Bioassay-guided isolation of secondary metabolite responsible for the biofilm inhibitor activity of *Marcgravia nervosa* ethanolic extracts

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ABSTRACT

Marcgravia nervosa ethanolic extracts showed significant inhibition of biofilm formation. Biofilm formation is a major concern in several health areas with few compounds currently available to prevent this. *M. nervosa* is a member of the Marcgraviaceae family of vines, native to Central and South America. This study examined the phytochemical components and biofilm inhibitory response of the plant's crude extracts from the leaves. Bioassay guided fractionation was employed to isolate and identify the active compound using different chromatographic methods, directed by results from *in vitro* biofilm inhibition screening. Structural elucidation of both the biofilm inhibitors and other secondary metabolites was carried out using UPLC-MS and NMR spectroscopy techniques. The phytochemical analysis led to the isolation of the bioactive compound while also disclosing the presence of pentacyclic triterpenes in the ethanolic leaf extract. The latter compounds are typical of the triterpenes found in all of the other members of the Marcgraviaceae studied by the research group. The major component responsible for inhibiting biofilm formation was identified by NMR analysis as 2-methoxy-l,4-naphthoquinone. This is the first report of the presence of a quinone in *Marcgravia nervosa* and within the entire Marcgraviaceae family. The investigation has identified a new type of structure capable of inhibiting the formation of biofilms and suggests the possibility of more potent analogs either of natural product or synthetic origin. It continues to illustrate the potential of plants to provide lead structures for medicinal purposes.

BACKGROUND

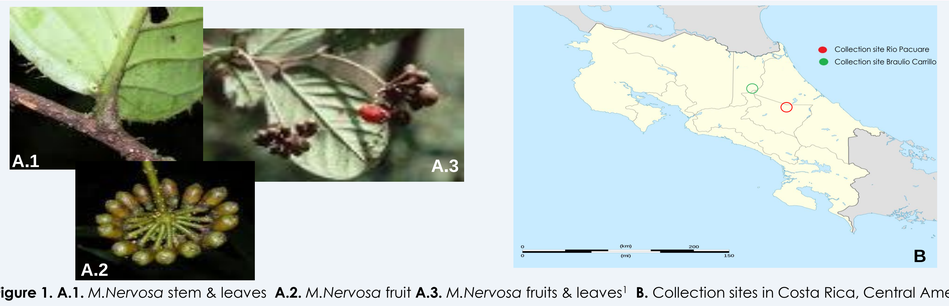

Figure 1. A.1. *M.Nervosa* stem & leaves **A.2.** *M.Nervosa* fruit **A.3.** *M.Nervosa* fruits & leaves[1] **B.** Collection sites in Costa Rica, Central America

- *Marcgravia nervosa* is a species within the Marcgraviaceae family.
- Native to the neotropics, Marcgraviaceae species range from Southern Mexico to Northern Bolivia and Eastern Brazil including the Antillean arc[2].
- *M.nervosa* is a rare species showing significant inhibition of biofilm formation, as well as some anti-fungal properties.
- Biofilm inhibition is important in medicine because biofilms create problems associated with lung diseases, medically implanted devices (i.e. prosthetics, pacemakers), as well as the fouling of equipment. Biofilms are also a leading cause for all human microbial infections[3].
- There is currently no phytochemistry reported in the literature for this species.
- This work is part of a larger research project wherein eleven Marcgraviaceae species are being studied to create a better understanding of the metabolites produced by the family.

METHODOLOGY

a. Plant Material

i. Samples of wild *Marcgravia nervosa* were collected under permit, from two locations: 1. Rio Pacuare, Costa Rica (lat: 09° 55' 06" N, long: 83° 33' 53" W) and 2. Braulio Carrillo, Costa Rica (lat: 10° 09' 36 N long: 83° 58' 36 W). The original sample from Pacuare was collected in the dry season and two more samples from both regions were collected for further comparative analyses during the rainy season. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2 mm mesh. Voucher specimens were identified by L. Poveda and M. Otarola and deposited in the JVR Herbarium, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 13157 [Pacuare], and 13489 [Carrillo]).

b. Bioassay-guided fractionation overview

i. The plant leaves were extracted via maceration for fluid extract. Three extractions were performed and combined for each solvent of different polarity used; yielding hexanes, ethyl acetate, and ethanolic extracts.
ii. The extracts, as well as the crude dry leaf material was analyzed using two bioassays:

Quorum Sensing (QS)

Bacteria use quorum sensing to regulate and control the process of biofilm formation, for QS is a stimulus-response system related to population density. A modified disk diffusion assay was used to determine whether the plant extracts can interfere with the QS of *C. violaceum*. *C. violaceum* produces a purple pigment, violacein, which is under QS control. The inhibition of violacein production will indicate the disruption of QS. Briefly, sterile paper disks loaded each with 1 mg of extract were placed onto TGY agar plates inoculated 100 µl of overnight cultures then incubated without agitation for 24 hours at 30°C. QS Inhibition was indicated by a colourless opaque halo around the disc and growth inhibition by a clear halo. Plates were examined under a dissecting microscope to confirm whether the extract has anti-QS and/or antibacterial activity. Two positive controls were used and each sample tested in triplicate[4].

Antifungal disc diffusion assay

Saccharomyces cerevisiae S288C was used for the initial screening of the plant extracts for antifungal activity. *S. cerevisiae* was inoculated into Sabouraud's broth medium and grown to an optical density of 600 nm of ~1.0 and diluted 1:100. Aliquots (100 µl) of the diluted broth culture were spread over the surface of Sabouraud's agar plates. Paper discs (7.0 mm diameter) were impregnated with crude extract (2 mg/disc), berberine (1mg/disc) or HPLC methanol and allowed to air-dry (berberine and MeOH acted as positive and negative controls). All treatments were subsequently incubated in the dark for 48h at 30°C. Inhibition zones from active extracts were then measured. Plates were stored at 4°C. Active extracts were then tested for anti-fungal activity on *C. neoformans* and *C. albicans* D10, using the same method described above.

iii. From the assay results, the bioactive compound was isolated and purified from the ethanolic plant extracts using column chromatography.

c. Structure elucidation

i. Upon isolation of the compound, various techniques were used for elucidation.
ii. Samples were prepared following regular procedure for proton nuclear magnetic resonance (1H-NMR) spectroscopy.
iii. To further separate the components of the compound, HPLC and UPLC-UV-MS technologies were used.
iv. Conditions for the HPLC include sample concentrations of 10 mg/mL, using HPLC grade methanol.
v. Using a Shimadzu UPLC-MS system, the compound was separated using a phenomenex Kinetex™ C18 column with a gradient elution method (95% 0.1% formic acid and 5% ACN). The flow was set at 0.6 ml/min with a column temperature of 55°C.

The structure of the metabolite was determined by close analysis of the information derived from NMR spectroscopy and from the fingerprinting data obtained from the UPLC-UV-MS. The isolate metabolite was then used as a standard to evaluate the presence of the compound in three different samples of *Marcgravia nervosa*. The samples vary in their location as well as the time of year when they were collected. This was done to confirm the presence of the metabolite in the plant.

RESULTS

Figures 1 and **2** illustrate the qualitative and quantitative results of the biofilm assays. **Figures 3** and **4** display the fingerprint of the crude extract using UPLC-UV-MS. **Figure 5** uses NMR spectroscopy to evaluate the presence of the metabolite in different *Marcgravia nervosa* samples. **Figure 6** depicts the NMR representing the elucidated structure of the isolated bioactive compound.

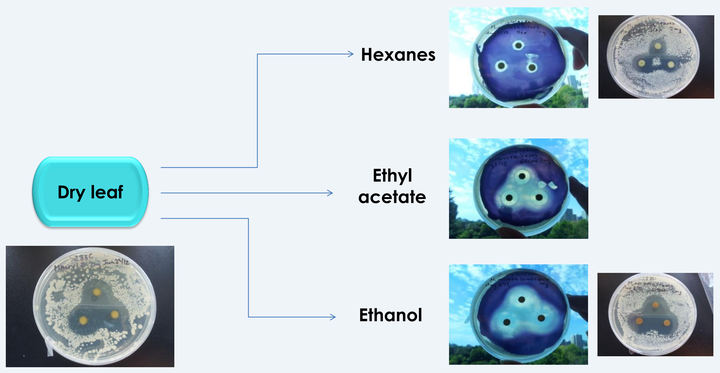

Figure 1. Bioassay guided isolation of novel bacterial biofilm inhibitors and antifungal compounds from *Marcgravia nervosa*

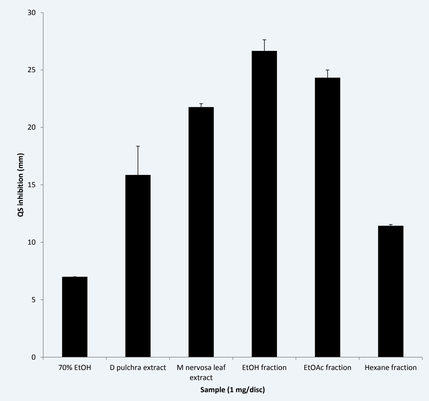

Figure 2. Quorum sensing (QS) bioassay

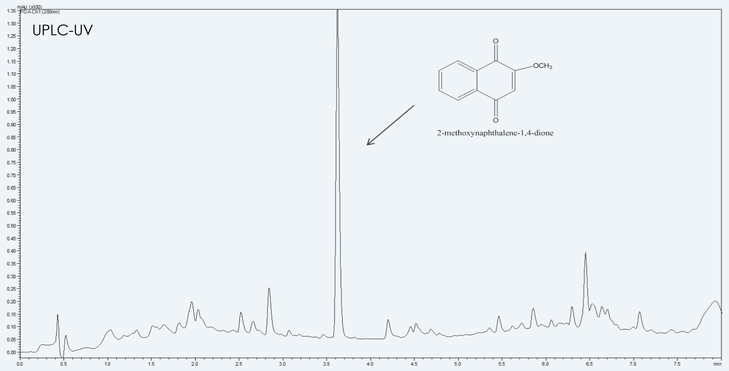

Figure 3. UPLC-UV chromatogram, 280nm absorbance wavelength

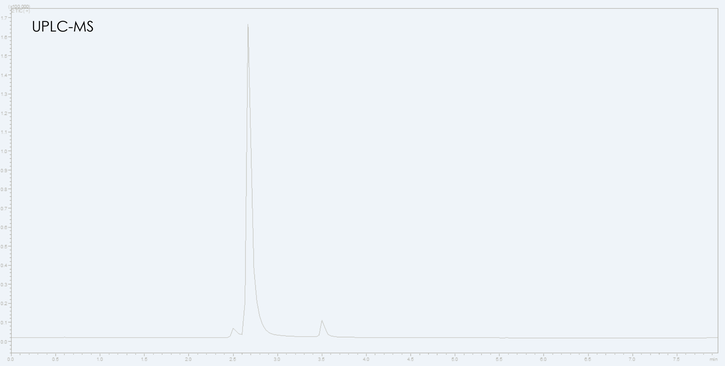

Figure 4. UPLC-MS chromatogram, selected ion at 189 UMAS (M+H)

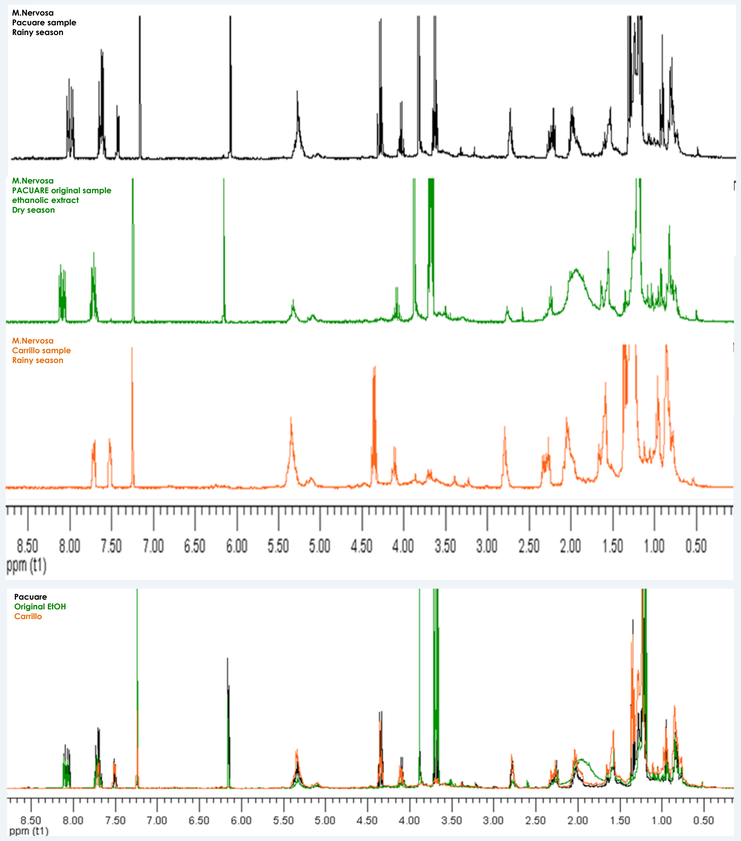

Figure 5. Qualitative NMR comparison confirming the presence of the metabolite in three different samples from different regions

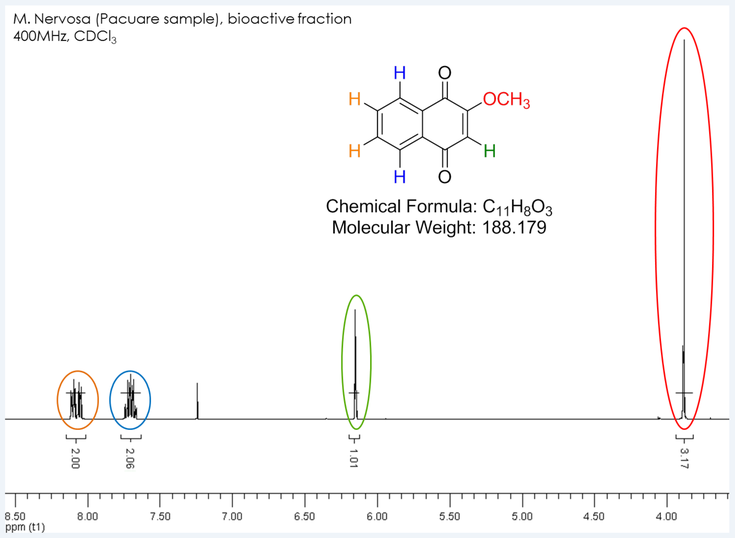

Figure 6. NMR of isolated bioactive compound, 2-methoxy-1,4-naphthoquinone

CONCLUSION

- The ethanolic extracts showed the most inhibition of biofilm formation for both the quorum sensing and antifungal disc diffusion assays.
- UPLC-UV-MS analyses show the presence of the compound with a retention time of 2.65 minutes and a mass of 189 g/mol (M+H) corresponding to the predicted molecular weight of 188 g/mol.
- Intense absorption is normal for napthoquinones because of their extensive conjugation system. The high UV absorption is matched to the MS data to ensure the proper molecule is being analyzed.
- Liquid chromatography analyses showed the presence of the metabolite in all the samples analyzed. The only variation is in the concentration of the metabolite in the sample.
- The major component responsible for inhibiting biofilm formation is identified as 2-methoxy-l,4-naphthoquinone.
- This is the first report of the presence of a quinone in *Marcgravia nervosa* and within the entire Marcgraviaceae family.
- The investigation has identified a new type of structure capable of inhibiting the formation of biofilms and suggests the possibility of more potent analogs either of natural product or synthetic origin. It continues to illustrate the potential of plants to provide lead structures for medicinal purposes.

REFERENCES

(1) [Images]: Vargas O. *Marcgravia nervosa*. Data collection #2472. Available from <http://sura.ots.ac.cr/local/florula3/en/list_images.php?key_species_code=LS001298>. Accessed: January 28, 2013.

(2) Dressler, Stefan. Neotropical Marcgraviaceae: Distribution in the Neotropics. Kew Royal Botanic Gardens, Senckenberg, Research Institute, Frankfurt am Mains, Germany. Available from <http://www.kew.org/science/tropamerica/neotropikey/families/Marcgraviaceae.htm>. Accessed: January 28, 2013.

(3) Bryers, James. Medical Biofilms. 2008. Biotechnol Bioeng 100(1): 1–18.

(4) Adonizio *et al*. Anti-quorum sensing activity of medicinal plants. 2006. J Ethnopharmacol 105, 427–435.

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