the present study demonstrates that different *T. vaginalis* isolates present hemolytic activity and heterogeneity in cytolysis against HVECs and in the profile of extracellular nucleotide hydrolysis. In addition, there was no relationship between *M. hominis* and TVVs infection of *T. vaginalis* isolates and hemolytic activity, cytolysis, and nucleotide hydrolysis. Importantly, a strong correlation was found between lyses of erythrocytes and HVECs, indicating hemolysis and cytolysis as key virulence factors played by *T. vaginalis*. Although no correlation between the parasite ability to exert cytolysis against HVECs and trichomonads ectonucleotidases activities was found, it can be noted that the formation of adenosine through *T. vaginalis* ecto-5'-nucleotidase may have an important role to decrease the cytolysis injury and consequently, the infection establishment. Therefore, the enzyme activity based on AMP hydrolysis may be important to determine the virulence of the parasite. More studies with a larger number of isolates are necessary to better understand the adenosine protective effect.

Conflicts of interest

All authors have declared that are no conflicts of interest.

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Figure captions

Figure 1. Hemolysis induced by *Trichomonas vaginalis* isolates (ATCC 30236, TV-LACM6, TV-LACM11, TV-LACM15, TV-LACM22, TC-LACM24, TV-LACH4 and TV-LACH6) in 24 hours. Positive control means erythrocytes treated with 0.2 % triton X-100. Hemolysis was measured at 540 nm. Bars represent the mean ± SD of at least three independent experiments. Data were analyzed by Student's t test.

Figure 2. Cytolysis of human vaginal epithelial cells induced by different *Trichomonas vaginalis* isolates. Positive control means HMVII cells treated with 0.18 % triton X-100. Parasites were exposed to cells monolayers for 6 hours. Data were expressed as a percentage of total lysis. Bars represent the mean ± SD of at least three independent experiments. Data were analyzed by Student's t test.

Figure 3. NTPDase and ecto-5'-nucleotidase activities among different *T. vaginalis* isolates. Data are expressed as the mean ± S.D. of at least three experiments. Specific enzymatic activities were expressed as nmol Pi released/h/mg protein.

Figure 4. Cytolysis induced by TV-LACM6 isolate without treatment and treat with adenosine (ADO) (500 μM) and EHNA (50 μM). Results were analyzed statistically by the Student's t-test ($P<0,05$). (*) indicates significant difference from control; (#) indicates significant difference from TV-LACM6 only.

Table 1. *Trichomonas vaginalis* isolates infected with *Mycoplasma hominis* and *Trichomonas vaginalis* virus (data from unpublished work).

Isolate	*M. hominis*	TVV 1	TVV2	TVV3	TVV4
ATCC 30236	+	+	-	+	-
TV-LACM6	+	+	-	-	-
TV-LACM11	+	-	+	+	+
TV-LACM15	-	-	-	-	-
TV-LACM22	+	-	-	-	-
TV-LACM24	+	+	+	+	-
TV-LACH4	-	+	+	+	+
TV-LACH6	+	+	-	+	-

Figure 1

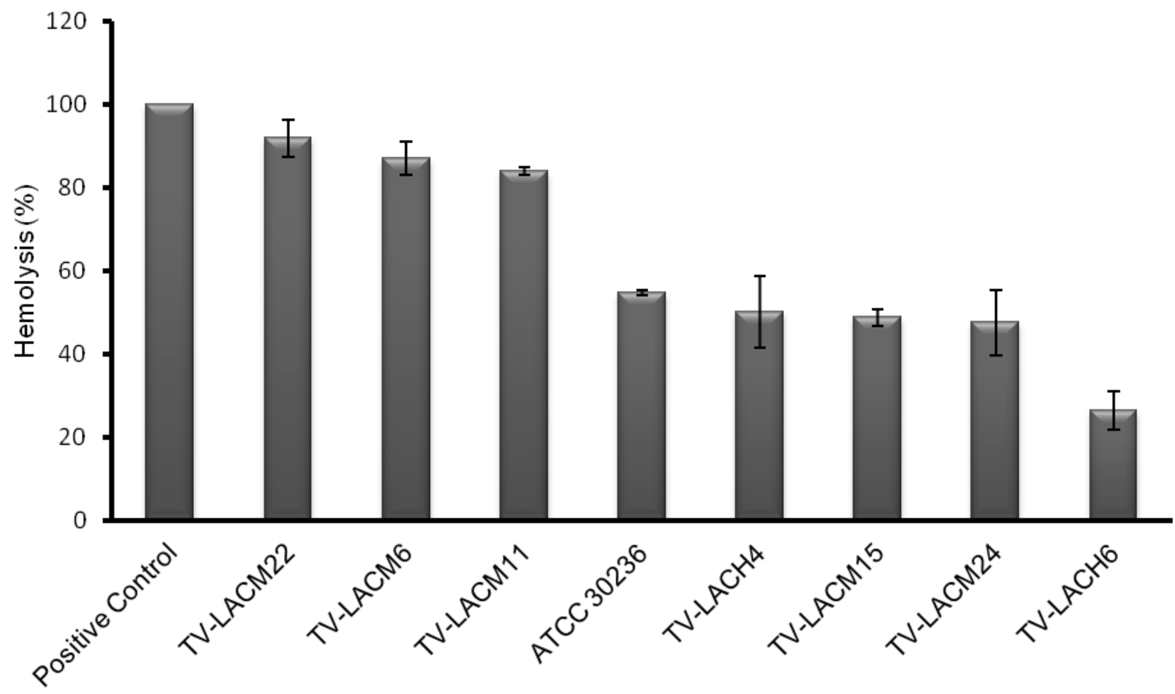

Figure 2

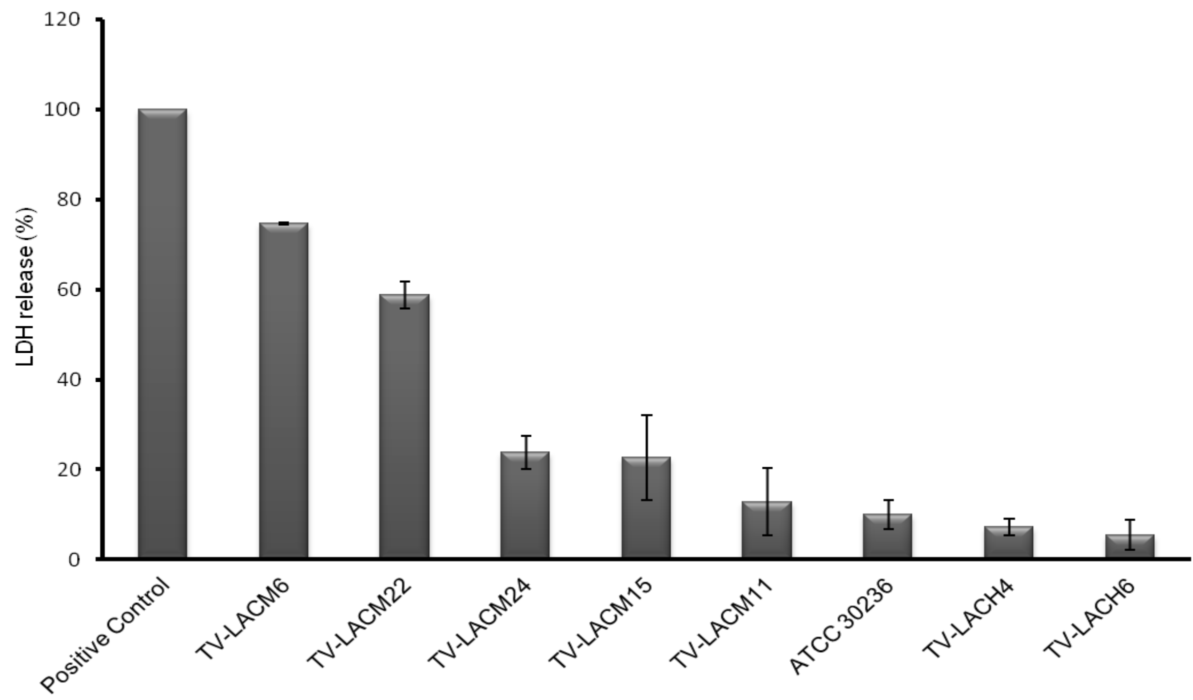

Figure 3

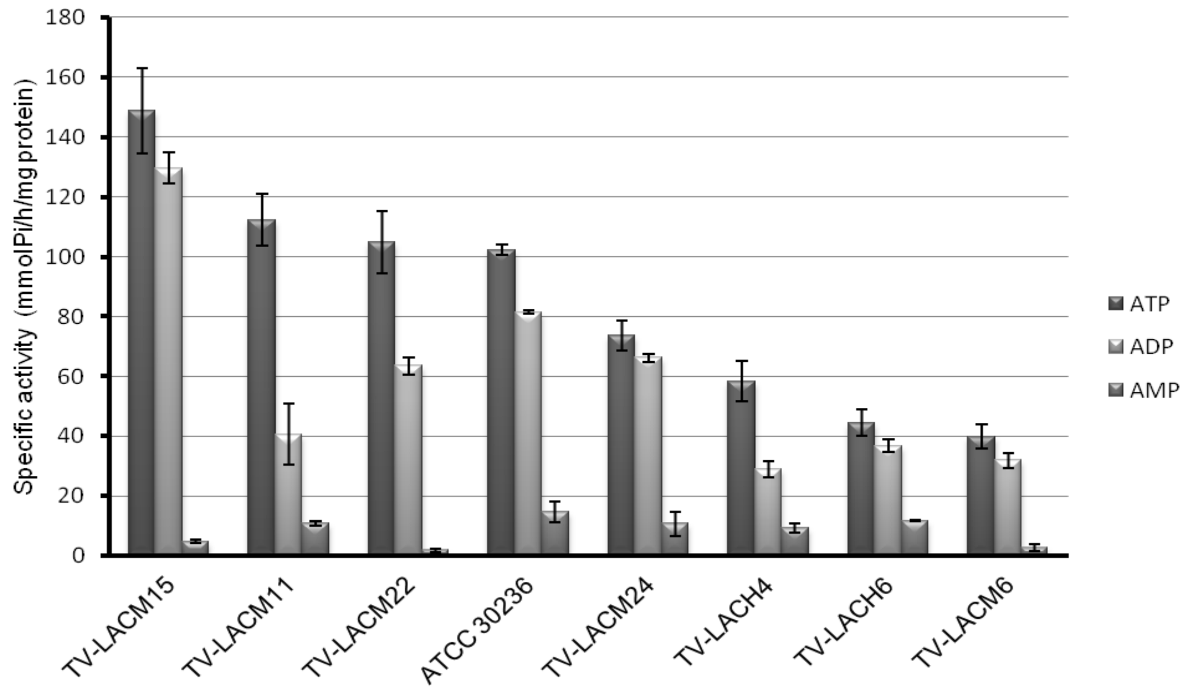

Figure 4