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ASSESMENT OF PLATELET ANTIAGGREGANT ACTIVITY OF A FRACTION FROM AN ETHANOLIC EXTRACT OF THE BARK OF *Nectandra amazonum* Nees

EVALUACIÓN DE LA ACTIVIDAD ANTIAGREGANTE PLAQUETARIA DE UNA FRACCIÓN DEL EXTRACTO ETANÓLICO DE CORTEZA DE *Nectandra amazonum* Nees

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ABSTRACT

Background: Some species of the *Lauraceae* family are known to produce secondary metabolites that have antiplatelet properties. Studies on the leaves of *Nectandra amazonum* Nees. have shown antiaggregant activity but the bark of this species has not been studied. **Objectives:** To assess the antiplatelet effect of the ethanolic fraction obtained from the bark of *Nectandra amazonum* Nees. [N.V. "*laurel amarillo*", *Lauraceae*] applying the "Born" turbidimetric method. **Methods**: The screening test compared the effects of a fraction of *N. amazonum* (0.1 mg/mL), acetylsalicylic acid (ASA, 0.5 mM, as reference standard) and dimethylsulphoxide (DMSO, 0.1%, as control) on human platelets stimulated with adenosine diphosphate (ADP, 2 μM), epinephrine (EPI, 1 uM (one micromolar)), collagen (COLL, 1 μg/mL) and arachidonic acid (AA, 0.2 mg/mL). Subsequently, the study focused on determining the antiaggregant potency of the *N. amazonum* fraction through concentration - response curves (from 1 μg/mL to 0.4 mg/mL), obtaining pIC_{50} (-log IC_{50}) values against the platelet agonists. **Results**: Control platelets attained the highest percentage values of aggregation (96% AA, 89% EPI, 85% COLL, and 77% ADP). The *N. amazonum* fraction significantly reduced the aggregation effects (6% AA, 45% EPI, 10% COLL, 21% ADP), with values close to those obtained with ASA (17% AA, 21% EPI, 10% COLL, 20% ADP). According to concentration - response curves, the pIC_{50} values of the ethanolic fraction indicated the following order of potency: AA, 4.90 > ADP, 4.51 > COLL, 4.33 > EPI, 3.85. **Conclusions**: These results suggest that the *N. amazonum* Nees. ethanolic fraction elicited antiplatelet effects mainly related to the inhibition of the arachidonic acid pathway.

Keywords: *Lauraceae*, platelet aggregation, arachidonic acid, adenosine diphosphate, epinephrine, collagen.

RESUMEN

Antecedentes: Algunas especies de la familia *Lauraceae* poseen metabolitos secundarios que ejercen efectos antiplaquetarios. Estudios de las hojas de *Nectandra amazonum* Nees. han mostrado esa actividad, pero no se conoce sobre las propiedades antiagregantes de su corteza. **Objetivos**: Evaluar el efecto antiagregante plaquetario de la fracción etanólica obtenida de la corteza de *Nectandra amazonum* Nees. [N.V. "*laurel amarillo*", *Lauraceae*] aplicando el método turbidimétrico de Born. **Métodos**: En el tamizaje

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antiagregante se comparó la fracción de *N. amazonum* (0,1 mg/mL) con ácido acetil salicílico (ASA 0,5 mM, como estándar de referencia) y dimetilsulfóxido (DMSO, 0,1%, como control) en plaquetas humanas estimuladas con adenosin difosfato (ADP, 2 μM), epinefrina (EPI, 1 uM (uno micromolar)), colágeno (COLL, 1 μg/mL) y ácido araquidónico (AA, 0,2 mg/mL). Posteriormente, el estudio se enfocó en la determinación de la potencia antiagregante de la fracción mediante curvas de concentración - respuesta (desde 1 μg/mL hasta 0,4 mg/mL) para obtener los valores respectivos de pIC_{50} (-log CI_{50}). **Resultados**: En el grupo control se alcanzaron altos valores de agregación plaquetaria (96% AA, 89% EPI, 85% COLL, 77% ADP). La fracción de *N. amazonum* redujo significativamente la agregación (6% AA, 45% EPI, 10% COLL, 21% ADP), con valores próximos a los obtenidos con ASA (17% AA, 21% EPI, 10% COLL, 20% ADP). De acuerdo con las curvas de concentración – respuesta, los valores de pIC_{50} arrojaron el siguiente orden de potencia: AA, 4,90 > ADP, 4,51 > COLL, 4,33 > EPI, 3,85. **Conclusiones**: Estos resultados sugieren que la fracción etanólica de *N. amazonum* Nees ejerce efectos antiplaquetarios relacionados especialmente con la cascada metabólica del ácido araquidónico.

Palabras clave: *Lauraceae*, agregación plaquetaria, ácido araquidónico, adenosin difosfato, epinefrina, colágeno.

INTRODUCTION

Coronary artery disease has become a leading cause of morbimortality, not only in highly industrialised countries but also in several developing countries such as Colombia [1-3]. The progression of this disorder involves closely related complications such as unstable angina (UA), myocardial infarction (MI), stroke, sudden death and renal failure [4]. Behavioural changes like stopping smoking, increasing physical activity and consuming a healthy diet are key factors in reducing cardiovascular risk. When pharmacological treatment is necessary, the objective is the inhibition of thrombus formation on the atheromatous plaque in order to mitigate the progression of cardiovascular complications [5]. Natural products are a possible source of new active compounds that can help to ameliorate the impact of coronary artery disease, as alternatives to commercial drugs [6].

The family *Lauraceae* includes some species, such as *Ocotea quixos*, *Laurus nobilis* and *Cinnamomum tenuifolium* among others, that produce metabolites such as trans-cinnamaldehyde, cinnamtannin B-1 and isotenuifolide that have antiplatelet properties in screening assays [7-9]. These compounds act on diverse targets, including components of the thromboxane A_2, thrombin and adenosine diphosphate pathways that end in platelet adhesion and aggregation [7-9].

Nectandra amazonum Nees. (*Lauraceae*) has shown antiplatelet properties in experimental models. Chalcone derivatives isolated from this species display inhibition of cyclooxygenase I (COX-1) and bicyclooctane neolignan metabolites, selectively inhibiting the platelet-activating factor (PAF) [10-12]. The antiagreggant potency of these compounds varies from the micromolar to the nanomolar range. All of these compounds are found in the leaves of the plant and until now, there have been no studies on *N. amazonum* Nees. bark. This study focuses on effects of a fraction from the ethanolic extract of the bark of *N. amazonum* Nees. against adenosine diphosphate (ADP), epinephrine (EPI), collagen (COLL) and arachidonic acid (AA), agonists implicated in key pathways of platelet aggregation.

MATERIALS AND METHODS

Extraction and Fractionation

The material used in this study was collected from "El Zafire", a biological station in the Amazon region belonging to the "Universidad Nacional de Colombia, Sede Amazonas" (S 4° 3' 7"; O 69° 59' 54", Engineer Adriana Aguilar). A voucher specimen (COL 518189) has been deposited in the Colombian National Herbarium of the Institute of Natural Sciences, Bogotá, Colombia (Botanist Adolfo Jara Muñoz). The bark of *N. amazonum* Nees. was dried, milled, and then extracted with ethanol (96%) at room temperature by percolation. The ethanolic extract obtained was fractionated by column chromatography with toluene - isopropyl acetate (7:3) and the major fraction of the ethanolic extract of the bark of *N. amazonum* Nees. was used for the experiments.

Experimental protocol

The study applied the turbidimetric method described by Born. A modified photometer (aggregometer) measures changes in the light transmission of platelet rich plasma (PRP) after stimulation with an agonist of platelet aggregation [13]. Eighteen millilitres of blood was obtained from each antecubital vein of 16 healthy volunteers aged 18 to 45, who had not received any medication in the preceding two weeks. The blood was collected in vacuum tubes containing 3.2% sodium citrate in a ratio of nine to one. Platelet enriched plasma (PRP) was obtained by centrifugation of the whole blood for 10 min at 1500 rpm. Platelet poor plasma (PPP) was then obtained by further centrifugation for 10 minutes at 3500 rpm. The equipment was calibrated so that PPP gave a 100% transmittance value, maintaining the temperature at 37 °C.

In order to identify the antiaggregant effect, the *N. amazonum* ethanolic fraction (100 μg/mL), ASA (0.55 mM, reference agent) or DMSO (0.1%, vehicle, as control,) were incubated with PRP for 30 min. ADP (2 μM), EPI (1 μM), COLL (1 μg/mL) or AA (0.2 mg/mL) were then added. To determine the antiaggregant potency of the ethanolic fraction and calculate IC_{50} values, the above procedure was followed applying a range of ethanolic fraction concentrations ranging from 1 to 400 μg/mL.

Data analysis

Platelet aggregation was expressed as a percentage of the maximum change in light transmission during the test period. The value of 0–100% aggregation was obtained from the changes in light transmission values between PRP and PPP. Concentration - percentage response curves were obtained from sigmoidal regression analysis of the data. The criterion used to obtain IC_{50} values with confidence intervals was the presence of at least three data points between approximately 20% and 80% of the response. The antiaggregant potency was expressed as the negative logarithm of the IC_{50} value (pIC_{50}).

In this work the results are shown as mean ± standard error (SEM). The non-parametric Kruskal-Wallis test was chosen after performing tests of variance homogeneity and normal distribution (Bartlett's and D'Agostino-Pearson tests, respectively). Dunn's multiple comparison test was performed to identify significant differences. A p value ≤ 0.05 was assumed to be significant. The GraphPad PRISM® version 5.01 package was used for statistical analysis.

RESULTS

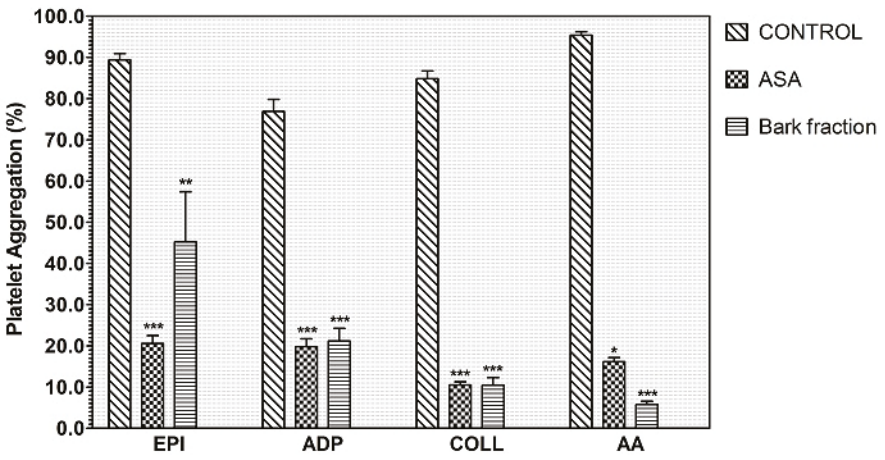

Figure 1. Percentage of platelet aggregation induced by *N. amazonum* bark fraction (100 μg/mL), acetylsalicylic acid (ASA 0.55 mM) and DMSO (control, 0.1%) in the presence of epinephrine (EPI, 1 μM), collagen (COLL1 μg/mL), adenosine diphosphate (ADP, 2 μM) and arachidonic acid (AA, 0.2 μg/mL). Each bar represents the mean ± SEM, n≥ 8. * p <0.05, ** p <0.01, *** p <0.001 compared with control.

The *N. amazonum* Nees. fraction significantly reduced the platelet aggregation induced by the four agonists tested as follows: AA: 6%, EPI: 45%, COLL: 10% and ADP: 21%, (Figure 1). The control group (0.1% DMSO) had high aggregation values (96, 89, 85 and 77%, respectively) whereas the reference agent, ASA, also markedly reduced the response (17, 21, 10 and 20%) (Figure 1).

According to the concentration – response curves and the IC_{50} values for the *N. amazonum* ethanolic fraction, the resulting pIC_{50} values indicated the following order of potency: AA 4.90> ADP 4.51> COLL 4.33> EPI 3.85. (Table 1, Figure 2).

Table 1. pIC_{50} values (negative logarithm of $IC_{50,}$ μg/mL) with fiducial limits ($p \leq 0.05$) generated by the bark ethanolic fraction of *N. amazonum* in the presence of epinephrine (EPI 1 μM), collagen (COLL1 μg/mL), adenosine diphosphate (ADP, 2 μM) and arachidonic acid (AA, 0.2 μg/mL).

Agonist	pCI_{50} [$-\log CI_{50}$, μg/mL]	CI_{50}[μg/mL]
EPI (1 μM)	3.85 [3.77 – 3.93]	14.2 [11.8 – 17.1]
COLL(1 μg/mL)	4.33 [4.29 – 4.36]	4.70 [4.35 – 5.08]
ADP (2 μM)	4.51 [4.43 – 4.58]	3.12 [2.61 – 3.74]
AA (0.2 μg/mL)	4.90 [4.85 – 4.95]	1.26 [1.13 – 1.41]

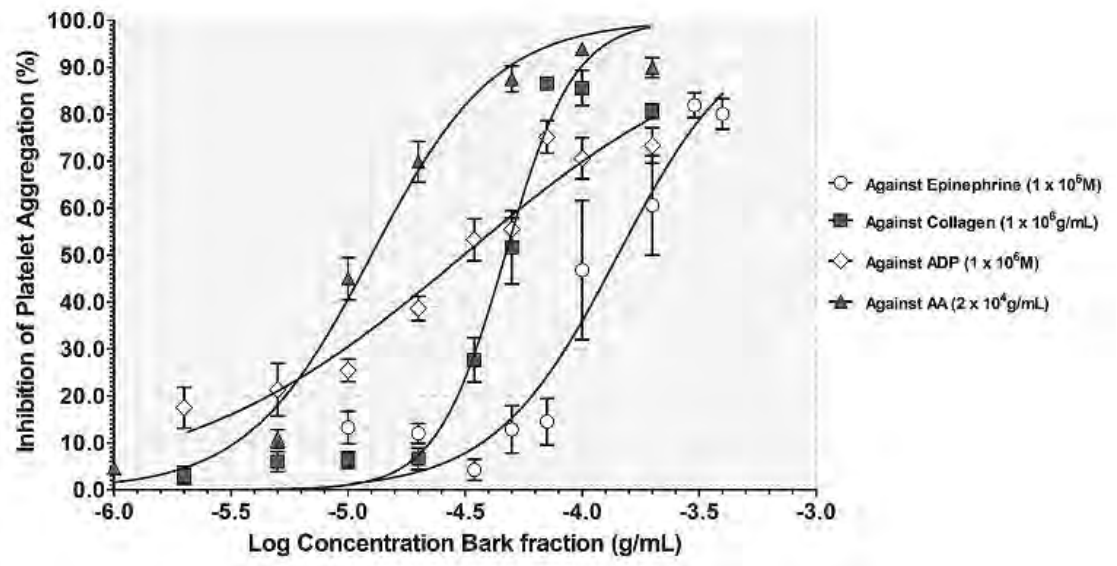

Figure 2. Percentage inhibition of human platelet aggregation induced by *Nectandra amazonum* bark fraction (1 – 400 μg/mL) in the presence of epinephrine (EPI, 1 μM), collagen (COLL, 1 μg/mL), adenosine diphosphate (ADP, 2 μM) and arachidonic acid (AA, 0.2 μg/mL). Each point represents the mean ± S.E.M. $n \geq 8$.

DISCUSSION

The results show that an ethanolic fraction from the bark of *N. amazonum* displays antiplatelet effects against the aggregation inducers EPI, COLL, ADP and AA suggesting that it has a broad-spectrum activity. However, given the different values of antiplatelet potency against the different aggregation inducers, the data suggest that the active metabolites of the bark extract could mainly inhibit mechanisms associated with the arachidonic acid pathway. In that case, because AA is a precursor of thromboxane A2 (TXA_2) autacoid, the *N. amazonum* bark fraction could be exerting modulatory effects on this key cascade of platelet aggregation. The activity might occur via inhibition of AA synthesis, by blocking TXA_2 receptors, by affecting an intermediate between them or by acting at the post TXA_2 receptor level. Further studies should be performed to clarify where the inhibition occurs.

The aggregation pathway mediated by arachidonic acid is such a key factor in thrombus formation that a therapeutic agent like acetylsalicylic acid, a selective inhibitor of the COX-1 platelet enzyme, has been able to reduce the risk of cardiovascular morbidity and mortality related to atherothrombotic disorders, at least in secondary prevention [14]. New alternative agents, including those obtained from natural sources, could aid the therapeutic management of atherothrombotic disease. According to the results of the present study, the bark of *N. amazonum* may contain active metabolites with antiplatelet activity that act at some level of the TXA_2 pathway. In fact, chalcone and dihydrochalcone related compounds isolated from leaves of *N. amazonum* display selectivity towards COX-1 inhibition in the range 1.6 – 36.1 μM [10]. Further studies on the bark of this species will determine the bioactive metabolites and their mechanisms of actions [10, 11].

The antiaggregant potency of the bark fraction of *N. amazonum* is rather modest (IC_{50}: 1.13 – 1.41 μg/mL) but phytochemical bioassay-guided fractionation of this fraction could eventually lead to the isolation of more potent active metabolites. In fact, *Kadsurenone,* a neolignan compound also isolated from the leaves of *N. amazonum,* inhibits the platelet activated factor (PAF) in the range 0.13 – 0.17 nM [11]. In any case, a marked antiaggregant potency would not be desirable because it would increase the risk of bleeding events [15].

Previous phytochemical analysis from *N. amazonum* has shown the presence of tannins, steroids, triterpenes, coumarins and flavonoids, although terpene lactones, neolignans and chalcone-related compounds seem to be the main source of the antiplatelet metabolites [10-12]. According to the results of the present work, active compounds are also present in the bark fraction of this species. Given that *N. amazonum* seems to be a source of several bioactive antiaggregant metabolites acting through different pathways of platelet activation, there are good reasons to propose this species as a probable source of phytotherapeutic agents.

CONCLUSIONS

The bark fraction of *N. amazonum* Nees. exerts antiaggregant effects in human platelets through signalling pathways related mainly to the inhibition of arachidonic acid. Further studies are required to identify its active metabolites and clarify its mechanism of action.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest in this research.

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