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Constituents of *Talisia nervosa* with Potential Utility against Metabolic Syndrome

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This study is focused on the isolation and characterization of bioactive secondary metabolites from the ethanolic extract of stems of the Panamanian plant *Talisia nervosa* Radlk**,** through a series of target-based cellular assays related to the metabolic syndrome (MetS): a combination of type 2 Diabetes Mellitus (T2DM), hypercholesterolemia, inflammation, and obesity. Bioassay guided fractionation allowed the isolation of four known compounds: (–)-catechin (**1**), methyl gallate (**2**), ethyl gallate (**3**), and ß-D-glucopyranose,1,4,6-tris(3,4,5-trihydroxybenzoate) (**4**). This is the first report of (–)-catechin (**1)** and ß-D-glucopyranose,1,4,6-tris (3,4,5-trihydroxybenzoate) (**4)** from *T. nervosa.* Among the isolates, **1** activated PPARγ, but had no effect on PPARα. Compounds **2** - **4** activated PPARα, PPARγ and LXR. Interestingly, **2** was stronger than **3** towards all three targets. Methyl gallate (**2)** showed the most potent effect toward both PPARα and PPARγ with an increase of 3.0 and 13-fold, respectively, while **4** was most potent in activating LXR with a fold induction of 5.3 at concentrations of 100 µg/mL. The nitric oxide (NO) production was reduced by compounds **2** and **3** with IC_{50} values of 7.0 and 7.5 µg/mL, respectively. ß-D-glucopyranose,1,4,6-tris (3,4,5-trihydroxybenzoate) (**4)** did not cause a significant increase in adipogenesis despite its strong PPARγ agonistic action (8.6-fold increase) and may represent a good candidate for the treatment of MetS without the undesirable side effect of weight gain.

Keywords: *Talisia nervosa*, Panamanian flora, Metabolic syndrome, PPARα, PPARγ, LXR, Nitric oxide, Adipogenesis.

Metabolic syndrome (MetS) is a complex, progressive disorder that can develop over many years as a cluster of conditions characterized by a constellation of metabolic abnormalities, which include abdominal obesity, hyperglycemia, insulin resistance, atherogenic dyslipidemia and hypertension [1-2]. This devastating disease represents a public health concern and Latin American populations exhibit a higher prevalence of MetS than developed countries [3-4].

Epidemiological studies have shown that MetS is associated with a greater risk of developing T2DM, cardiovascular disease (CVD) and stroke [5-6]. Due to the inter-connection between the multiple defects associated with MetS, there has been an increased interest in the molecular targets such as peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), nuclear factor-*κ*B (NF-*κ*B), and inducible nitric oxide synthase (iNOS) because of their close association with metabolic disorders such as T2DM, hypercholesterolemia, inflammation, and obesity. As a part of the inflammatory pathway, activation of NF-*κ*B leads to insulin resistance and by blocking this pathway, insulin resistance and the resultant T2DM can be prevented [7]. In contrast, activators of PPARα and PPARγ are effective in lowering blood lipids and glycemia, and have been considered useful in the treatment of obesity and diabetes [8]. LXR, in the same manner as the PPARs, suppresses production of inflammatory mediators in a manner reciprocal to its regulation of lipid metabolism [9]. Plant secondary metabolites affecting multiple targets are emerging as an important class of therapeutic agents. In fact, new anti-diabetic drugs that do not display undesirable side effects, such as hepatotoxicity, edema and weight gain, are in great demand.

In our ongoing efforts to discover the potential of Panamanian flora as a source of bioactive molecules toward the cluster of metabolic disturbances, a screening of 75 plants against different molecular targets was carried out. The most active extract of *Talisia nervosa* Radlk (stems) was chosen for bioassay guided fractionation and isolation and identification of its bioactive constituents. *T. nervosa*, also called "*mamón de monte*", belongs to the Sapindaceae family. This plant is distributed in the tropical moist forest and tropical wet forest of the Central and Caribbean parts of Panama. The pulp of this plant is edible and is consumed by Panamanian Choco Indians [10]. The genus *Talisia* contains diverse constituents such as catechins, flavonoids and hydroxycinnamic acids [11-12]. Previous chemical investigation of stems of *T. nervosa* in CIFLORPAN yielded ethyl gallate and methyl gallate, which showed weak antimalarial activity against chloroquine resistant strains of *Plasmodium falciparum* with IC_{50} of 35.3 µM and 38.0 µM, respectively [13].

Our preliminary evaluation showed that the ethanolic extract of *T. nervosa* exhibited dual activation of PPARα and PPARγ with an increase of 5.25 and 9.52-fold in PPARα and PPARγ activities compared with the control, at a concentration of 100 µg/mL, respectively (Table 1). Furthermore, the ethanolic extract also increased the insulin-stimulated glucose uptake and, unlike rosiglitazone, it did not cause significant increase in adipogenesis despite its PPARγ agonistic action, as shown in Figure 1. On the other hand, *T. nervosa* did not show inhibition of NF-*κ*B, showing its specificity toward PPARs as the nuclear receptor targets. This plant extract also increased the LXR activity with an increase of 1.5-fold, at a concentration of 50 µg/mL (Table 1). These data suggest that *T. nervosa* may play an important role in preventing MetS.

Table 1: Fold activation of PPARα, PPARγ and LXRα in HepG2 cells by the ethanolic extract of *T. nervosa.*

Plant Species / Part	PPARα			PPARγ			LXRα		
	100	50	25	100	50	25	100	50	25
	(µg/mL)			(µg/mL)			(µg/mL)		
***Talisia nervosa* Radlk (Stem)**	5.25	2.34	2.51	9.52	2.02	1.84	1.36	1.53	1.53
Ciprofibrate[a] (30 µM)			3.09						
Ciglitazone[a] (30 µM)						5.88			
25-hydroxycholesterol[a] (25 µM)									3.04

[a]Positive control

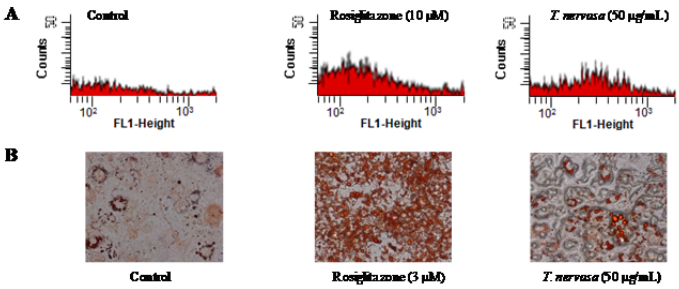

Figure 1: Flow cytometric analysis of glucose uptake by *T. nervosa* in HepG2 cells **(A)**. Effect of *T. nervosa* on adipocyte differentiation in 3T3-L1 cells by oil red O staining **(B)**.

In order to discover the active constituents, the ethanolic extract of stems of *T. nervosa* was successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, BuOH and H_2O. This procedure generated five fractions which were subjected to bioassay guided fractionation. Their activities were monitored by a reporter gene assay for PPARα and PPARγ transcriptional activities.

Among the 5 fractions, the EtOAc fraction was the most effective in increasing PPARα and PPARγ directed luciferase expression with 6.5 and 7.5 fold increase at 100 µg/mL in comparison with the vehicle control, respectively. The other four fractions, hexane, $CHCl_3$, BuOH and H_2O, showed less activation of both PPARα and PPARγ signaling (an increase of 2.4, 2.2, 3.0, and 1.4-fold for PPARα, and 3.0, 1.8, 4.5 and 1.7-fold for PPARγ compared with the vehicle control, respectively).

Bioassay guided fractionation of the EtOAc fraction led to the isolation of four known compounds: (–)-catechin (**1,** 9.5 mg), methyl gallate (**2**, 67.1 mg), ethyl gallate (**3**, 11.0 mg), and ß-D-glucopyranose,1,4,6-tris(3,4,5-trihydroxybenzoate) (**4**, 5.3 mg). Their structures were elucidated by spectroscopic methods including ^{1}H NMR, ^{13}C NMR, COSY, and HSQC, and by comparing with published spectral data [13].

Table 2: Inhibition of NF-κB in SW1353 cells and iNOS in RAW 264.7 cells and fold activation of PPARα, PPARγ, LXRα in HepG2 cells by compounds 1-4 from *T. nervosa.*

Compound	NF-κB	iNOS	PPARα			PPARγ			LXRα		
	IC_{50}	IC_{50}	100	50	25	100	50	25	100	50	25
	(µg/mL)			(µg/mL)			(µg/mL)			(µg/mL)	
1	NA	NA	1.24	1.23	1.17	1.99	1.85	1.69	1.74	2.15	1.71
2	NA	7.0	3.05	1.23	1.18	13.0	3.02	1.83	3.16	1.99	1.99
3	NA	7.5	1.66	1.24	1.18	4.58	2.32	2.13	2.62	1.53	1.45
4	NA	>50	2.26	1.33	1.25	8.62	2.31	2.37	5.32	2.12	1.49
Parthenolide[a] (µM)	1.20	0.35									
Ciprofibrate[a] (30 µM)					3.31						
Ciglitazone[a] (30 µM)								4.44			
25-hydroxycholesterol[a] (25 µM)											4.67

[a]Positive control; NA: No activity.

Out of 4 isolates, compound **2** (methyl gallate) showed the most potent effect toward both PPARα and PPARγ with an increase of 3.0 and 13-fold in their activity compared with the vehicle control, at a concentration of 100 µg/mL, respectively (Table 2). This compound has been previously isolated from *T. nervosa* and showed a weak *in vitro* antiplasmodial activity against *Plasmodium falciparum* and antileishmanial activity against *Leishmania mexicana* amastigotes [13]. However, this is the first report of compound **2** as a dual modulator of PPARα and PPARγ. On the other hand, the PPARα and PPARγ activations were followed by compounds **4** and **3** (an increase of 2.3 and 1.7-fold for PPARα, and 8.6 and 4.6-fold for PPARγ compared with the vehicle control, at a concentration of 100 µg/mL, respectively) as shown in Table 2.

The dual activation of PPARα and PPARγ exhibited by compound **4** (a gallotannin) can be compared with the activity of another gallotannin 2,3,6-tri-*O*-galloyl-β-D-glucose reported earlier [14]. Compound **1** [(–)-catechin] was less potent (1.2 and 2.0 fold activation for PPARα and PPARγ, respectively) compared with compounds **2, 3** and **4**. It is consistent with a previous report that showed very weak activation of PPARα by catechin [15]. In addition, earlier studies have shown that catechin possesses a potential agonistic effect on PPARγ [16]. Our results are in agreement with previous reports and support the hypoglycemic effect of catechin mediated by PPARγ activation [17].

In order to evaluate whether the isolated compounds, acting as PPARγ agonists, also induce adipogenesis similar to rosiglitazone, their effect on adipocyte differentiation was examined on insulin-stimulated 3T3-L1 adipocytes. A concentration-dependent increase in adipocyte differentiation was seen with compounds **2** and **3** as determined by oil red O staining (Figure 2). This result was consistent with the increase in lipid accumulation, determined by Nile Red fluorescent reagent. At the highest concentration of 50 µg/mL, the lipid content of adipocytes was increased to 58% and 68% by compounds **2** and **3**, respectively, while rosiglitazone at a concentration of 3 µM increased adipogenesis by 100%, compared with the vehicle control. However, in a recent study, an inhibition of lipid accumulation in the early stages of adipogenesis by methyl gallate has been reported [18]. In contrast, compound **4** did not exhibit a significant increase in adipogenesis (Figure 2) despite its PPARγ agonistic action. This is similar to the effect of another gallotannin 2,3,6-tri-*O*-galloyl-β-D-glucose which did not enhance adipocyte differentiation in 3T3-L1 cells [14]. This suggests that compound **4** may represent as potential candidate for the treatment of MetS without the undesirable side effect of weight gain.

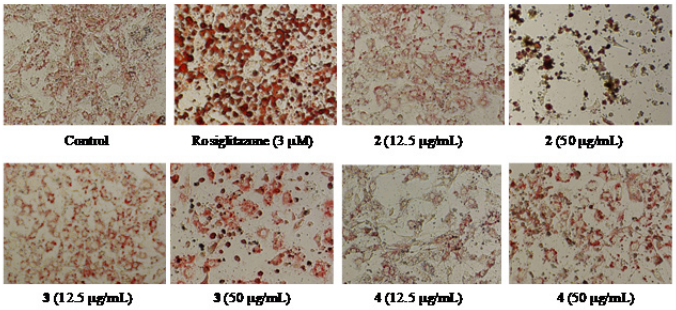

Figure 2: Effect of compounds **2-4** on adipocyte differentiation in 3T3-L1 cells by oil red O staining.

Subsequently, these compounds were also evaluated for NF-κB and iNOS inhibition, in order to investigate their anti-inflammatory potential. As a result, compound **1** did not show inhibition of phorbol 12-myristate 13-acetate (PMA) induced NF-κB activation, which is in agreement with the previous report related to the effect of catechin on NF-κB [19]. In addition, no inhibition of iNOS activity in LPS induced macrophages was seen, even though a weak effect of catechin on iNOS was reported earlier [20]. However, catechin has been shown to exhibit anti-inflammatory effects in

microglial cells by suppressing the production of pro-inflammatory mediators and mitigating NF-*κ*B activity [21]. Furthermore, compounds **2** and **3** decreased the nitric oxide (NO) production in macrophages with IC_{50} values of 7.0 and 7.5 μg/mL, respectively (Table 2). These data are consistent with the previous studies, which reported that methyl gallate and ethyl gallate inhibited NO production and iNOS expression in macrophages stimulated by LPS [22-23]. On the other hand, these two compounds (**2,3**) did not show any inhibition of NF-*κ*B in PMA induced chondrocytes, as shown in Table 2.

The effect of these compounds on the activity of LXR was also evaluated. Compound **4** was the most effective with a 5.32 fold activation of LXRα at a concentration of 100 μg/mL, as shown in Table 2. At a concentration of 100 μg/mL, compounds **1**, **2**, and **3** showed 1.74, 3.16, and 2.62-fold activation of LXRα, respectively. The results of molecular docking in an earlier study suggest catechin as a potential inhibitor of atherogenesis through activating LXR signaling [24].

In conclusion, these compounds may have potential to work against metabolic disorders due to their effects on carbohydrate and lipid metabolism, as well as their anti-inflammatory effect.

Experimental

Plant material: Stems of *T. nervosa* were collected from National Park Altos de Campana, Buena Vista, Bejuco, Chame, Panama (N 08° 41' 27.2'' W 079° 58' 02.2'') in January 2011 by botanist Alex Espinosa. Its taxonomic identity was established by Prof. Mireya D. Correa, at the Herbarium of the University of Panama (PMA), where the voucher specimen (Florpan No. 8735) is deposited.

Extraction and isolation of active constituents: The dried powdered stems (1628 g) of *T. nervosa* were extracted exhaustively with 95% EtOH at room temperature for 24 h. After filtration, the solvent was removed in a rotary evaporator under reduced pressure to yield 86.5 g of crude dry extract, which was successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, BuOH and H_2O. This procedure gave hexane (4.79 g), $CHCl_3$ (2.81 g), EtOAc (18.38 g), BuOH (24.05 g) and H_2O fractions (11.87 g). The EtOAc fraction (10 g) was subjected to column chromatography using polyamide (50-160 μm, 310 g) with gradient elution using MeOH-H_2O (1:1 → 5:1) and MeOH-acetone (3:1 → 1:3). Fractions (20 mL) were collected and combined on the basis of their TLC profiles. Separated compounds were detected by UV before and after spraying with vanillin-H_2SO_4. The bioassay-guided fractionation for PPARα and PPARγ was monitored. The combined fractions were further separated on Sephadex LH-20 with MeOH (100%), which led to the isolation of: (–)-catechin (9.5 mg), methyl gallate (67.1 mg), ethyl gallate (11.0 mg), and ß-D-glucopyranose,1,4,6-tris(3,4,5-trihydroxybenzoate) (5.3 mg).

PPARs and LXRα transcriptional activity: Activation of PPARα, PPARγ and LXR was carried out following the method described previously, with some modification [14, 25]. Briefly, human hepatoma (HepG2) cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. For the identification of PPARα and PPARγ agonists, HepG2 cells were transfected with pSG5-PPARα and PPRE X3-tk-luc or pCMV-rPPARγ and pPPREaP2-tk-luc plasmid DNA (25 μg of each/1.5 mL cell suspension), respectively, by electroporation at 160 V for single 70 msec pulses using a BTX Square Electroporator T820. In a similar manner, cells were transfected with pCMX-hLXRα and LXRE-tk-luc plasmid DNA, for the LXRα transcriptional activity. Transfected cells were plated at a density of 5×10^4 cells/well in 96-well tissue culture plates and grown for 24 h. After 24 h, the cells were treated with either test samples or positive controls (ciprofibrate, ciglitazone or 25(*R*)-hydroxyl-cholesterol). After incubation for 24 h, the cells were lysed and the luciferase activity was measured. Light output was detected on a SpectraMax M5 plate reader.

Adipocyte differentiation assay: Mouse embryo preadipocyte (3T3-L1) cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. For the differentiation assay, preadipocytes were seeded in 48-well plates at a density of 2×10^4 cells/well and maintained until 2 days post-confluence. After 2 days of confluence (day 0), medium was replaced with DMEM containing 10% FBS, 10 μg/mL insulin, 1 μM dexamethasone and test samples at various concentrations (12.5, 25 and 50 μg/mL in case of pure compounds) or positive control rosiglitazone (3 μM). After 2 days, the medium was replaced with DMEM containing 10% FBS, 10 μg/mL insulin and test samples (day 4). Cells were then maintained in 10% FBS/ DMEM for an additional 4 days (day 8) [26]. The extent of adipogenesis was determined in differentiated adipocytes by quantification of lipid content and oil red O staining as described below.

Quantification of lipid content in 3T3-L1 cells: Lipid content was estimated with an AdipoRed Assay Kit according to the manufacture's protocol. On day 8 after differentiation in the presence of samples as described above, cells were carefully rinsed with 400 μL PBS. PBS (400 μL) and 12 μL AdipoRed reagents were added to each well and the cells were incubated at 37°C for 10 min. The fluorescent intensity was measured in a SpectraMax M5 plate reader with excitation at 485 nm and emission at 572 nm. The percent lipid content of cells was calculated compared with the vehicle control (100%).

Oil red O staining: In order to visualize the morphological changes, differentiated adipocytes were stained with Oil red O according to a previously described method, with some modification [27]. Differentiated 3T3-L1 cells were rinsed twice with PBS and fixed with 70% ethanol for 30 min. The fixed cells were stained with Oil Red O solution (0.6% Oil Red O in isopropanol : water; 3:2) for 1 h at room temperature and washed twice with distilled water. The images of stained cells were captured by a Nikon DS-Ri1 camera attached to an Olympus IX50 microscope.

NF-κB inhibition: Human chondrosarcoma (SW1353) cells were cultured as described earlier, with some modifications [28]. In brief, SW1353 cells were transfected with pBIIXLUC luciferase plasmid construct and plated in 96 well plates at a density of 1.25×10^5 cells/well. After 24 h, the cells were exposed to different concentrations of pure compounds (1, 10, 100 μg/mL) or positive control parthenolide (0.125, 1.25, 12.5 μM) for 30 min and then induced with 70 ng/mL of PMA for 8 h for the activation of NF-*κ*B. Percent decrease in luciferase activity was calculated relative to the vehicle control (DMSO).

iNOS inhibition: The assay was performed in mouse macrophage (RAW 264.7) cells, as described previously, with some modification [29]. RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 96-well plates and incubated for 24 h. After incubation, the cells were treated with either pure compounds or positive control (parthenolide) for 30 min. LPS (5 μg/mL) was added to induce the cells and the plates were incubated for 24 h. The activity of iNOS was determined by measuring the level of nitrite in the cell culture supernatant with Griess reagent.

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