ARTÍCULO ORIGINAL

Antiplatelet but not anticoagulant activity of *Morinda citrifolia* L. leaf extracts

Actividad antiagregante plaquetaria pero no anticoagulante de extractos de *Morinda citrifolia* L

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ABSTRACT

Introduction: *morinda citrifolia* L (Noni) is a medicinal plant species that has gained popularity among Cuban population, suggesting the need to characterize the pharmacological activities of noni preparations developed in Cuba.
Objective: assessing the effects of Noni leaf and fruit extracts on platelet aggregation and blood coagulation, as well as the possible influence of plant age, leaf maturity and extract total phenol and flavonoid contents on it.
Methods: green and yellow leaves and light yellow fruits were collected from three- and six- year old Noni trees*.* Leaf (30 g/100 mL w/v) and fruit (100 g/100 mL w/v) extracts were prepared by maceration in 50 % alcohol and water respectively. The concentrations of total soluble solids (TSS), phenolic compounds (PHEN) and flavonoids expressed as quercetin (FLV) in the extracts were determined. The *in vitro* effects of leaf (0,4 and 0,8 mg TSS/mL) and fruit (0,6 and 1,2 mg TSS/mL) extracts on ADP-induced platelet aggregation (PA), prothrombin time (PT) and partial activated thromboplastine time (PATT) in human plasma was determined. Furthermore, the *ex vivo* effects of a leaf extract (42 and 378 mg TSS / kg i.p. and 630 mg/kg p.o.) on these variables of rat plasma were assessed.
Results: fruit extracts showed higher TSS, but lower PHE and FLV values than leaf extracts. Leaf but not fruit extracts inhibited ADP-induced PA that was independent on

plant age and leaf maturity. A significant reduction of PA was *ex vivo* induced by a leaf extract in rats. TP and PATT were not modified during *in vivo* or *ex vivo* experiments.
Conclusion: noni leaf hydro-alcohol extracts developed in Cuba have antiplatelet but not anticoagulant effect.

Key words: *Morinda citrifolia* L. (Noni), medicinal plant, platelet aggregation, blood coagulation, flavonoid, Cuba.

RESUMEN

Introducción: *morinda citrifolia* L (Noni) ha ganado popularidad entre la población cubana, lo que sugiere la necesidad de caracterizar farmacológicamente las preparaciones de noni desarrolladas en Cuba.
Objetivo: evaluar los efectos de extractos de hojas y frutos de Noni sobre la agregación plaquetaria y la coagulación sanguínea, así como la influencia de la edad de la planta, la madurez de la hoja y los contenidos de fenoles totales y flavonoides.
Métodos: hojas verdes y amarillas y frutos ligeros amarillos fueron recolectados de plantas de noni de tres y seis años de edad. Extractos de hojas (30 g/100 mL w/v) y frutos (100 g/100 mL w/v) estuvieron preparados por maceración en alcohol al 50 % y agua, al respecto. Las concentraciones de sólidos totales (TSS), compuestos fenólicos (PHEN) y flavonoides expresados como quercetina (FLV) fueron determinadas. Los efectos de los extractos de hojas (0,4 y 0,8 mg TSS/mL) y de frutos (0,6 y 1,2 mg TSS/mL) sobre la agregación plaquetaria (PA) inducida por ADP, el tiempo de protrombina (PT) y el tiempo parcial de tromboplastina parcial activado (PATT) fueron determinados en plasma humano *in vitro* y los efectos de un extracto de hojas (42 y 378 mg TSS/kg i.p. y 630 mg/kg p.o.) sobre estas variables de plasmas de ratas fueron evaluados *ex vivo*.
Resultados: los extractos de frutos tuvieron mayor contenido de TSS pero menores de PHEN y FLV que los de hojas. Estos últimos inhibieron la PA *in vitro* en plasma humano independiente de la edad de la planta y la madurez de las hojas. Un extracto de hojas indujo significativa reducción de PA *ex vivo* en ratas. TP y PATT no fueron modificados en ninguna situación experimental.
Conclusión: extractos hidroalcohólicos de hojas de Noni desarrollados en Cuba tienen efecto antiagregante plaquetario pero no anticoagulante.

Palabras clave: *Morinda citrifolia* L. (Noni), planta medicinal, agregación plaquetaria, coagulación sanguínea, flavonoide, Cuba.

INTRODUCTION

Morinda citrifolia Linn. (Noni) which belongs to the Rubiaceae family, has been traditionally used by Polynesian healers to treat different kinds of illnesses[1,2] and has gained popularity among Cuban population during the recent years.
The influence of cultivation conditions on chemical composition of noni fruits that could affect its biological properties has been demonstrated,[3] suggesting the need to characterize the pharmacological potentials of noni preparations developed in Cuba.

The antiplatelet activity of rutin,[4] one of the major quercetin derivatives identified in noni fruits and leaves,[2] could be present in products derived from these parts of this plant species.[4] Furthermore, the inhibition of phospholipase A2 activity by a methanol extract of *M. citrifolia* fruits[5] and the reduced cyclooxygenase 1 (COX 1) activation in the presence of a noni juice or the hydro-alcohol extracts of noni leaves or fruits[6] suggest that these preparations could bring antiplatelet potentials that have not been evaluated. Nevertheless, a contradictory noni inhibitory effect on the anticoagulant/antiplatelet drug group efficacy has been reported.[7] Therefore, the aim of the present study was to assess the effects of leaf and fruit extracts from *M. citrifolia* plants grown in Cuba on platelet aggregation and blood coagulation, as well as the possible influence of plant age, leaf maturity and total phenol and flavonoid contents on it. Aqueous and not hydro-alcohol fruit extracts were assessed in this work because it is the way this part of the plant is usually consumed in traditional medicine.[1,2]

METHODS

Plant material

Green and yellow (mature) leaves and light yellow (unripe) fruits of were collected from three- and six - year old noni trees grown in a fruit farm (Unidad Básica de Producción Agropecuaria, La Vereda, La Lisa, Havana, May, 2006), with 25 m separation between each other and under natural agronomic treatment. Voucher samples of leaves and fruits (No.85695 and 85696, respectively) were deposited at the Herbarium of the National Botanic Garden of Havana, Cuba. Plant materials were collected early in the morning, free of microbial contamination and without affecting the ecosystem.

Preparation of noni leaf and fruit extracts

The extraction procedures were developed in "Mario Muñoz" Pharmaceutical Laboratory. A 30 % (w/v) extract was prepared. Ground fresh *M. citrifolia* leaves (450 g) were macerated with 50 % ethanol (1.5 L) at room conditions during 15 days. Afterwards, the supernatant was separated and stored in a closed dark bottle at 8 to 10 °C during 7 days followed by paper filtration. Rota-evaporation (at 40 °C and 27 mmHg reduced pressure) of the homogeneous liquid obtained was performed for ethanol elimination from it in order to avoid possible bias of the results of the pharmacological evaluations. The remaining liquid was stored in a closed dark bottle at 4 to 8 °C.

Fresh unripe fruits were kept in a close flask at room conditions to complete natural maturation (4-5 days). Afterwards, they were mashed, the seeds discarded and a 100 % (w/v) extract prepared from the pulp (2 Kg) macerated with deionized water (2L) at 8 a -10 °C for 14 days , followed by vacuum filtration.
The supernatant was kept at 8 a -10 °C during 7 days, paper filtrated and the homogenous liquid obtained was stored in closed a dark bottle at 4 to 8 °C.

Identification of Noni Leaf and fruit extracts

A: Green leaves from a three year old noni plant.

B: Green leaves from a six year old noni plant.

C: Yellow leaves from a three year old noni plant.

D: Yellow leaves from a six year old noni plant.

E: Fruits from a three year old noni plant.

F: Fruits from a six year old noni plant.

Determination of Total soluble solids (TSS) content: it was gravimetrically determined in dried 1 mL aliquots of each extract (N = 5) by using a Sartorius MA40 balance and was expressed as g/100 mL.

Total phenol content (PHE): it was estimated by the tungstate-molybdic-phosphoric acid method[8](N= 3) and expressed as mg/100 mL.

Total flavonoid content (FLV): it was estimated by the spectrophotometric method for quantification of total flavonoids.[9] Chromatographic grade quercetin was used as standard. Results were expressed as mg quercetin/100 mL.

Preparation of human platelet suspensions

Human blood samples were taken from adult subjects of both genders who were participating in a routine health study at the National Institute of Angiology and Vascular Surgery and gave their informed consent. All of them denied having consumed any drug with known antiplatelet activity for at least 2 weeks before the phlebotomy. Human venous blood was obtained from an antecubital vein using a syringe with a 19 gauge needle and a tourniquet. The collected blood was anticoagulated with 3.8 % (w/v) trisodium citrate (1 volume to 11 volumes of blood) and centrifuged at 150 x *g* for 10 min at room temperature. After isolation of supernatant (platelet-rich plasma; PRP), the remaining plasma was re-centrifuged at 1000 x *g* for 20 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to about 2.5×10^8 platelets/mL by dilution with autologous PPP. Both PRP and PPP were used within 3 h after preparation.

Platelet Aggregation (PA) in human PRP

Adenosine diphosphate (ADP) was obtained from CPM (CPM S.A.S, Rome, Italy) and prepared according to the instructions provided. The turbidimetric method [10] was applied to measure platelet aggregation, using a CLOT 2S Aggregometer (Seac and Radim Group, Rome, Italy).

The concentration of noni leaf and fruit extracts reported with COX 1 inhibiting activity (3,4 mg/mL)[6] was a reference information for *in vitro* experiments. Herb preparations were always tested at the highest concentrations attainable in the incubation medium according to their TSS contents expressed as mg/mL.

Five and ten microliters of leaf extracts (0.4 and 0,8 mg TSS/mL in the incubation medium respectively) and fruit extracts (0.6 and 1, 2 mg TSS/mL in the incubation medium respectively) or water (control) were added to 280 μL aliquots of PRP in Aggregometer cuvettes. Successively, 15 μL of ADP (5 μmol/L in the incubation medium) were added after 2 min of pre-incubation at 37 °C. Platelet aggregation

was monitored for 5 min. The results are expressed as percentages of aggregation as provided by the instrument. The percentage inhibition of platelet aggregation was calculated as follows: percentage inhibition (%) [1 - (Platelet aggregation of sample/platelet aggregation of control)] × 100 %. Each sample was measured in triplicate. IC50 values (the concentration necessary to reduce the induced platelet aggregation by 50 % with respect to control) were obtained from concentration-effect curves.

Preparation of rat platelet suspensions

Animals. Male Wistar rats (250-300 g) were obtained from the National Center for Laboratory Animals (Havana, Cuba). The animals were housed in a controlled environment, and free access to feed and water was allowed.

Rat treatment and PRP preparation

C extract was selected to assess its effect on rat platelet activation since it showed the highest (though not statistically) levels of total phenols and flavonoids (table 1). C (test groups) or water (control group) was randomly given to rats (1 mL/100 g b.w.) by intraperitoneal (42 and 378 mg TSS/kg) or intra-gastric (630 mgTSS/kg) via after an 18-hour fasting period. These doses corresponded to 1,26; 11,34 and 18,9 mg flavonoid/kg, respectively and were selected according to a report of quercetin *ex vivo* antiplatelet effect on platelets from volunteer subjects who consumed 150 and 300 mg of this flavonoid,[11] that are equivalent to 2,14 and 4,28 mg/kg, considering an average 70 kg b.w for adult humans. Thus, C doses were about 0,3 (i.p. via); 2,6 and 4,4 (intragastric via) times the highest dose of quercetin ingested by humans.[11] The known drug resistance of rats with respect to human beings was taken into account.

Table 1. Total soluble solids (TSS), phenolic compounds (PHE) and flavonoid (FLV) contents in *Morinda citrifolia* L. extracts

	Morinda citrifolia L. extracts					
	A	B	C	D	E	F
TSS (g/100 mL)	2.09 ± 0.20	1.88 ± 0.19	1.62 ± 0.08	1.61 ± 0.09	3.70 ± 0.04	3.50 ± 0.25
PHE (mg/100 mL)	297.99 ± 10.97	309.32 ± 17.44	327.37 ± 24.21	310.93 ± 6.00	179.42 ± 3.43	160.71 ± 1.47
FLV (mg quercetin/100 mL)	31.00 ± 13.77	40.04 ± 10.48	50.93 ± 8.25	41.56 ± 0.24	13.58 ± 0.50	12.,09 ± 2.19

Data are expressed as the media ± DS

The animals were anesthetized with ether two hours later the intraperitoneal or intra-gastric treatment. Blood (9 volumes) was withdrawn from the cava vein using a syringe with a 20 Gx 1 ½ gauge needle. It contained 3.8 % (w/v) trisodium citrate (blood/anticoagulant = 9/1). Anticoagulated blood was poured into a plastic tube and centrifuged at 100 x *g* for 5 min at room temperature. After isolation of the PRP, the remaining plasma was re-centrifuged at 1000 x *g* for 20 min to obtain the PPP.

Platelet Aggregation in rat PRP

Twenty six microliters of ADP (5 µmol/L in the incubation medium) were added to 400 µL aliquots rat PRP in Aggregometer cuvettes and aggregation was followed by five minutes. Results were obtained and expressed as explained above.

Determination of prothrombin time (PT) and partial activated thromboplastine time (PATT) in human and rat plasma

Kits for PT and PATT measurements were obtained from CPM (CPM S.A.S, Rome, Italy) and used according to the instructions provided for manual determinations.

Human PPP (100 µL) was pre-incubated for 2 min with 10 µL of A, B, C, D (0, 8 mg TSS/mL), E and F (1, 2 mg TSS/mL) or water (control) in a water bath at 37 ˚C for *in vitro* experiments. Afterwards, PT or PATT stimulating reagent (100 µL) was added, the clot formation visually followed and the time needed for it manually recorded with a chronometer. The results are expressed as a relation = time (seconds) of extract/time (seconds) of control.

PPP from C administered or control rats (see above) were used to determine C *ex vivo* effect on PT and PATT. The procedure was performed as explained in the previous paragraph. The results are expressed in seconds.

Statistical Analysis

Data of TSS and FLV are reported as the means (SD) of five and three determinations, respectively. Experimental results are expressed as the means (SEM and are accompanied by the number of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared with those for controls by the Student's *t* test, and *p* values of <0.05 were considered to be statistically significant.

Ethics

All procedures described were carried out using a protocol approved by the Institutional Research Ethics Committee of the National Institute of Angiology and Vascular Surgery, according to the national and international guidelines for the human use of laboratory animals.

RESULTS

TSS, PHE and FLV contents in *M. citrifolia* leaf and fruit extracts

As can be seen in the table 2, there were not statistical differences of the absolute values of TSS, PHE and FLV contents and percentages of PHE and FLV with respect to TSS and FLV related to PHE neither between leaf extracts (A, B, C and D) nor between fruit extracts (E and F). Fruit extracts showed higher TSS, but lower PHE and FLV values than leaf extracts (table 1), consequently, its percentages of PHE and FLV with respect to TSS and the percentages of FLV related to PHE were lower too (table 2).

Table 2. Percentages of phenol and flavonoid contents with respect to total soluble solids and flavonoid with respect to phenol content in *Morinda citrifolia* L. extracts

	Morinda citrifolia L. extracts					
	A	B	C	D	E	F
PHE/TSS (%)	19.3	17.0	20.2	19.4	9.9	9.6
FLV/TSS (%)	2.6	2.1	3.0	3.1	0.37	0.40
FLV/PHE (%)	10.9	12.9	15.6	13.4	7.6	7.5

In vitro effect of *M. citrifolia* leaf and fruit extracts on ADP-induced platelet aggregation in human plasma

Leaf but not fruit extracts inhibited ADP-induced PA in a concentration-dependent manner. There was not a statistical difference between the percentages of inhibition induced by the highest concentration of extract tested (p = 3983, ANOVA).
IC5o values were 0,505; 0, 46; 0,505 and 0, 55; mg/mL for A, B, C, D, respectively (table 3).

Ex vivo effect of a *M. citrifolia* leaf extract on ADP- induced platelet aggregation in rat plasma

There was not statistical differences (p = 0. 7375, Student´s t test for independent data) between the means of the percentages of PA in the group of rats treated with C 42 mg/kg i.p. (N = 10) and control (N = 32; % PA = 67. 7 ± 4.3).
The administration of 378 mg/kg i.p. was able to significantly reduce PA to 28.5 ± 5.1 (p = 0.0009), corresponding to 58 ± 6.5 % inhibition, while an oral dose of 630 mg/kg (N = 10) induced a lower but statistically significant decrease of PA to 45.0 ± 2,2 % with respect to the control group (p = 0.014, Student´s t test for independent data), corresponding to 35.5 ± 3.7 % inhibition.

Table 3. *In vitro* effect of *M. citrifolia* leaf and fruit extracts on ADP-induced platelet aggregation in human plasma

	Morinda citrifolia L. extracts					
	A	B	C	D	E	F
Conc. 1 [a]	32.8 ± 9.4	41.7 ± 7.8	42.0 ± 5.5	5.0 ± 5.9	49.0 ± 7.1 n.s.	15.8 ± 7.1 n.s.
Conc. 2 [b]	85.8 ± 4.2*	90.7 ± 2.4*	70.8 ± 10.7*	95.0 ± 2.2*	27.5 ± 5.3 n.s.	29.2 ± 5.6 n.s.

0,4 mg TSS/mL for A,B,C and D; 0,6 mg TSS/mL for E and F [a]
0,8 mg TSS/mL for A,B,C and D; 1,2 mg TSS/mL for E and F [b]
The data ate the percentages of inhibition of platelet aggregation (mean ± esm, N = 5)
p < 0,05*; n.s = non statistical difference (Student t test for paired data)

In vitro effect of *M. citrifolia* leaf and fruit extracts on coagulation times in human plasma

The relation of the coagulation times (PT and PATT) with respect to control after incubation of human plasma with yellow or green leaf extracts (1.1 mg/mL) or fruit extracts (1.7 mg/mL) around 1.0 in all cases that means no effect (table 4).

Table 4. *In vitro* effect of *M. citrifolia* leaf and fruit extracts on coagulation times in human plasma

	Morinda citrifolia L. extracts					
	A	B	C	D	E	F
	0.8 mg TSS /mL				1.2 mg TSS /mL	
TP	1.02 ± 0.02	0.97 ± 0.01	0.99 ± 0.01	1.04 ± 0.02	0.98 ± 0.01	1.08 ± 0.01
TATT	1.06 ± 0.01	0.93 ± 0.01	1.0 ± 0.02	0.98 ± 0.02	1.23 ± 0.01	1.11 ± 0.01

The data ate the relations extract time/control time, (mean ± e.s.m, N = 5)

Ex vivo effect of a *M. citrifolia* leaf extract on coagulation times in rat plasma

There were not statistical variations of the coagulation times of rats treated with extract C 378 mg/kg i.p with respect to the control group (PT = 12.1 ± 0.6 s *vs* 12.4 ± 0.5 s; P = 0.7203 and PATT = 24.1 ± 1.3 s *vs* 25.8 ± 0.7 s; P = 0.2640). Extract C 630 mg/kg p.o was ineffective too (PT = 13.5 ± 0.4 s *vs* 12.4 ± 0.5 s; P = 0.0808 and PATT = 25.3 ± 0.9 s *vs* 25.8 ± 0.7 s; P = 0.7022).

DISCUSSION

This investigation has demonstrated the inhibition of ADP-induced platelet aggregation in human plasma *in vitro* as well as in rat plasma *ex vivo* by leaf hydro-alcohol extracts but not fruit aqueous extracts from noni plants grown in Cuba. Flavonoids, mainly a quercetin derivative like rutin, may have contributed to with its antiplatelet activity,[4] since they were present in higher quantity in leaf extracts. However, other polyphenol compounds could be involved too.

Prothrombin and partial activated thromboplastine times are classical and sensitive methods for detecting any faults of coagulation factors activities. They were unaffected in the concentrations and doses of the extracts used in this work, thus it not support any influence of noni products on this hemostatic events.

These study findings could be reference for the development of *M. citrifolia* products in Cuba the future. For instance, though the relation fruit/solvent was higher than the proportion leaf/ solvent, fruit extracts had lower phenol and flavonoid contents and were ineffective on platelet aggregation. It could be a consequence of the use of water in the manufacture process, since mixtures of ethanol and water, between 50 and 75 %, have been shown to be effective in the extraction of flavonoids.[12-14] Therefore, some modifications of the procedure used for fruit extract production could be convenient to develop an antiplatelet product from noni fruits.

The practical relevance of the lack of pharmacological activity showed by the extracts of this part of the plant is that it suggests that the probability of a reduction of platelet reactivity after fruit aqueous preparations intake is low and the increase of bleeding hazard for people on hypo-coagulability states should not be expected.

The demonstration that the antiplatelet effects of leaf extracts *in vitro* were independent on noni plant age and leaf maturity and that this pharmacological activity was induced by single intraperitoneal and intragastric administrations to rats is crucial for future studies aimed to the pharmacological characterization and standardization of a new natural platelet inhibitor and determine the possible bleeding risk associated to the intake of noni products.

This research has provided *in vitro* and *ex vivo* evidence of the antiplatelet but not anticoagulant activity of *M. citrifolia* leaf hydro-alcohol extracts developed in Cuba.

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