Ultrasound-assisted Extraction of Ursolic Acid from the Flowers of *Ixora coccinia* Linn (Rubiaceae) and Antiproliferative Activity of Ursolic Acid and Synthesized Derivatives

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ABSTRACT

**Background**: *Ixora coccinea* Linn (Rubiaceae) is an evergreen shrub with bright scarlet colored flowers found in several tropical and subtropical countries. It is used as an ornamental and medicinal plant. Phytochemical studies revealed that its major special metabolites are triterpene acids, such as ursolic and oleanolic acid. **Objective**: To evaluate the isolation of ursolic acid (UA) (1) from methanol extracts of *I. coccinea* flowers through two methodologies, to prepare four derivatives, and to evaluate the cytotoxic effect against six cancer cell lines. **Materials and Methods**: The UA was isolated from vegetal material by percolation at room temperature and by ultrasound-assisted extraction. The preparation of derivatives was performed according to literature methods, and the cytotoxic effects were evaluated using the MTT (3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay. **Results**: The most efficient extraction was achieved through ultrasound irradiation with a yield of 35% after KOH-impregnated silica in chromatography column. Furthermore, four derivatives (3, 5, 6, 7) of UA were prepared and evaluated, including 1, against two lung cancer (A549 and H460) and four leukemia (K562, Lucena, HL60, and Jurkat) cell lines. Generally, results showed that 1 and 7 were the most active compounds against the assayed cell lines. Also, the cytotoxic effects observed on terpenes 1 and 7 were higher when compared with cisplatin, used as positive control, with the exception of Jurkat cell line. **Conclusion**: The efficacy of such an extraction method led to the principal and abundant active component, 1, of *I. coccinea*, thus representing a considerable contribution for promising triterpenoid in cancer chemotherapy.

**Key words**: Cytotoxic activity, *Ixora coccinea*, ultrasound-assisted extraction, ursolic acid

**SUMMARY**

- The ultrasound-assisted extraction of *Ixora coccinea* flowers improved the ursolic acid isolation.
- Methanolic extract from flowers of *I. coccinea* provided, by ultrasound irradiation, after KOH-impregnated silica in chromatography column, the ursolic acid in 35% yield.
- The ursolic acid and four derivatives were prepared and assayed against two lung cancer and four leukemia cell lines.
- The ursolic acid and their 3-oxo-derivative, in general, were more cytotoxic when compared to cisplatin, used as positive control.

**INTRODUCTION**

The *Ixora* genus of the tribe Ixorae in the subfamily Ixoroidae (Rubiaceae) is represented by ca. 150 species widespread in tropical areas of Asia, Africa, and South America. Many of them are extensively used in folk medicine and Ayurveda, the traditional Indian system of medicine, in the treatment of several diseases, such as dysentery, dysmenorrhea, hypertension, bronchitis, chronic ulcers, and skin diseases, among others. *Ixora coccinea* Linn species is a small evergreen shrub that shows eye-catching and colorful flowers, which are used in the treatment of dysentery, leucorrhoea, dysmenorrhea, bronchitis, and microbial infections. The hexane extract of these flowers has displayed antiproliferative activity against different types of leukemia cell lines and enhanced the survival of mice inoculated with Dalton lymphoma and Ehrlich carcinoma.

Phytochemical studies have shown that the major metabolites present in *I. coccinea* flowers are ursolic acid (UA), oleanolic acid, stearic acid, oleic acid, linoleic acid, lupeol, and sitosterol. Literature has also reported the presence of ixoroid, D-mannitol, 5-O-caffeoyl-quinic acid, and 5-caffeoyl-quinic acid. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

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of this institution, where a voucher specimen (no. 4243c) has been deposited.

**Preparation of the crude extract and UA isolation**

**Methodology A-percolation method and traditional silica gel column chromatography for UA isolation**

The methanol extract (ME-F; 26.3 g; 8.4%) from *in natura* flowers of *I. coccinea* (315 g) was prepared by percolation at room temperature. The obtained crude extract was submitted to chromatographic separations by classical phytochemical procedures. ME-F extract afforded 340 mg (0.12%) of a rich mixture of several nonpolar constituents (hydrocarbons and steroids), which was obtained from the fraction groups F1-19 (eluted with Hexane: EtOAc 10:0 - 9:1); 570 mg (2.17%) of UA [from F20-39 eluted with Hexane:EtOAc (8:2-5:5)] and 143 mg (0.04%) of the sugar mannitol [from F40-46 eluted with EtOAc:MeOH (100:0 - 50:50)].

**Methodology B-ultrasound-assisted extraction and KOH-impregnated silica gel column flash filter for UA isolation**

The methanol crude extract was prepared from 400 g of fresh flowers subjected to solvent-soaking for 30 min; then, the tube with the mixture was immersed into the water bath of the ultrasonic device and irradiated for 40 min. After the extraction, the sample was concentrated under vacuum, and the methanol extract was compared with a standard sample of UA by CCDA.

KOH-impregnated silica gel column flash filter was used for the isolation and purification of the UA. The column preparation was carried out as follows: 100 g of silica gel (230–400 mesh) in 1000 mL iPrOH (1000 mL) were mixed with 200 mL of a saturated solution of KOH (25 g) for 10 min. This mixture was then transferred into a glass column and washed with 400 mL of hexane. Crude extract (388.1 mg) was applied to the top of this column and successively eluted with hexane, CHCl₃, and MeOH in order of increasing polarity. The fractions were combined in eight groups using analytical thin-layer chromatography (TLC) compared with standard UA. The detection and quantification of this natural product in these fractions as well as the extract was conducted by HPLC.

**Instrument and chromatographic conditions**

The ultrasound bath (Cleaner-Unique USC 2500) with ultrasonic power of 155 Watts RMS, operating at a frequency of 60 Hz with controlled temperature and time, was used to obtain the crude extract from *I. coccinea* flowers.

HPLC consisted of Shimadzu LC-10A pump with UV detector (205 nm)-a loop of 20 µL was used for injection. The standard UA and fractions were analyzed by RP-18 column 250 × 46 mm × 5 µm and acetonitrile (isocratic) was used as mobile phase at a flow rate of 1.7 mL min⁻¹.

**Preparation of the standard solution and validation of HPLC method**

A stock solution in acetonitrile containing 1 mg/mL of UA was prepared in order to obtain the standard curve. A total of 20 µL of each of the successive dilutions, 10, 15, 25, 50, and 70 µg/mL in acetonitrile was used to obtain the standard curve and the area corresponding to each concentration. The analyses were performed in triplicate. After plotting the peak area versus concentration of analyte, the linear regression treatment provided the correlation coefficient and the standard deviation values. Each sample of extract was analyzed in quintuplicate, and the average area of these measurements replaced the value of y in Equation 1.

\[
y = 67,285.32 + 2,358.93 \times x
\]
Synthesis

Chemicals

All commercially available reagents were used without further purification unless otherwise stated. The progress of the reactions was monitored by analytical TLC performed on Merck 254 nm silica gel 

Extraction, isolation, and purification of UA from I. Coccinea flowers

Not only did the present study investigate the effects of two methods in the extraction efficiencies of UA content from I. coccinea flowers, but it also evaluated their isolation technique. Initially, the methanol extract was prepared by percolation at room temperature, and it afforded 8.4% of the crude extract. The traditional isolation of UA by silica gel chromatographic column furnished 2.2% of triterpenic acid, characterized by melting point, IR, NMR 1H, and 13C spectroscopies. However, the crude extract was obtained in 19.4% yield using the technique of ultrasonic-assisted extraction. Interestingly, the UA isolated from the crude extract of the ultrasound-assisted method proved very efficient for obtaining this bioactive natural product with high yield after the use of KOH-impregnated silica gel column chromatography; it furnished pure 1, which represents 35.0%. This methodology resulted in an increase of the yield of the isolated bioactive natural product as well as in the decrease of the organic solvent usage and the amounts of time; it is efficient for obtaining this bioactive natural product with high yield.

Cell culture

A549, H460 (lung cancer), JURKAT, HL60, K562, and Lucena1 (a MDR vincristine derivative of K562) cells were maintained in RPMI 1640 medium supplemented with fetal calf serum, 50 µM of 2-mercaptoethanol, 100 IU/mL of penicillin and 100 µg/mL of streptomycin. The cultures were incubated at 37 °C in humidified atmosphere with 5% CO₂, and the medium changed twice a week.

Drugs and MTT assay

Stock solutions of compounds 1, 3, 5, and 6 were prepared in DMSO. The maximum DMSO percentage in assays was 0.5% (v/v) in saline solution. Drug cytotoxicity assays were performed using MTT for viable cell measurements. Aliquots of 104 cells/mL were seeded onto 96-microtiter flat well plates and incubated for 24 h. After that, cells were treated with medium, different concentrations of the drugs (6.25, 12.5, 25, or 50 µM), or the DMSO. 48 h later, the cultures were treated with MTT (5 mg/mL), incubated for 4 h in the dark, the formazan produced by live cells solubilized with DMSO, and the absorbance was read at 570 nm. Cisplatin was used as positive control. Results represent mean ± standard deviation of at least three experiments performed in triplicate. IC₅₀ values were obtained by a linear regression analysis of the absorbance percentage versus the log of the drug concentration.

RESULTS AND DISCUSSION

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3-Oxo-urs-12-en-28-oic acid (7)

UA (200 mg, 0.43 mMol) in acetone (20 mL) was treated with pyridinium chlorochromate (PCC) (280 mg). After being stirred at room temperature for 48 h, the mixture was treated using the reported procedure to afford 2.
Synthesis of UA derivatives

In this work, three UA derivatives (5, 6, and 7) were prepared in an attempt to search for more active compounds. The structure modification of the UA was done at the carbinolic carbon atom (C-3) and carboxylic carbon atom (C-28).

Initially, the UA was acetylated with anhydride acetic and pyridine to afford its acetyl ester 3. This compound (3) was treated with oxalyl chloride to obtain the corresponding acid chloride 4 as intermediate, which was not isolated. After this, a condensation reaction with 4 and aniline as well as with p-toluidine, each separately, was performed. The obtained derivatives were purified by silica gel chromatography column and afforded compounds N-[phenyl]-3-O-acetylursolamide (5) and N-[4-tolyl]-3-O-acetylursolamide (6), respectively [Figure 3].

Antiproliferative assays

MTT procedure, with minor modifications, was used to evaluate the antiproliferative activity of 1, 3, 5, 6, and 7 against the two lung cancer (A549 and H460) and four leukemia (K562, Lucena, HL60, and Jurkat) cell lines, and IC\textsubscript{50} values were determined in M, at least in three independent experiments. Table 1 shows the IC\textsubscript{50} values observed to each cell line.

In general speaking, the results indicated that the UA and the oxidized derivative were the most active compounds against the assayed cell lines. For the A549 lung cancer cell line, the best result was observed to 1 (IC\textsubscript{50} = 13.12 µM), while for the H460, to 7 (IC\textsubscript{50} = 17.58 µM). The results for leukemia cell lines presented similar values of IC\textsubscript{50} for 1 and 7, with the exception of HL60, against which derivative 7 was more active. Furthermore, the cytotoxic effects observed to terpenes 1 and 7 were higher when compared with cisplatin, a well-known chemotherapeutic agent used in clinic treatment, with the exception of Jurkat cell line.

The results suggested the importance of structure modification in C-3 position and the maintenance of the carboxylic acid group that keeps the acid character of compounds 1 and 7 when compared with 5 and 6 with amide moieties. In addition, we calculated the lipophilic parameter log \textit{P} by ACD Labs software package (version 12.0) for all assayed terpenes, and the values indicated the possible reason for this difference in the cytotoxicity observed. Terpenes 1 and 7, the most active compounds, presented higher \textit{log P} values, 9.41, 10.78, and 11.24, respectively, thus reinforcing the importance of the polar carboxylic group in the structures.

Table 1: IC\textsubscript{50} in µM values of 1, 5, 6, and 7 against two lung cancer (A549 and H460) and four leukemia (K562, Lucena, HL60, and Jurkat) cell lines

| Cell line | 1       | 5       | 6       | 7       | CIS*  |
|-----------|---------|---------|---------|---------|-------|
| A549      | 13.12 ± 0.36 | 31.70 ± 2.33 | 18.71 ± 1.33 | 23.56 ± 1.9 | 25.00 ± 2.12 |
| H460      | 21.04 ± 0.98 | 45.71 ± 3.69 | 33.57 ± 3.00 | 17.58 ± 0.59 | 33.33 ± 1.87 |
| HL60      | 23.77 ± 1.36 | 40.46 ± 3.69 | 29.99 ± 1.99 | 12.82 ± 0.98 | 36.65 ± 2.34 |
| JUKART    | 22.91 ± 0.33 | 52.36 ± 0.56 | 89.54 ± 1.36 | 23.93 ± 2.10 | < 17.00          |
| K562      | 10.45 ± 0.69 | > 100    | 92.26 ± 0.96 | 11.99 ± 0.36 | 22.27 ± 0.86 |
| LUCENA    | 13.09 ± 0.45 | 45.29 ± 1.26 | 47.64 ± 2.56 | 12.59 ± 1.25 | 18.32 ± 0.76 |

*CIS = Cisplatin used as positive control
CONCLUSION

This study investigated the different extraction methods for the isolation of triterpene UA from Ixora coccinea flowers, a species widely used in traditional medicine, and verified that the ultrasound-assisted extraction is the best methodology with a remarkable 35% yield. Ursolic derivatives were prepared and characterized at reasonable yields, and the cytotoxic effects against two lung cancer (A549 and H460) and four leukemia (K562, Lucena, HL60, and Jurkat) cell lines showed that the most active compounds were the UA and the oxidize derivative, thus indicating the importance of hydrophilic moieties. Finally, we concluded that among the semisynthetic derivatives, 7 improved or was similar to UA 1 antiproliferative effects in leukemia and lung cancer cell lines. Furthermore, the importance of using alternative methods of extraction led us to the principal and abundant active component, 1, of I. coccinea, which represents a considerable contribution for drug discovery in cancer.

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Conflicts of interest

There are no conflicts of interest

REFERENCES

1. Arnaut-Mouly A, Razafimandimbison SG, Khodabandeh A, Bremer B. Phylogeny and classification of the species-rich pantropical showy genus Ixora (Rubiaceae-Ixoreae) with indications of geographical monophyletic units and hybrids. Am J Bot 2009;96:698-706.
2. Baliga MS, Kurian PJ. Ixora coccinea Linn: Traditional uses, phytochemistry and pharmacology. Chin J Integr Med 2012;18:72-9.
3. Sankaranarayanan S, Balam P, Ramachandran J, Kalachelian PT, Deccaraman M, Vijayalakshimi M, et al. Ethnobotanical study of medicinal plants used by traditional users in Villupuram district of Tamil nadu, India. J Med Plants Res 2010;4:1089-101.
4. Annappurn J, Amarnath PV, Kumar DA, Ramakrishna SV, Raghavan KV. Antimicrobial activity of Ixora coccinea leaves. Fitoterapia 2003;74:291-3.
5. Momin FN, Kalai BR, Shikalgar TS, Naikwade NS. Cardioprotective effect of methanolic extract of Ixora coccinea leaves on doxorubicin-induced cardiac toxicity in rats. Indian J Pharmacol 2012;44:178-83.
6. Lattha PG, Panikkar KR. Cytotoxic and antitumor principles from Ixora coccinea flowers. Cancer Lett 1988;130:197-202.
7. Ayammar M, Ignacimuthu S. Herbal medicines for wound healing among tribal people in Southern India: ethno botanical and scientific evidences. Int J Appl Res Nat Prod 2009;2:29-42.
8. Lattha PG, Nayyar MNS, Singh O, George V, Panikkar KR, Pushpangadan P. Isolation of antigenotoxic ursolic acid from Ixora coccinea flowers. Actual Biol 2001;23:1-4.
9. Versiani MA, Ikram A, Khalid S, Fauz S, Tahiri IA. Ursolic: a new triterpenoid from the flowers of Ixora coccinea. Nat Prod Commun 2012;7:831-4.
10. Idowu TO, Ogundaini AO, Salau AO, Obuotor EM, Bezahib M, Abegaz BM. Doubly linked, α-type proanthocyanidin trimmer and other constituents of Ixora coccinea leaves and their antioxidant and antibacterial properties. Phytochemistry 2010;71:2092-6.
11. Ngs SN, Williams DB, Heed RJ. Rosemary and cancer prevention: prediclinical perspectives. Crit Rev Food Sci Nutr 2011;51:946-54.
12. Zhang P, Cheng Y, Duan RD. Ursolic acid inhibits acid sphingomyelinase in intestinal cells. Phytother Res 2013;27:173-8.
13. Tavano MF, Micali N, Montfor MT, Tazkou O, Galati EM. Ursolic acid plays a role in Nepeta sibirilnna Bentham CNS depressing effects. Phytother Res 2007;21:382-6.
14. Ferreira DS, Esperandim VR, Tolda MPA, Kuehn CC, Prado-Júnior JC, Cunha WR, et al. In vivo activity of ursolic and oleanolic acids during the acute phase of Trypanosoma cruzi infection. Exp Parasirol 2013;134:455-9.
15. Fontany S, Grare M, Mayer J, Finance C, Duvale R. Ursolic, oleanolic and betulinic acids: antibacterial spectra and selectivity indexes. J Ethnopharmacol 2008;120:272-6.
16. Huang CY, Lin CY, Tsai CW. Mei-Chin. Inhibition of cell proliferation, invasion and migration by ursolic acid in human lung cancer cell lines. Toxicol in vitro 2011;25:1234-80.
17. Shanmugam MK, Dai X, Kumar AP, Tan BKH, Sethi G, Bisthaye A. Ursolic acid in cancer prevention and treatment: Molecular targets, pharmacokinetics and clinical studies. Biochem Pharmacol 2013;85:1579-87.
18. Yang YC, Weib MC, Huang HC. Optimization of an Ultrasound-assisted extraction followed by RP-HPLC separation for the simultaneous determination of oleanolic acid, ursolic acid and oridonin content in Rabdosia rubescens. Phytochem Anal 2012;23:627-36.
19. Xia EO, Yu YY, Xu XR, Deng GF, Guo YJ, Li HB. Ultrasound-assisted extraction of oleanolic acid and ursolic acid from Ixora coccinea: isolation and antioxidant activity. RSC Adv 2011;25:1272-6.
20. Maciel MAM, Martins JR, Pinto AC, Kaser CR, Esteves-Souza A, Echevarria A. Natural and semi-synthetic clerodanes of Cotone cauraca and their cytotoxic effects against Ehrlich carcinoma and human K562 leukemia cells. J Braz Chem Soc 2007;18:391-6.
21. Pinto AC, Braga WF, Razeendar CM, Gamido FMS, Veiga Jr VF, Bergter L, et al. Separation of acid diterpenes of Copaifera cearensis Huber ex Duke by flash chromatography using potassium hydroxide impregnated silica gel. J Braz Chem Soc 2000;11:355-60.
22. Gnaotta SCB, Dassonville-Klimpt A, Nascimento S, Galera P, Boudemedene K, Gossmann G, et al. Evaluation of ursolic acid isolated from Ilex paraguariensis and derivatives on aromatase inhibition. Eur J Med Chem 2008;43:1865-77.
23. Ma CM, Cai SQ, Cui JR, Wang RQ, Tu PF, Hatton M, et al. The cytotoxic activity of ursolic acid derivatives. Eur J Med Chem 2005;40:582-9.
24. Rumjaknek VM, Trinidad GS, Wagner-Souza K, Meletti-de-Oliveira MC, Marques-Santos LF, Maia RC, et al. Multidrug resistance in tumor cells: characterization of the multidrug resistant cell line K562-Lucena 1. Ann Acad Bras Ci 2001;73:5749.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
26. Nagaraj S, Baskar L, Papiya MM, Muthukumar K, Simon SJ, Ashok KP. Evaluation of wound healing and antimicrobial potentials of Ixora coccinea root extract. Asian J Trop Med 2011;4:969-73.