Isolation and Bioactivity of a Xanthone Glycoside from *Peperomia pellucida*

Alam Khan[1*], Moizur Rahman[2], Md Shariful Islam[3]

[1]Department of Pharmacy, University of Rajshahi, Rajshahi 6205, Bangladesh.
[2]Department of Animal Husbandry and Veterinary Science, University of Rajshahi, Rajshahi 6205, Bangladesh.
[3]Department of Chemistry and Bioscience, Kagoshima University, Kagoshima 890-0065, Japan.

*Correspondence to: Alam Khan, alamkhan792002@yahoo.co.in; alamkhan@ru.ac.bd

Published online: January, 2010

Abstract

Patuloside A (3-β-D-glucopyranosyloxy-1,5,6-trihydroxy-9H-xanthene-9-one) is a xanthone glycoside isolated from *Peperomia pellucida* using chromatographic techniques (TLC, PTLC, GC) and the structure was confirmed on the basis of spectral data (liquid chromatography/electrospray-mass spectroscopy, ^{1}H and ^{13}C NMR including JMOD, COSY, NOESY, HMBC, HSQC). *In vitro* antibacterial, antifungal and cytotoxic activities of the compound were studied. Disc diffusion technique was used to determine *in vitro* antibacterial and antifungal activities. Cytotoxicity was determined against brine shrimp nauplii. In addition, minimal inhibitory concentration (MIC) was determined using serial dilution technique to find out antibacterial potency. The compound showed significant antibacterial activity against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escheichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonus aeruginosa*, *Salmonella typhi*). The MIC values against these bacteria were ranged from 8 to 64 µg/mL. The compound showed weak antifungal activities against *Aspergillus flavus* and *Candida albicans*. In cytotoxicity determination, LC_{50} of the compound against brine shrimp nauplii was 18.24 µg/mL.

Keywords: *Peperomia pellucida*; Patuloside A; Antibacterial activity; Antifungal activity; Cytotoxicity.

Introduction

The frequency of life threatening infections caused by pathogenic microorganisms is increased worldwide and is becoming an important cause of morbidity and mortality in immune-compromised patients in the developing countries [1]. Although huge numbers of antimicrobial agents have been discovered, the pathogenic microorganisms are developing resistance against these agents day by day. In third world countries like Bangladesh irrational use of antimicrobial agents is a major cause of such resistance [2]. In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases. Research in the field of indigenous plant is a significant aspect to develop a safer antimicrobial principle through isolation, characterization, identification and biological studies [3].

Peperomia pellucida (Linn.) HBK (Fam. Piperaceae), locally known as Luchi Pata, is an annual herb [4]. *Peperomia pellucida* is widely distributed in many South American and Asian countries [5-8]. The plant is refrigerant, its leaves are used in the treatment of headache, fever, eczema, abdominal pains and convulsions [4]. According to Manila Medical Society *P. pellucida* is used to relieve arthritic pains, but can cause CNS depression [9]. Evaluations of antibacterial, anti-inflammatory and analgesic activities were reported for *P. pellucida* [8, 10, 11]. Isolation of antifungal constituents such as arylpropanoids [12] and anticancer constituents such as peperomins [13] were also reported for this plant. Although, the plant is reported for its antibacterial activity, its antibacterial constituent has not yet been isolated. Therefore, in the present study an attempt was made to isolate antibacterial constituent(s) from the plant and to evaluate its antibacterial activity. Since isolation of other constituents having antifungal [12] and anticancer [13] properties were also reported for this plant therefore we also determined antifungal and cytotoxic activities of the isolated compound.

Methods

Plant materials

The leaves of the plant was collected in July, 2004 from various part of Lakshmipur district of Bangladesh and identified by Prof. ATM Naderuzzaman, Department of Botany, University of Rajshahi, Bangladesh where its voucher specimen (No. KM6543) was deposited. The leaves were air-dried and ground into powder.

Plant materials extraction and fractionation

The powdered materials (450 g) were extracted with ethanol (3 L) in a Soxhlet apparatus (Quickfit, England) at 65^0C for 72 h [14]. The extract was filtered and the solvent were evaporated to dryness *in vacuo* with rotary evaporator at 40-50 degrees celsius to afford a blackish green mass (32 g) which was further extracted with petroleum ether (3 x 50 mL), ethyl acetate (3 x 50 mL) and methanol (3 x 50 mL) to afford petroleum ether (9 g), ethyl acetate (7 g) and methanol (11 g) fractions, respectively [15].

Isolation of compound

Among all fractions (petroleum ether, ethyl acetate and methanol), methanol soluble fraction was observed for better antibacterial activity. The methanol soluble fraction (3 g) was subjected to column chromatography using chloroform and methanol of increasing polarity. Column chromatography fractions eluting with 40-60% chloroform in methanol showed good antibacterial activity were subjected to preparative TLC (Silica gel PF_{254}) with solvent system chloroform : methanol (4 : 1) to afford compound 1 (32.5 mg). Its structure was confirmed based on various spectroscopic methods (IR, liquid chromatography/electrospray-mass spectroscopy (LC/ES-MS), ^{1}H and ^{13}C NMR including JMOD, COSY, NOESY, HMBC, and HSQC). The liquid chromatography/electrospray-mass spectroscopy (LC/ES-MS) in the positive ion mode of compound 1 have showed molecular $[M+H]^+$ peak at *m/z* 423.2 corresponding to a molecular formula $C_{19}H_{18}O_{11}$. ^{1}H and ^{13}C NMR data of compound 1 were good agreement with ^{1}H and ^{13}C NMR data of patuloside A that published in literature [16].

Organisms

Antibacterial activity and MIC were determined against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escheichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonus aeruginosa*, *Salmonella typhi*). These organisms were available in the Microbiology Research Laboratory of Pharmacy Department, Rajshahi University, Bangladesh. The pure cultures of these bacteria were collected from the Microbiological Laboratory of the Institute of Nutrition and Food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh. Antifungal screening was carried out against four fungi (*Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Rhizopus aurizae*). These organisms were available in the Microbiology Research Laboratory of Pharmacy Department, Rajshahi University, Bangladesh. The pure cultures of these fungi were collected from the Department of Botany, University of Rajshahi, Bangladesh. Cytotoxicity was determined against brine shrimp nauplii. Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial seawater (3.8% NaCl solution) for 48 h.

Media

Nutrient agar medium (Difco laboratories, USA) pH 7.2, Nutrient broth medium (Difco laboratories, USA) pH 6.8, Sabouraud dextrose agar medium (Biolife Viale Monza, Italy) pH 5.6 and artificial sea-water (3.8% NaCl solution) pH 8.4

were used for antibacterial screening, MIC determination, antifungal screening and cytotoxicity determination, respectively.

Antibacterial screening

In vitro antibacterial screening was carried out by disc diffusion method [17, 18], which is a qualitative to semi quantitative test. Briefly, 20 mL quantities of nutrient agar were plated in petridish with 0.1 mL of a 10^{-2} dilution of each bacterial culture (18 h old). Filter paper discs (6 mm in diameter) impregnated with various concentration of patuloside A were placed on test organism seeded plates. Methanol was used to dissolve the compound and was completely evaporated before application on test organism seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 12 h of incubation at 37^0 C. The diameters of zone of inhibition produced by the patuloside A were then compared with the standard antibiotic kanamycin 30 µg/disc. Each sample was used in triplicate for the determination of antibacterial activity.

Minimum inhibitory concentration (MIC) determination

Serial tube dilution technique [2, 19, 20] was used to determine MIC of the compound against these bacteria. Patuloside A (1.024 mg) dissolved in 2 mL distilled water (few drops Tween 80 was added to facilitate dissolution) to obtain stock solution having concentration of 512 µg/mL. In serial dilution technique, 1 mL prepared stock solution was transferred to test tube containing 1 mL nutrient broth medium to give concentration 256 µg/mL from which 1 mL was transferred to another test tube containing 1 mL nutrient broth medium to give concentration 128 µg/mL and so on up to concentration 2 µg/mL. After preparation of suspensions of test organisms (10^7 organism per mL), 1 drop of suspension (0.02 mL) was added to each broth dilution. After 24 h incubation at 37^0 C, the tubes were then examined for the growth. The MIC of patuloside A was taken as the lowest concentration that showed no growth. Growth observed in those tubes where the concentration of the patuloside A was below the inhibitory level and the broth medium was observed turbid (cloudy). Distilled water with few drops of Tween 80 and kanamycin were used as negative and positive control, respectively.

Antifungal screening

In vitro antifungal screening was carried out by disc diffusion method [17, 18]. In this method, 20 mL quantities of Sabourand dextrose were plated in petridish with 0.2 mL of a 10^{-2} dilution of each fungal culture (10 h old). Filter paper discs (6 mm in diameter) impregnated with various concentration of patuloside A was placed on test organism seeded plates. Methanol was used to dissolve the compound and was completely evaporated before application on test organism seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 72 h of incubation at 30^0 C. The diameter of zone of inhibition produced by the patuloside A, were then compared with the standard antibiotic nystatin 30 µg/disc. Each sample was used in triplicate for the determination of antifungal activity.

Cytotoxicity assay

The cytotoxicity assay was performed on brine shrimp nauplii using Mayer method [21, 22]. Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial seawater (3.8% NaCl solution) for 48 h. Dissolution of compound was performed in artificial seawater using DMSO. Each 5 mL solution of different concentrations (0.5, 1, 2, 5, 10, 20 and 40 µg/mL) of the compound was taken in different vials where brine shrimp nauplii were given and their mortality was observed for 24 h. The resulting data were transformed to probit analysis [20, 23] for the determination of LC_{50} values of the compound. Artificial seawater medium containing

DMSO that used for the analysis used as control. Gallic acid and vincristine sulfate were used as standards in this assay.

Results

The structure of the compound has shown in figure 1 and its 1H and 13C NMR data have shown in table 1. The results of antibacterial activity of patuloside A against the test bacteria are presented in Table 2. In comparison to reference standard kanamycin (30 μg/disc), patuloside A exhibited significant antibacterial activity at 160 μg/disc. Patuloside A showed highest activity against *Streptococcus-β-haemolyticus* and lowest against *Shigella sonnei*. The MIC values against these Gram-positive bacteria were ranged from 8 to 32 g/mL and Gram-negative bacteria were ranged from 16 to 32 g/mL (Table 3).

Patuloside A showed very weak antifungal activity against *Aspergillus flavus* and *Candida albicans* (Table 4). It was also observed that patuloside A inactive against *Rhizopus aurizae* and *Aspergillus niger*. In cytotoxicity assay with brine shrimp nauplii, the LC_{50} value of patuloside A was 18.24 μg/mL. The cytotoxicity of patuloside A was compared with cytotoxicity of standard gallic acid and vincristine sulfate where LC_{50} values were 4.23 and 0.62 μg/mL, respectively (Table 5). No mortality was found in the control group. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on graph paper.

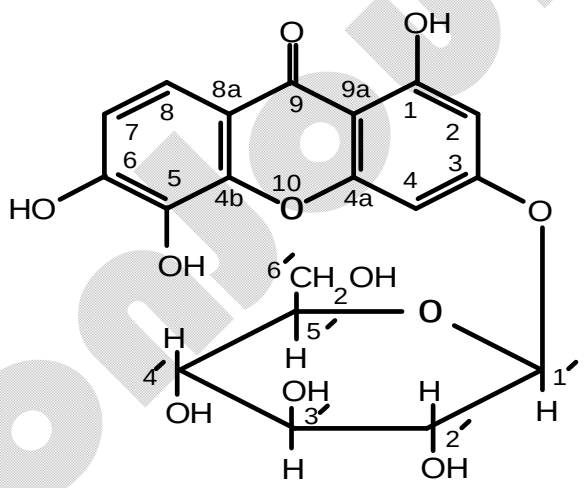

Figure 1. Structure of Patuloside A.

Discussion

Patuloside A is the first report of isolation from the genus *Peperomia* as well as from the family Piperaceae. Patuloside A was previously isolated from root of *Hypericum patulum* Thunb. (Guttiferae) [16]. Though it showed activity against all tested bacteria, activities against Gram-positive bacteria were better than Gram-negative bacteria. Antibacterial potency of patuloside A against these bacteria expressed in MIC are presented in Table 3 also indicate patuloside A effective against Gram-positive bacteria at lower concentration than that against Gram-negative bacteria. Overall, this compound has shown significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Khan and Omoloso (2002) reported the antibacterial activity of crude extract of *Peperomia pellucida,* support the finding of antibacterial activity of Patuloside A isolated from the plant.

It's very weak antifungal activity against *Aspergillus flavus* and *Candida albicans* and inactivity against *Rhizopus aurizae* and *Aspergillus niger* indicates that its antifungal application clinically insignificant. Although many scientists have shown a correlation between cytotoxicity and activity against the brine shrimp nauplii [24-27], however due to very weak activity of patuloside A against brine shrimp nauplii (LC_{50} 18.24) it is cytotoxic application also clinically insignificant. These antibacterial, antifungal and cytotoxic studies are the first report for this compound.

Table 1. ^{1}H and ^{13}C NMR data for patuloside A.

Carbon/Proton No	^{1}H NMR (*J* in Hz)	^{13}C NMR
1		163.0
2	6.15 d (2.5)	98.5
3		164.5
4	6.42 d (2.5)	96.4
4a		153.4
4b		146.6
5		133.2
6		152.9
7	6.67 d (8.5)	114.1
8	7.25 d (8.5)	116.0
8a		113.7
9		176.8
9a		105.3
1-OH	11.92 s	
Glucose moiety		
1′	4.76 d (10)	101.7
2′	3.10-3.31 m	75.4
3′	3.10-3.31 m	77.3
4′	3.10-3.31 m	73.5
5′	3.38-3.56 m	79.0
6′	3.68 dd (5.5, 10)	62.2

Conclusions

The results obtained in this study suggest that patuloside A is important for its antibacterial activity. However, more studies are needed to focus on mechanism of action, structure activity relationship, toxicological evaluation and to identify other active constituents of the plant *Peperomia pellucida*.

Competing Interests

The authors declare that they have no competing interests.

Table 2. *In vitro* antibacterial activities of patuloside A isolated from *Peperomia pellucida*.

Test organism	Diameter of zone of inhibition (mm)				
	Methanol Fraction 160 µg/disc (Mean ± SEM)	1 30 µg/disc (Mean ± SEM)	1 80 µg/disc (Mean ± SEM)	1 160 µg/disc (Mean ± SEM)	Kanamycin 30 µg/disc (Mean ± SEM)
Gram-positive					
Bacillus subtilis	17 ± 1.1	13 ± 1.6	18 ± 2.1	23 ± 1.4	31 ± 2.6
Bacillus megaterium	18 ± 1.3	12 ± 0.8	16 ± 1.6	20 ± 1.3	29 ± 1.5
Staphylococcus aureus	15 ± 1.0	11 ± 1.1	21 ± 1.7	22 ± 1.9	32 ± 1.7
Streptococcus-β-haemolyticus	18 ± 1.2	14 ± 1.3	18 ± 1.3	24 ± 1.5	26 ± 2.4
Gram-negative					
Escheichia coli	11 ± 1.6	10 ± 1.1	15 ± 1.4	20 ± 1.3	26 ± 2.0
Shigella dysenteriae	14 ± 1.4	9 ± 1.5	12 ± 1.5	16 ± 1.8	30 ± 1.9
Shigella sonnei	12 ± 1.7	10 ± 0.7	13 ± 1.2	15 ± 1.5	33 ± 1.5
Shigella flexneri	13 ± 0.8	8 ± 1.6	12 ± 1.5	17 ± 1.7	31 ± 2.3
Pseudomonus aeruginosa	11 ± 0.9	10 ± 1.4	14 ± 1.7	17 ± 1.2	30 ± 2.1
Salmonella typhi	14 ± 1.3	9 ± 1.5	13 ± 1.3	18 ± 0.7	29 ± 1.8

The control disc used for solvent (dried off before application) had no zone of inhibition, so their data was omitted from the above data. Data are represented in the form of mean of three tests ± SEM of the standard kanamycin group. 1 = patuloside A.

Authors' Contributions

AK developed the project and supervised preparation of the manuscript. At remaining works of this article all authors (AK, MR and MSI) have similar contribution.

Acknowledgement

The authors wish to thank Professor ATM Naderuzzaman, Department of Botany, University of Rajshahi, for identification of the plant.

Table 3. Minimum inhibitory concentration of Patuloside A isolated from *Peperomia pellucida*.

Bacteria	MIC values of Methanol Fraction (µg/mL)	MIC values of patuloside A (µg/mL)	MIC values of kanamycin (µg/mL)
Bacillus subtilis	64	16	2
Bacillus megaterium	32	32	4
Staphylococcus aureus	64	8	8
Streptococcus β-haemolyticus	32	8	8
Escheichia coli	128	16	8
Shigella dysenteriae	32	32	2
Shigella sonnei	64	64	4
Shigella flexneri	32	16	16
Pseudomonus aeruginosa	128	32	16
Salmonella typhi	64	16	4

The negative control containing solvent had no MIC values, so their data was omitted from the above data.

Table 4. *In vitro* antifungal activity of patuloside A isolated of *Peperomia pellucida*.

Test organism	Diameter of zone of inhibition (mm)		
	Patuloside A 80 µg/disc (Mean ± SEM)	Patuloside A 160 µg/disc (Mean ± SEM)	Nystatin disc 30 µg/disc (Mean ± SEM)
Aspergillus flavus	7 ± 1.2	10 ± 0.8	16 ± 1.7
Aspergillus niger	0	0	18 ± 1.3
Candida albicans	8 ± 0.6	9 ± 1.8	17 ± 1.3
Rhizopus aurizae	0	0	16 ± 1.4

The control disc used for solvent (dried off before application) had no zone of inhibition, so their data was omitted from the above data. Data are represented in the form of mean of three tests ± SEM of the standard nystatin group.

Table 5. Cytotoxicity of Patuloside A isolated from *Peperomia pellucida*.

Sample	LC_{50} (µg/mL)	95% confidence limits (µg/mL)	Regression equation	x^2 value
Patuloside A	18.24	10.72 - 29.04	Y = 2.17 + 1.09 X	1.54
Gallic acid	4.23	3.43 - 5.21	Y = 3.19 + 2.88 X	3.02
Vincristine sulfate	0.62	0.49 - 0.77	Y = 2.95 + 2.58 X	2.87

References

1. Al-Bari MAA, Sayeed MA, Rahman MS, Mossadik MA, 2006. Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis* a novel specis collected in Bangladesh. Research Journal of Medicine and Medical Science, 1: 77-81.

2. Al-Bari MAA, Khan A, Islam MR, Kudrat-E-Zahan M, Rahman MMS, Mosaddik MA, 2007. Isolation and *in vitro* antimicrobial activities of ethyl acetate extract from *Streptomyces bangladeshiensis*. Research Journal of Microbiology, 2: 272-277.

3. Rahman MM, Wahed MII, Biswas MHU, Sadik GMG, Haque ME, 2001. *In vitro* antibacterial activity of the compounds of *Trapa bispinosa* Roxb. Pakistan Journal of Medical Science, 1: 214-216.

4. Ghani A, 1998. *Medicinal plants of Bangladesh*. Bangladesh, Asiatic Society of Bangladesh, 77-78.

5. Bayma JD, Arruda MS, Müller AH, Arruda AC, Canto WC, 2000. A dimeric ArC_2 compound from *Peperomia pellucida*. Phytochemistry, 55: 779-782.

6. Santos PR, Moreira DL, Guimaraes EF, Kaplan MA, 2001. Essential oil analysis of 10 Piperaceae species from the Brazilian Atlantic forest. Phytochemistry, 54: 547-551.

7. Mde FA, Oliveira RL, Mandes SS, 2002. Seed germination, phenology, and antiedematogenic activity of *Peperomia pellucida* (L.) HBK. BMC Pharmacology, 2: 12-19.

8. Arrigoni-Blank MF, Dmitrieva EG, Franzotti EM, Antoiolli AR, Andrade MR, Marchioro M, 2004. Anti-inflammatory and analgesic activity of *Peperomia pellucida* (L.) HBK (Piperaceae). Journal of Ethnopharmacology, 91: 215-218.

9. Calimag MMP. *Herb-Drug Interactions*. Manila, Manila Medical Society, [http://www.geocities.com/mmsi1902/herbal_aware.htm]

10. Aziba PI, Adedeji A, Ekor M, Adeyemi O, 2001. Analgesic activity of *Peperomia pellucida* aerial parts in mice. Fitoterapia, 72: 57-58.

11. Khan MR, Omoloso AD, 2002. Antibacetrial activity of *Hygrophila stricta* and *Peperomia pellucida*. Fitoterapia, 73: 251-254.

12. Ragasa CY, Dumato M, Rideout JA, 1998. Antifungal compounds from *Peperomia pellucida*. ACGC Chemical Research Communications, 7: 54-61.

13. Xu S, Li N, Ning MM, Zhou CH, Yang QR, Wang MW, 2006. Bioactive compounds from *Peperomia pellucida*. Journal of Natural Product, 69: 247-250.

14. Bhal BS, Bhal A, 1992. *A text book of organic chemistry*. India, Schand and Company Ltd, 5-14.

15. Jeffery GH, Bassett J, Mendham J, Denney RC, 2000. Vogel's textbook of quantitative chemical analysis. 5th ed., England, Longman Group UK Ltd, 161-163.

16. Ishiguro K, Yamamoto R, Oku H, 1999. Patuloside A and B, novel xanthone glycoside from cell suspension cultures of *Hypericum patulum*. Journal of Natural Product, 62: 906-908.

17. Carson CF, Hammer KA, Riley TV, 1995. Broth microdilution method for determination susceptibitity of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Melaleuca altenifolia* (tea tree oil). Microbios, 82: 181-185.

18. Dash S, Nath LK, Bhise S, Bhuyan N, 2005.: Antioxidant and antimicrobial activities of *Heracleum nepalense* D Don root. Tropical Journal of Pharmaceutical Research, 4: 341-347.

19. Rahman MM, Mosaddik MA, Wahed MII, Haque ME, 2000. Antimicrobial activity and cytotoxicity of *Trapa bispinosa*. Fitoterapia, 71: 704-706.

20 Mosaddik MA, Haque ME, 2003. Cytotoxicity and antimicrobial activity of goniothalamin isolated from *Bryonopsis laciniosa*. Phytotherapy Research, 17: 1155-1157.

21. Islam MA, Sayeed MA, Islam MA, Khan GRMAM, Mosaddik MA, Bhuyan MSA, 2002. Terpenes from bark of *Zanthoxylum budrunga* and their cytotoxic activities. Revista Latinoamericana de Química, 30: 24-28.

22. Hossain MS, Hossain MA, Islam R, Alam AHMK, Kudrat-e-Zahan, Sarkar S, Farooque MA, 2004. Antimicrobial and cytotoxic activities of 2-aminobenzoic acid and 2-aminophenol and their coordination complexes with Magnesium (Mg-II). Pakistan Journal of Biological Science, 71: 25-27.

23. Finney DJ, 1971. Probit Analysis. 3rd ed., Cambridge, University Press, 37-77.

24. Gurkan E, Tuzun OT, Hirlak F, 1995. Cytotoxicity assay of some Papaver alkaloids using *Artemia salina* (Brine shrimp). Fitoterapia, LXVI: 544-545.

25. Martin-Cordero G, Saenz MT, Ayuso MJ, 1995. Cytotoxic activity of *Retama spaerocarpa*. Fitoterapia, XVI: 495-498.

26. Mongelli E, Desmarchelier C, Giulietti A, 1995. Bioactivity of certain medicinal latexes used by the Eséejas. Journal of Ethnopharmacology, 47:159-163.

27. Desmarchelier C, Mongelli E, Coussio J, Ciccia G, 1996. Studies on the cytotoxicity, antimicrobial and NA-binding activities of plants used by the Eséejas. Journal of Ethnopharmacology, 50: 91-96.