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Uncaria tomentosa alkaloidal fraction reduces paracellular permeability, IL-8 and NS1 production on human microvascular endothelial cells infected with Dengue virus

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Dengue is the major arbovirus in the world annually causing morbidity and death. The severe dengue is associated with changes on the endothelial barrier function due to the production of inflammatory mediators by immune cells and by the endothelium. Dengue virus (DENV) replicates efficiently in human endothelial cells *in vitro* and elicits immune responses resulting in endothelial permeability. *Uncaria tomentosa* (Willd.) DC.(Rubiaceae), known as cat's claw, has been used in folk medicine for the treatment of a wide-array of symptoms and several scientific studies reported its antiviral, immunomodulatory, anti-inflammatory and antioxidant properties. Here we infected a human lineage of dermal microvascular endothelial cells (HMEC-1) with DENV-2 and treated it with an alkaloidal fraction from *U. tomentosa* bark (AFUT). We showed antiviral and immunomodulatory activities of *U. tomentosa* by determining the NS1 antigen and IL-8 in supernatant of DENV-2 infected HMEC-1. Furthermore, by measurement of transendothelial electrical resistance (TEER) we demonstrated for the first time that a plant derivative contributed to reduce paracellular permeability in DENV-2 infected HMEC-1. We also showed that IL-8 contributed significantly to the induction of permeability. Further studies are still required until a new drug can be indicated, but our *in vitro* results support the evidences that AFUT could be potentially useful to preventive treatment of severe dengue.

Keywords: *Uncaria tomentosa*, dengue, antiviral, oxindole alkaloid, endothelial cells, permeability.

Dengue is the most important mosquito-borne viral disease of the world causing annually enormous death numbers as well as significant economic losses [1]. The Dengue virus (DENV) has 4 serotypes (1-4) and infection by any one of them can result in a wide spectrum of clinical manifestations that can be classified according to the severity level. Severe dengue is characterized by plasma leakage that may lead to shock, besides organ impairment and occasionally severe hemorrhages [2].

The endothelium is the primary barrier of the circulatory system and plays an important role in regulating vessel permeability and maintaining hemostasis. During certain diseases, such as dengue, the endothelial cell (EC) function can be changed leading to plasma leakage [3]. DENV infected ECs may contribute directly to pathogenesis by increasing viremia, secreting cytokines, modulating complement pathways, or transforming the endothelium into an immunologic target of cellular and humoral immune responses ~~[3]~~. During innate immune responses against dengue, factors such as MCP-1, MMP, MIF, and TNF-α play an important role in endothelial cell activation being detected at increased circulating levels in severe patients [3, 7]. Interleukin-8 (IL-8) is among the main factors that contribute to endothelial permeability [8].

Currently, patients are treated only symptomatically, since there is neither an effective approved vaccine for use nor a specific therapeutic agent for dengue [9]. Natural products remain one of the most important sources on the development of new drugs, however their potential against DENV infection has not been well explored [10]. *Uncaria tomentosa* (Willd.) DC (Rubiaceae), known as "cat's claw", is a woody vine distributed from the Amazonian to Central American rain forest. This plant, commonly used by folk medicine has antiviral, immunomodulatory, anti-inflammatory and antioxidant properties scientificaly studied [11-15]. For DENV-infected human monocytes, an aqueous-ethanol extract and its alkaloid enriched fraction of *Uncaria tomentosa* bark presented inhibitory activities in virus load and cytokine production [13].
In the present work we further investigated the effect of this same alkaloidal fraction on human dermal microvascular endothelial cell in an *in vitro* infection model demonstrating its antiviral activity as well as the reduction of endothelial permeability.

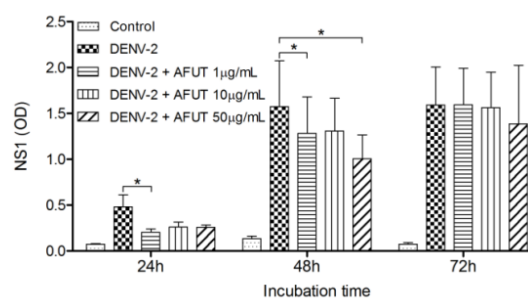

Figure 1: Antiviral effect of *U. tomentosa* in DENV-2 infected HMEC-1. DENV-2 infected HMEC-1 were incubated with alkaloidal fraction from *Uncaria tomentosa* (AFUT) at concentrations 1-50 μg/mL during 3 days. Inhibitory activity against DENV-2 was detected by NS1 antigen quantification ELISA assay in supernatant of DENV-2 infected HMEC-1, treated or not with AFUT. The bars represent the mean ±SE of three independent experiments in triplicate each. OD = optical density at 620 nm. (*)$P < 0.05$, indicates statistical significance calculated by paired *t*-test .

The total alkaloid content in the crude aqueous-ethanol extract of *U. tomentosa* was calculated as 29.1 mg/g (± 1%). The HPLC analysis

of this alkaloidal fraction (AFUT) revealed the six pentacyclic oxindole alkaloids that are currently considered the chemical markers of the species: speciophylline, mitraphylline, uncarine F, pteropodine, isomitraphylline and isopteropodine [13, 16].

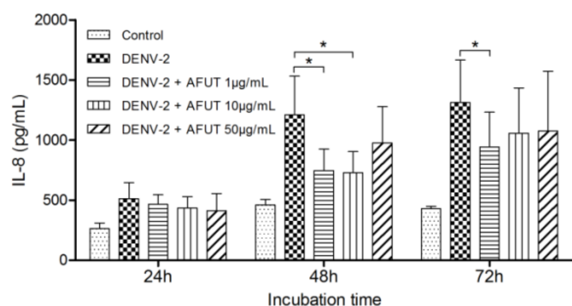

Figure 2: *U. tomentosa* modulating IL-8 production in DENV-2 infected HMEC-1. DENV-2 infected HMEC-1 was incubated with alkaloidal fraction from *Uncaria tomentosa* (AFUT) at concentrations 1-50 μg/mL during 3 days. Inhibitory effect against IL-8 in supernatant of DENV-2 infected HMEC-1, treated or not with AFUT. The bars represent the mean ±SE of three independent experiments in triplicate each. (*) $P < 0.05$, calculated by Wilcoxon signed rank test

All concentrations of AFUT used in the following experiments presented no cytotoxicity on HMEC-1 cell cultures according to the the tetrazolium salt (MTT) reduction assay.

The antiviral activity was detected by the reduction of NS1 antigen secretion in DENV-2 infected HMEC-1 at 24 and 48 h post-infection (p.i.) at AFUT concentrations from 1- 50 μg/mL (Figure 1). The decrease in NS1 antigen was statistically significant when the infected HMEC-1 were treated with AFUT 1 μg/mL (24 and 48 h p.i.) or with 50 μg/mL (48 h p.i). As observed by Reis *et al*. [13] that used DENV-2 infected monocytes, we also demonstrated that AFUT 1 μg/mL induced significant antiviral effect.

An immunomodulating effect was detected by the inhibition of IL-8 production in supernatant from DENV-2 infected HMEC-1.
Significant modulation of this cytokine was observed when infected HMEC-1 were treated with AFUT 1 μg/mL (48 and 72h p.i.) or 10 μg/mL (48 h p.i.). As observed for the quantification of NS1 antigen, the AFUT 1 μg/mL revealed the most effective inhibitory activity. (Figure 2).

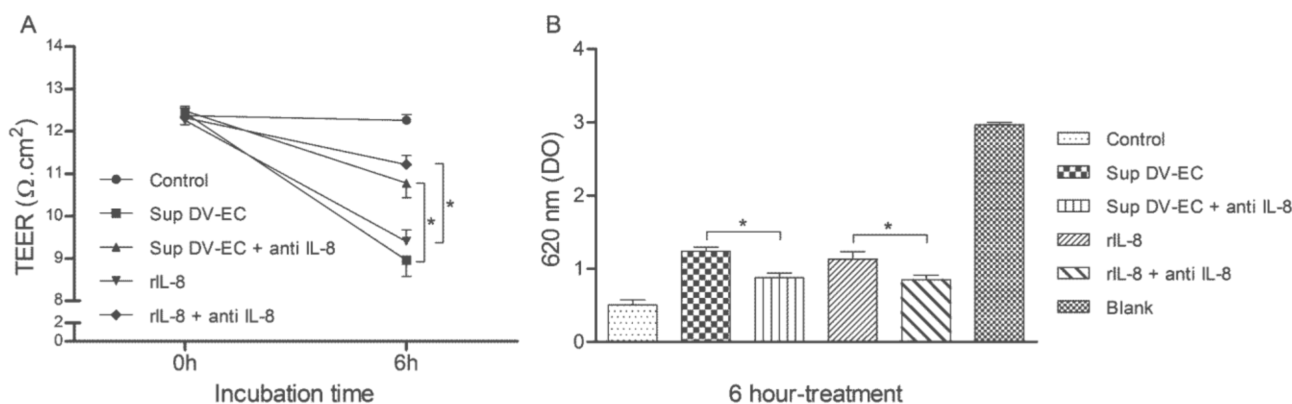

Figure 3: Effect of IL-8 in the permeability of HMEC-1. Confluent monolayers in inserts were incubated for 6 h with culture medium (Control), supernatant from DENV-2 infected HMEC-1 (Sup DV-DC) with or without neutralizing antibody to IL-8 (Sup DV-DC + anti IL-8), and culture medium containing recombinant IL-8 (rIL-8) with or without neutralizing antibody to IL-8 (rIL-8 + anti IL-8). The effect of IL-8 in inducing endothelial permeability was evaluated by TEER assay (A) and by transendothelial migration of streptavidin-HRP (B). Horse Radish Peroxidase (HRP) activities were measured and O.D. values higher than the control were interpreted as an alteration in endothelial barrier function. Blank are inserts containing culture medium without cells. The bars represent the mean ±SE of two independent experiments in triplicate each. (*) $P < 0.05$.indicates statistical significance by paired *t*-test.

IL-8 has been found in patients with severe dengue and it is produced by endothelial cells infected *in vitro* with DENV [17, 18] . Recombinant IL-8 induced paracellular endothelial permeability *in vitro* as evaluated by by albumin transendothelial migration assay [5, 19], whereas recombinant TNF-α treateatment presented permeability, detected by transendothelial electrical resistance (TEER) assay[4],.
In the present study we could definitely confirm the IL-8 involvement in endothelial permeability using two different assays, TEER and streptavidin-HRP transendothelial migration (Figure 3). Moreover, when supernatants from previously DENV-2 infected HMEC-1 were added to the EC monolayer, a more effective permeability induction was observed. Most probably a synergism between IL-8 and other molecules present in cell culture supernatant, such as IL-6 and RANTES [17, 20], may be contributing to further increase permeability. The IL-8 role in changing the endothelium barrier function was confirmed when IL-8 neutralizing antibody was added to the permeability assays and partially recovering the TEER.

Knowing that IL-8 and other cytokines play an important role in the modulation of the endothelium, much effort has been made to identify inhibitors of pro-inflammatory cytokine production [8].

Endothelial monolayer cultures provide an excellent model to study the endothelial barrier function and paracellular permeability *in vitro* [21] and the Endohm chamber enables reproducible and accurate measurements of the TEER [4]. We showed here that HMEC-1 infected with DENV-2 present a time-dependent increase in endothelial permeability that started after 24 h p.i. (Figure 4). An alkaloidal fraction from *Uncaria tomentosa* (AFUT) added daily at 1 μg/mL immediately after viral adsorption during 5 days. This treatment significantly reduced the endothelial permeability in the third and fourth days p.i. No significant effect was detected with single treatment even at higher doses. Despite that 1 μg/mL used once has shown significant reduction in the quantification of NS1 antigen and IL-8, the permeability induction is a late event in cell

cultures and also in the patient. It seems reasonable to treat cells continuously avoiding that compounds remaining long in culture lose their effectiveness permiting that virus replication and cytokine release may be reverted. Most antiviral and immunosuppressive drugs are administrated at multiple doses *in vivo* in order to be effective.

The endothelium is the primary fluid barrier of the vasculature and the direct infection by DENV, permit virus replication and induce immune-enhancing effects that increase vascular permeability and can result in severe dengue [3]. We confirmed here our previous results [13] on the *in vitro* antiviral and immunomodulatory activities of the alkaloidal fraction from *U. tomentosa* by applying a different experimental approach by studying endothelial cell line infection. We used here a simple method for detecting virus load that consists in the detection of a nonstructural protein - NS1.

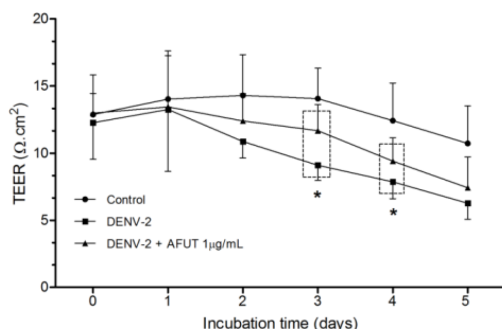

Figure 4: *U. tomentosa* reduce the transendothelial electrical resistance (TEER) in DENV-2 infected HMEC-1. DENV-2 infected HMEC-1 were incubated with alkaloidal fraction from *Uncaria tomentosa* (AFUT) at concentrations 1 μg/mL added daily. The TEER was measured before infection and treatment with AFUT (0 day) and after daily during 5 days. The bars represent the mean ±SE of three independent experiments in triplicate. (*) indicates statistical significance between TEER values of non-treated and AFUT treated DENV-2 infected HMEC-1 calculated by paired *t*-test (doted rectangles, $P < 0.05$).

Natural products are capable of reducing endothelial permeability induced by TNF-α and LPS [22, 23]. The data presented here show for the first time the role of a medicinal plant in the attenuation of endothelial permeability *in vitro* during DENV infection. We added an important concept that is the effect of *U. tomentosa* in endothelial cells altering permeability functions.

DENV-2 infected endothelial cells has been demonstrated to undergo an increasing permeability [4, 5]. Endothelial permeability induced by DENV infection is a complex system that involves many factors; therefore it would be unlikely to solely assign the IL-8 to effect the endothelium modulation . TNF-α, IL-8 and other factors such as MCP-1, MMP and MIF are likely acting synergistically and are all well known by their role in vascular permeability and shock [3]. *U. tomentosa* inhibited TNF-α production by regulating the NF-κB transcription factor [14]. NF-κB may regulate the expression of several other pro-inflammatory cytokines including IL-1, IL-2, IL-6 and IL-8 [24].
It may be hypothesized that the immunomodulating activity may be a result of viral load reduction. Alternatively induction of pathways that inhibit pro-inflammatory responses such as NF-κB and stimulate antiviral mechanisms such as interferons and nitric oxide production may be occurring. For these reasons, further studies are necessary to elucidate the effects observed in our study. Surely, the future establishment of compounds acting on the vascular permeability during dengue may represent a great breakthrough in treating the severe clinical manifestations of this infection.

Experimental

Plant material: The alkaloidal fraction from *Uncaria tomentosa* (AFUT) used in this study belongs to the same lot used in previous studies [12, 13, 16] and its pentacyclic oxindole alkaloids (POA) content was determined by high-performance liquid chromatography (HPLC). Briefly, stem bark of wild specimen of *U. tomentosa*, collected in Cruzeiro do Sul, Acre, Brazil (voucher data in Miranda *et al*. [25]), was exhaustively extracted with aqueous-ethanol 1:1 at room temperature [26]. The crude hydroalcohol extract was sonicated (10 min) with 0.1 N HCl and then partitioned with ethyl acetate. The resulting aqueous fraction was treated with NH_4OH until pH 9-10 and extracted with ethyl acetate. This alkaloid-rich ethyl acetate fraction was dried and filtered and the solvent removed under low pressure at 37 ºC. The POA present in the alkaloidal fraction were identified on the TLC plate by comparison to alkaloid standards previously isolated and characterized [26]. The additional characterization of the POA profile was carried out by reverse-phase HPLC system with a Lichrocart Lichrospher 5 μm, 125 × 4.6 mm i.d. column with a UV-detector at 245 nm under the same conditions described previously [27], system II. The identification of the POA signals was made by comparing their relative retention times to the corresponding alkaloid profile described by those authors. The total alkaloid content was determined by HPLC under the indicated chromatographic conditions through external standardization relative to isopteropodine. Five independent points for the external calibration curve were established from triplicate injections of isopteropodine solution in methanol. The curve presented linearity ($R^2 = 0.9846$) in the range of 4.75 to 76.0 μg/mL with standard deviation of 1.0 μg/mL, the minimum detectable concentration estimated as 1.0 μg/mL.

Cell culture: The human lineage of dermal microvascular endothelial cells (HMEC-1) was cultured in EGM-2 MV complete medium (Lonza) or MCDB-131 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor, 1 μg/mL hydrocortisone, 2mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin.

Virus stock preparation and titration: The DENV serotype 2 strain 16681was propagated tittered as described before [13].,. The supernatant containing the virus particles was ultracentrifuged (100,000 g) for 1 hour at 4 °C. The pellet was suspended stored at -70 ºC. Virus titer was calculated as 50 percent tissue culture infection dose ($TCID_{50}$) resulting in a concentration of 1.6 x 10^9 $TCID_{50}$/mL.

Cell viability assay: HMEC-1 were seeded in quadruplicate in a 96-well tissue culture plate (BD Falcon) and incubated with AFUT at different concentrations (1, 10, 50 and 100 μg/mL) at 37 ºC under 5% CO_2 for 2-5 days., The cytotoxicity effect of AFUT on the HMEC-1 conducted using Vybrant® MTT Cell Proliferation Assay (Molecular Probes) and the optical density was measured at 540-570 nm.

Antiviral effect by NS1 antigen determination: HMEC-1 were seeded in 24-well plates in MCDB-131 medium and, after 2 days of growth at 37 ºC under 5% CO_2, infected with DENV-2 at dilution of 1:10 for 2 h. Then, cells were washed, fresh medium (with 5% FBS) added and some wells containing infected cells were treated with different concentrations of AFUT (1, 10 and 50 μg/mL). After 24 h, 48 h and 72 h supernatants were stored in aliquots at -70 ºC. The Platelia Dengue NS1 AG kit (Bio-Rad), ~~[28],~~ was used to quantify

the DENV pruduction in HMEC-1 cultures. Supernatants were diluted 1:10 and the assay was further performed according to manufacturer's instructions.

IL-8 determination by ELISA assay: The IL-8 concentration was determined by employing the standard ELISA kit according with the manufacturer's instruction (PeproTech), using streptavidin-HRP (Zymed) and TMB solution (Invitrogen) as the substrate. The absorbance was read at 620 nm.

Endothelial permeability assay: HMEC-1 were seeded (1.5 x 10^5 cells/insert) on fibronectin-coated (Sigma-Aldrich, 15 µg/mL) 24-well polyester membrane inserts (6.5 mm diameter, pore size 0.4 µm, Corning) with complete EGM-2 MV medium. Inserts were placed into lower chambers containing each 1100 µL of the same medium and incubated at 37 ºC under 5% CO_2 atmosphere. The paracellular permeability was measured using the Endohm chamber (WPI) connected to an electrical resistance system (Millicell-ERS, Millipore).. Membrane inserts containing no cells were used for blank measurements. The TEER values were calculated using the following formula that considers the area of the insert membrane and the resistance value of the blank: $(R_{exp} - R_b)*0.33\ cm^2$. Once the monolayer was grown to confluence HMEC-1 were infected with DENV-2 inoculum (70 µL diluted 1:1 in medium) and incubated at 37 ºC under 5% CO_2 during 2 h. Changed EGM-2 MV medium, contained 1µg/mL of AFUT, in some inserts with infected cells (treated inserts). TEER measurements were performed every 24 h and then more AFUT (1µg/mL) was added. Uninfected cells were used as a control.
The effect of IL-8 in the permeability was studied using basically the same proceedures. Recombinant IL-8 (4 ng/mL; PeproTech) or supernatant from DENV-2 infected HMEC-1 that were pre-incubated (over-night at 4 ºC) or not with neutralizing antibody to IL-8 (6 µg/mL; PeproTech). After 6 h of incubation the TEER was measured and immediately the medium in the both chambers was changed to RPMI without phenol red and streptavidin-HRP (3,13 µg/mL, Invitrogen) added to the upper chamber. After 30 min aliquots (30 µL) from the lower chamber were assayed with TMB substrate and O.D. measured at 620 nm.

Statistical analysis: For data that fitted into normal Gaussian distribution we used paired *t*-test and for those that did not fit we used Wilcoxon signed rank test. All statistical analyses were performed using the GraphPad Prism software.

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