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Artículos

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Bioquímica

Urinary Elimination of Ortho-substituted Benzene in Rats Taking *Bauhinia megalandra* Leaves Aqueous Extract and Changes on glycaemia and glycosuria

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The intestinal absorption of plant secondary metabolites is required in order to exert effects on intermediary metabolism. Male rats received an aqueous extract of *Bauhinia megalandra* leaves (experimental) instead of drinking water (control). Their urine was subjected to differential solubility and column chromatography on silica gel; from this, a compound was partially purified, its substitution pattern, established by NMR of ^{1}H and ^{13}C, corresponds to benzene ortho-substituted. The compound was not present in the plant extract nor in the urine of the controls; in consequence do we suggest that it is the product of the metabolism of a secondary metabolite present in *B. megalandra* leaves. The rats that received the plant extract showed a discrete reduction in glycaemia of 9 %, statistically significant, and a 3.6-fold increase in the amount of glucose eliminated in the urine, which was interpreted as a renal glycosuria. The implications of these results are discussed.

Palabras Claves:*Bauhinia megalandra*; Benceno orto-substituido; glicemia; glicosuria.

Title

Eliminación Urinaria de Benceno Orto-substituido en Ratas Que Ingieren un Extracto Acuoso de Hojas de *Bauhinia megalandra* y Cambios en la Glicemia y en la Glicosuria

Abstract

La absorción intestinal de los metabolitos secundarios presentes en las plantas es un requisito para que los mismos puedan ejercer un efecto en el metabolismo intermediario. Ratas macho recibieron un extracto acuoso de las hojas de *Bauhinia megalandra* en lugar del agua de bebida (grupo experimental). El grupo control solo recibió agua. La orina de ambos grupos de animales fue sometida a solubilización diferencial y cromatografía en silica gel, a partir de lo cual, se purifico parcialmente un compuesto cuyo patrón de substitución, establecido por RMN de ^{1}H y ^{13}C, corresponden a un benceno orto-substituido. Dicho compuesto no estaba presente en el extracto de las hojas de la planta ni en la orina de las ratas controles, en consecuencia nosotros sugerimos que corresponde al metabolismo de un metabolito secundario presente en las hojas de *B. megalandra.* Las ratas que recibieron el extracto acuoso de la planta mostraron una reducción de la glicemia de un 9 %, la cual fue estadísticamente significativa y un in cremento de 3,6 veces en la eliminación urinaria de glucosa lo cual fue interpretado como una glucosuria renal. Se discuten la implicaciones de estos resultados.

Key Word

Bauhinia megalandra, Benzene ortho-substituted; Glycaemia; Glycosuria.

Introduction

The empirical treatment of different diseases with plant extracts is of worldwide distribution [1,2]. There are a large number of compounds present in vegetable matter, the flavonoids being an

important group among them. The flavonoids are polyphenols that constitute part of the non-energetic diet of humans [3]; they are absorbed at the intestine level, metabolized, and later on, eliminated by the bile, feces and/or urine as degraded products. Estrada *et al.* [4] have reported the presence of several flavonoids in *B. megalandra* leaves.

The digestion and intestinal absorption of flavonoids greatly depend on their chemical characteristics in particular on the presence of sugars and in their esterification with different acids [5,6]. In general the flavonoids are deglycosylated before being absorbed although some of them can get intact into the enterocyte and once into the cell can undergo chemical modifications such as: methylation, sulphation and/or glucuronidation [7], via the circulation they reach the liver where they are further metabolized [3]. The flavonoids that are not absorbed by the small intestine reach the colon, where they can be metabolized by the intestinal bacteria producing more simple phenolic compounds such as: acids phenylacetic, phenylpropionic, hydoxibenzoic and lactones[8]. The absorbed polyphenol compounds can reach the liver, may go back to the intestine, by the bile, as a different chemical from that ingested and eliminated in the feces. Some flavonoids or their metabolites can reach the kidney and are eliminated by the urine.

At present, there are not evidences of the intestinal absorption of the secondary metabolites present in *B. megalandra* leaves aqueous extract; in consequence we investigate the appearance of compounds present in the urine of rats that drink *B. megalandra* leaves aqueous extract that were not present in the urine of control animals in order to show a direct evidence of the intestinal absorption of the compounds present in the plant extract.

Materials and methods

Vegetal material. - Fresh *B. megalandra* leaves were collected at the Campus of the Universidad Central de Venezuela during May 2012, the plant was identified by Dr. S. Tillett and a specimen was deposited at the Herbarium “Manuel Ovalles” Faculty of Pharmacy of the Universidad Central de Venezuela under the number: MYF29251. The washed and dried leaves were cut into small pieces, 5 ml of distilled water were added per g, and the whole brought to the boil, cooled, then filtered and stored at -20º C until their use.

Animals and their treatment. - Sprague Dawley male rats of 200 ± 20 g were used, they were divided into 2 groups and kept in metabolic cages; the control group having free access to water and food and the experimental group receiving, during 7 consecutive days, the *B. megalandra* leaves extract, prepared as described above, in place of water, and received the same type of food as the control group. The urine of both groups of rats was collected, during the same 7 consecutive days, and stored at – 20º C until use.

Urine treatment. – Following Zhao *et al*. [9], the urines of both groups of animals were acidified with HCl (0.274 M) and 5 volumes of ice-cold acetone were added; later they were centrifuged at 1000 g for 15 min. at 4º C, the resulting precipitate was discarded and the supernatant after drying was stored at -20º C. An aliquot of the plant extract was treated with the same procedure.

The solid material obtained from the supernatant was fractionated using: chloroform, dichloromethane, acetone, ethanol, methanol and water respectively. For that, the solid material was resuspended in 100 ml of the solvent, sonicated (MSE, England) centrifuged at 2500 g for 15 min. at 4º C, the supernatant corresponding to each solvent fraction being dried and the precipitate treated with the next solvent using the same procedure. Each solvent fraction was dried using a rotatory evaporator Yamamoto (Japan) or a lyophilizer Virtis (USA). The solvent fractions were analyzed using TLC on silica gel 60 with fluorescence indicator UV_{254}, the mobile phase was dichloromethane-methanol (8:2; v/v), once the plates were dried, they were observed under a UV/Vis lamp or developed with ceric sulphate.

The acetone fractions of the urine of the experimental rats, showed a spot under TLC that was not present in the urine of control animals nor in the *B. megalandra* leaves extract (see results): in consequence we decided to fractionate it.

Treatment of the acetone fraction. – When the dry acetone fraction was resuspended in 15 ml of acetone a precipitate was formed which was discarded by centrifugation as above, due to the fact that the compound of interest was in the soluble fraction. When this fraction was dry some crystals were formed, extracted with hot acetone (45º C) and separated by filtration, again the compound of interest was in the soluble fraction which was dried at ambient temperature.

The last fraction of the above procedure was resuspended in a small volume of methanol, mixed with a small amount of silica gel, dried at room temperature and seeded on top of a dry silica gel chromatographic column and the elution was performed using dichloromethane-methanol (8:2; v/v) and 26 fractions of 2 ml were obtained. By TLC it was shown that the compound of interest was in fractions 17-23 which were combined, dried at room temperature, resuspended in dichloromethane, centrifuged as above and the precipitate washed with acetone in the same

way. The precipitate of acetone was resuspended in methanol, centrifuged as above and the compound of interest was in the soluble fraction, which by concentration at room temperature yielded a yellow-brown solid. This solid was resuspended in 5 ml of methanol, centrifuged as above, the precipitate was discarded and the supernatant constituted the Fr_{17-23}, fraction enriched with the compound of interest and mixed with urea.

Spectroscopic methods. – The ^{1}H and ^{13}C NMR of Fr_{17-23} were carried out using a JEOL spectrometer, Eclipse 270 model with an application camp of 270 MHz for ^{1}H and 67.5 MHz for ^{13}C and a spectrometer Bruker ^{1}H (500 MHz) and ^{13}C (125 MHz).

Changes in glycaemia and glycosuria during the administration of the *B. megalandra* leaf extract. - To a control group of animals and other group of treated rats similar to those used in the previous experiment (b) food was withdrawn for 24 h., the urine was collected during that time and a sample of the tail blood was taken. In the urine and blood the glucose was measured by the glucose oxidase-peroxidase method [(10)].

g. All experiments were carried out following the ethical criteria for experimental animals of the Institute of Experimental Medicine of the Medicine Faculty of the Venezuela Central University.

Results

Identification of the compound eliminated by the urine. - The fraction soluble in acetone from the urine of the experimental animals showed a blue-gray spot at 365 nm in TLC with an Rf of 0.28 that was not present in the urine of the control rats nor in the *B. megalandra* leaves aqueous extract. The blue-gray compound was partially purified by using column chromatography and differential solubilization and was partially identified by ^{1}H and ^{13}C NMR.

In the ^{13}C spectrum (Table 1) six signals between 122 and 110 ppm were observed and they were assigned to benzene carbons being two of them probably quaternary. Furthermore signals were observed at: 175.94 ppm that may correspond to a carboxylic acid, ester or amide; 162.29 ppm that correspond to the carbonyl carbon of the urea; a signal at 62.12 ppm and three signals of aliphatic carbons at 24.56, 19.67 and 13.10 ppm.

In the ^{1}H spectrum (Table 1) many signal were observed, however certain of them are of interest: four signals at low field, coupled between them at 7.7 and 6.9 ppm that correspond to aromatic protons and define the substitution pattern AA'BB'. Additionally, a broad and intense singlet was observed at 5.7 ppm that corresponds to the urea protons. At high field a triplet was observed at 1.23 ppm (J= 7.2 Hz) that is coupled to a quartet in 4.09 (J= 7.2 Hz) that defines as an ethoxy (EtO-), one of the substituent groups.

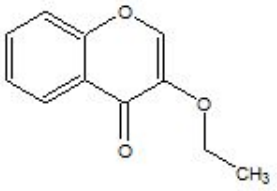
Table 1

Major signals observed for Fr_{17-23} in ^{1}H and ^{13}C NMR (270 MHz, MeOD-$_{d4}$).

Carbon number	δ ^{1}H (ppm)	δ ^{13}C (ppm)
1		175,94
2	5,71 *sa*[a]	162,29[b]
3	7,28 (*dd*; 8 and 2 Hz)	121,34
4	6,98 (*td*; 8 and 2 Hz)	118,63
5	7,07 (*td*; 8 and 2 Hz)	117,64
6		114,53
7		114,35
8	7,62 (*dd*; 8 and 2 Hz)	110,95
9	4,09 (*q*; 7,2 Hz)	62,12
10	NA[c]	24,56
11	NA[c]	19,67
12	1,23 (*t*; 7,2 Hz)	13,10

[a] urea protons ; [b] urea carbonyl; [c] NA= not assigned

The coupling pattern in the aromatic region of the ^{1}H NMR coincides with a benzene ortho-substituted and it is explained thus: two doublets of doublets centered at 7.62 and 7.28 ppm with a coupling ortho-meta (*J*= 8; 8 and 2 Hz). Additionally, a singlet at 7.9 ppm was observed, possibly these signals can be attributed to an aromatic system poly-substituted. A possible structure that fits the majority of the NMR signals could be:



Changes in glycaemia and glycosuria during the administration of the *B. megalandra* leaf extract. – As it can be observed in Table 2 the administration of the plant extract produces a decrease of approximately 9 % in the glycaemia which was statistically significant at $p< 0.01$. On the other hand, the 24 h. glycosuria increases by approximately 3.6 times this being statistically significant at $p< 0.005$

Table 2

Changes in glycaemia and glycosuria during the ingestion of *B. megalandra* aqueous leaves extract

Sample (n)	Glycaemia (mg/dL)	Glycosuria (mg/24 h)
Control (8)	77,31 ± 5,21	2,98 ± 0,23
Experimental (6)	70,26 ± 1,17	10,49 ± 1,20

The experimental rats received *B. megalandra* aqueous leaves extract in place of drinking water for 7 days. During a 24 h fast period the urine was collected, after which a sample of blood was taken, the glucose was measured in the plasma and urine by the GOD/POD [(10)]. The values represent the mean ± standard deviation. The differences between controls and experimental was significant at $p< 0.01$ for the glycaemia and $p< 0.005$ for the glycosuria.

Discussion

The ^{13}C NMR spectrum (Table 1) showed a signal at 162.29 ppm that corresponds to the carbonyl carbon of the urea, in the same way, the ^{1}H NMR spectrum showed a signal at 5.7 ppm assigned to the $–NH_2$ of urea; both values very similar to those reported in Spectral Data Base System for Organic Compounds

The six signals observed between 122 and 110 ppm in the ^{13}C spectrum were assigned to carbons of a benzene ring two of them being quaternary, which is in agreement with that reported by Marcano and Hasegawa [(11)] and Carey [(12)]. The signals assigned to the benzene carbons are in concordance with the signals observed in the ^{1}H NMR spectrum between 7.7 and 6.9 ppm and correspond to the zone of the aromatic protons and agree with that cited by Marcano and Hasegawa [(11)] and Carey [(12)]. In the same way, the protons coupling patron without doubt strongly suggest that the benzene is ortho-substituted [(13)].

To the best of our knowledge there are no reports of urinary elimination of benzene orhto-substituted in rats that ingest flavonoids which are compounds abundant in *B. megalandra* leaves [(4)].

Due to the fact that the compound was only partially purified it was not possible to know the exact substituents. However, the benzene ortho-substituted was not present in the urine of the control rats nor in the plant extract. In consequence it is possible to suggest that it is the product of the metabolism of a compound presents in the plant, possible a flavonoid; at present we are not able to suggest a metabolic pathway to its production.

The urinary elimination of a benzene ortho-substituted, during the ingestion of *B. megalandra* leaves extract, is evidence of the intestinal absorption of a secondary metabolite present in the plant.

The administration of the plant extract produces a small decrease, but significant, in the glycaemia (Table 2) that might be explained by:

a. Decrease in gluconeogenic and glucose-6-phosphatase activity of the liver [(14)].

b. Decrease in the glucose intestinal absorption by inhibition of the sodium-glucose cotransporter (SGLT) [(15,16)].

c. Renal glycosuria reported in the present paper (see below).

The increase in urinary elimination of glucose (Table 2) in the presence of a discrete decrease in glycaemia is a clear indication that there is a renal glycosuria, which might be due to the effects of the flavonoids, present in the plant extract, that were absorbed by the intestine and by the blood reaching the kidney where they inhibit the SGLT as they do in the intestine [(14,15)].

The renal glycosuria, produced during the ingestion of *B. megalandra* leaves extract, reported in this paper is indirect evidence of the intestinal absorption of the flavonoid present in *B. megalandra* leaves extract.

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