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Short Report

Application of an Ionic Liquid in the Microwave Assisted Extraction of Cytotoxic Metabolites from Fruits of *Schinus terebinthifolius* Raddi (Anacardiaceae)

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This work reports the application of an ionic liquid (1-butyl-3-methylimidazolium bromide, BMImBr) in the microwave assisted extraction (MAE) of metabolites from fruits of *Schinus terebinthifolius*. Dried fruits were individually extracted using BMImBr:H_2O 1:1, v/v (experiment 1) and pure H_2O (experiment 2) by MAE (10 min at 60 °C). After partition using EtOAc, the yield to experiment 1 was about 23% while to experiment 2 was 0.1%. The EtOAc fraction obtained from experiment 1 was purified by chromatographic methods to afford 3-oxotirucalla-7,24*Z*-dien-27-oic acid, 3α-hydroxytirucalla-7,24*Z*-dien-27-oic acid, 3α-acetoxytirucalla-7,24*Z*-dien-27-oic acid, gallic acid, and ethyl gallate, being the first occurrence of the third compound as natural product. Cytotoxic activity was evaluated *in vitro* against cancer cell lines (A2058, HeLa, SiHa, HCT, SKBR-3, U87, and B16F2Nex2), being 3α-acetoxytirucalla-7,24*Z*-dien-27-oic acid the more active metabolite with IC_{50} ranging from 10.9 ± 1.3 to 17.3 ± 1.4 μg mL^{-1}, lower than that determined to positive control cisplatin.

Keywords: *Schinus terebinthifolius*, tirucallane triterpenoids, gallic acid derivatives, ionic liquid, MAE, cytotoxic activity

Introduction

Schinus terebinthifolius Raddi (Anacardiaceae), popularly known as "aroeira-pimenteira", is geographically distributed throughout Brazil,[1,2] where it has been used by native communities to treat several diseases.[3] The scientific investigations have highlighted the importance of *S. terebinthifolius* extracts due to their antiulcer, anti-inflammatory, antiseptic, and antitumor properties.[4] Chemically, *S. terebinthifolius* extracts are rich in triterpenoids,[5-8] especially tirucallane derivatives (masticadienoic acid and schinol) with anti-inflammatory,[4] antifungal[9] and antiparasitic[10] activities. Beside these compounds, phenolic derivatives such as gallic acid, methyl and ethyl gallates as well as flavonoids *trans*-catechin, miricetrin, miricetin, quercitrin, and afzelin, which displayed antiradical[11] and cytotoxic[12] activities, were also isolated.

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As reported in previous papers,[4-12] the extraction procedures of bioactive compounds involve the use of toxic organic solvents such as MeOH, acetone, $CHCl_3$ and hexane in large quantities, leading to a significant environmental impact. The growing interest in adopting more environmentally compatible technologies make these processes inadequate. In this aspect, ionic liquids can be considered potential substitutes for organic solvents used in these extractions due to their interesting properties, such as negligible vapor pressure and non-flammability, high solubilization capacity, high chemical and thermal stabilities, possibility of reuse and reduced toxicity.[13-16] Moreover, ionic liquids have modular properties, since there are numerous possible combinations of cations, anions and substituents, which originate salts with different densities, viscosities, polarities and hydrophilicities.[16]

Recently, the use of ionic liquids in the natural products chemistry has gained importance.[17] Among the various applications, the use of ionic liquids as extracting agents of compounds with different polarities from aqueous

extracts such as organic acids, amino acids, phenols and alkaloids emerged as an alternative process of the high efficiency.[17] Additionally, ionic liquids in combination with more efficient extraction processes, such as microwave or ultrasound, have been successfully employed to extract various types of natural products including alkaloids, phenolic derivatives, terpenes and other metabolites.[18-29] Due to the high viscosity of the ionic liquids, usually they are used dissolved in water, which lowers the cost of the process. Finally, it is noteworthy that ionic liquids are compounds that absorb microwave very efficiently, making them excellent solvents when this technology is required.[18,30] Microwave heating is a very efficient process due to the microwaves couple directly with the molecules that are present in the reaction/extraction mixture, leading to a more efficient procedure. The two fundamental mechanisms for transferring energy from microwaves to the substance are dipole rotation and ionic conduction. Due to the ionic character, ionic liquids absorb microwave irradiation extremely well and transfer energy quickly by ionic conduction.[31] It is worth to mention that the use of microwave is considered a leading technology and sustainable, as it decreases the extraction time, minimizes energy expenditure, increases process efficiency and hence promotes cost reduction of the final product.

In order to minimize the problems associated to the use of conventional methods in the extraction of bioactive compounds of interesting plant species, this work has two main objectives: report the application of an ionic liquid (1-butyl-3-methylimidazolium bromide, BMImBr) in the microwave-assisted extraction process of metabolites from fruits of *S. terebinthifolius* and evaluate the cytotoxic potential of obtained EtOAc fraction, as well as purified metabolites, against several tumor cell lines.

Experimental

General experimental procedures

Microwave assisted extraction (MAE) experiments were performed with a MAS-I microwave oven (2450 MHz, Sineo Microwave Chemistry Technology Company) with a maximum delivered power of 1000 W. The temperature was monitored by an infrared probe inside the microwave oven. Sephadex LH-20 (Sigma-Aldrich) was used for the column chromatographic separation, while silica gel 60 PF_{254} (Merck) and silica gel 60 RP-18 F_{254} (Merck) were used for analytical and preparative thin-layer chromatography (TLC), respectively. High performance liquid chromatography (HPLC) analysis were performed in a Dionex Ultimate 3000 chromatograph, using a Luna Phenomenex RP-18 column (3 μm, 150 × 5 mm) and an UV-diode array detector (DAD). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 300 and 75 MHz, respectively, in a Bruker Ultrashield 300 Avance III spectrometer using $CDCl_3$ (Tedia Brazil) or CD_3OD (Aldrich) as solvents.

Plant material

Fruits of *S. terebinthifolius* were randomly collected from an individual tree at Mogi-Guaçu region (São Paulo State, Brazil) on February 2010 by Dr. Maria Claudia Marx Young from Instituto de Botânica (São Paulo, Brazil), where a reference specimen (SP272591) was deposited.

Preparation of BMImBr and the extraction using this ionic liquid

1-Butyl-3-methylimidazolium bromide (BMImBr) was prepared as previously described in the literature, with minor modifications.[32] Succinctly, the molar ratio was 1:1.2 of 1-methylimidazole (99%, Aldrich) and the 1-bromobutane (99.9%, Aldrich), respectively. Residual 1-bromobutane was removed by distillation under reduced pressure. Before extraction, the fruits of *S. terebinthifolius* were dried at 40 °C during 48 h. After grinding, the extractions were conducted in triplicate. To each procedure, the plant material (approximately 2.0 g) was extracted by microwave-assisted extraction (MAE, Figure 1) with 20 mL of mixture containing H_2O and BMImBr 1:1 (v/v)

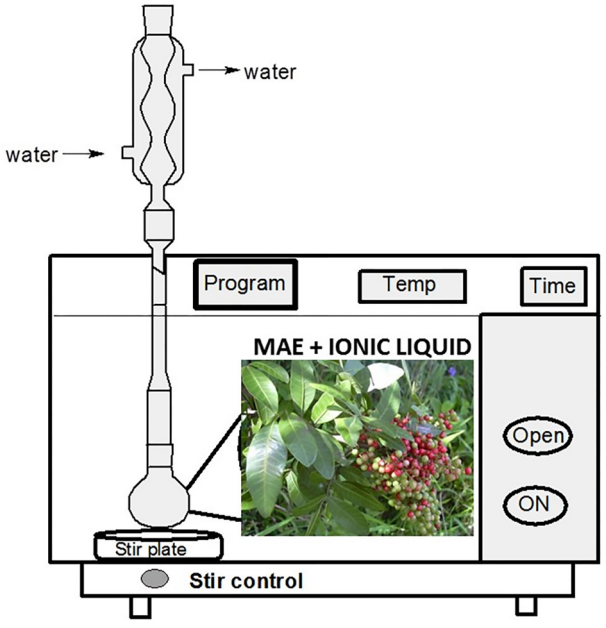

Figure 1. MAE apparatus used for the extraction of fruits of *S. terebinthifolius*.

(experiment 1). Additionally, the same procedure was conducted using distillated H_2O as solvent (experiment 2). To all MAE experiments, the extraction time was 10 min and extraction temperature was 60 °C.

The obtained aqueous extracts were filtered and then diluted to 5 mL with deionized H_2O. Sequentially, each aqueous solution was extracted using EtOAc (3 × 25 mL). After dried over Na_2SO_4 and distillation of the solvent under reduced pressure, were obtained 460 ± 12 mg (experiment 1) and 2.0 ± 0.3 mg (experiment 2) of each EtOAc fractions.

Isolation of bioactive compounds

Part of EtOAc fraction from experiment 1 (400 mg) was subjected to fractionation by Sephadex LH-20 column chromatography (3 × 50 cm, flow 1.0 mL min^{-1}) eluted with MeOH to afford 83 fractions (6 mL each). After analysis by TLC, these fractions were pooled into six groups (I-VI). Group III (122 mg) was purified by reverse phase prep. TLC (RP-18) eluted with MeOH:H_2O (9:1) to afford 3-oxotirucalla-7,24*Z*-dien-27-oic acid (**1**, 45 mg) and 3α-acetoxytirucalla-7,24*Z*-dien-27-oic acid (**3**, 4 mg). Group IV (95 mg) was subjected to separation on a Sephadex LH-20 column (3 × 60 cm, flow 0.7 mL min^{-1}) eluted with MeOH, to yield 28 fractions (5 mL each), which, after monitoring by TLC, were pooled into three groups (IV-1 to IV-3). Group IV-3 (12 mg) was composed by 3α-hydroxytirucalla-7,24*Z*-dien-27-oic acid (**2**). Group VI (61 mg) was subjected to fractionation on a Sephadex LH-20 column (3 × 50 cm, flow 1.0 mL min^{-1}) eluted with MeOH, yielding 52 fractions (5 mL each), which were pooled into five groups (VI-1 to VI-5) after analysis by TLC. Groups VI-2 (16 mg) and VI-4 (12 mg) were purified by semi-preparative RP-18 HPLC, eluted with MeOH:H_2O (8:2, flow rates 3.7 mL min^{-1}, UV 218 nm) to afford, respectively, gallic acid (**4**, 8 mg) and ethyl gallate (**5**, 6 mg).

Cell lines

The murine melanoma cell line B16F10 was originally obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). The murine melanoma B16F10-Nex2 sub-line is characterized by low immunogenicity and moderate virulence. Human melanoma (A2058), glioblastome (U87), colon carcinoma (HCT), cervical cancer (SiHa), and breast cancer (SKBR-3) cell lines were obtained from the Ludwig Institute for Cancer Research. Human cervical carcinoma (HeLa) was acquired from Dr. Hugo Pequeno Monteiro, UNIFESP. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) (Sigma, St. Louis, MO), 24 mM sodium bicarbonate (Sigma), 40 mg L^{-1} gentamycin (Schering-Plough, São Paulo, Brazil), pH 7.2, and 10% fetal calf serum (Invitrogen).

In vitro cytotoxic activity

Compounds **1**-**5** as well as the EtOAc fraction from fruits of *S. terebinthifolius* (experiment 1) were suspended in DMSO at final concentration of 10 mg mL^{-1} and then added to complete RPMI medium supplemented with 10% fetal calf serum. Different concentrations of the compounds **1**-**5** and EtOAc extract, ranging from 100 to 0 μg mL^{-1}, were incubated with 1 × 10^4 cells in a 96-well plate at 37 °C and 5% CO_2. After 24 h of incubation, cell viability was assessed using the Cell Proliferation Kit I (MTT) (Sigma), an MTT-based colorimetric assay as previously described.[33,34] Readings were made in a plate reader at 570 nm with a reference of 650 nm. All experiments were performed in triplicates using cisplatin (Sigma) and DMSO 1% as positive and negative controls, respectively.

Results and Discussion

Schinus terebinthifolius produces tirucallane triterpenes, flavonoids and gallic acid derivatives, which display important pharmacological activities.[4-12] Aiming the extraction of cytotoxic derivatives from fruits of this species, in this work was employed an alternative extraction method based in the use of ionic liquid extraction assisted by microwave. Using this approach, extracting plant material with BMImBr aqueous solution at 3 mol L^{-1} (experiment 1), followed by partition using EtOAc, were obtained 460 ± 12 mg (yield 23%) of EtOAc fraction. The same procedure was conducted using pure distillated H_2O (experiment 2) to afford 2.0 ± 0.3 mg (yield 0.1%) of EtOAc fraction, at the same MAE conditions. Each experiment was conducted in triplicate. This result showed that the use this ionic liquid affected the extraction of *S. terebinthifolius* metabolites, which could be explained by the dissolution of BMImBr in H_2O to form ionic liquid clusters due to its strong interactions, such as ionic interaction and hydrogen bonding. Therefore, it is possible to assume that these clusters could accommodate the metabolites and establish interactions with the ions, optimizing the extraction process. In addition, based in the fact that ionic liquids are microwave absorber, this

heating method showed to be more efficient than that using exclusively H_2O. Moreover, the efficiency of ionic liquids containing bromide anion in dissolving organic compounds have been indicated by the previously reported results.[18,30,35] Thus, using reduced amount of BMImBr in H_2O was observed an efficacy in extraction of metabolites from fruits of *S. terebinthifolius*.

After this extraction procedure, EtOAc fraction obtained from experiment 1 was purified by chromatographic techniques and the structures of isolated compounds (Figure 2) were confirmed as 3-oxotirucalla-7,24*Z*-dien-27-oic acid (*Z*-masticadienoic acid, **1**), 3α-hydroxytirucalla-7,24*Z*-dien-27-oic acid (*Z*-schinol, **2**), 3α-acetoxytirucalla-7,24*Z*-dien-27-oic acid (**3**), gallic acid (**4**), and ethyl gallate (**5**) by NMR spectral data analysis and comparison with

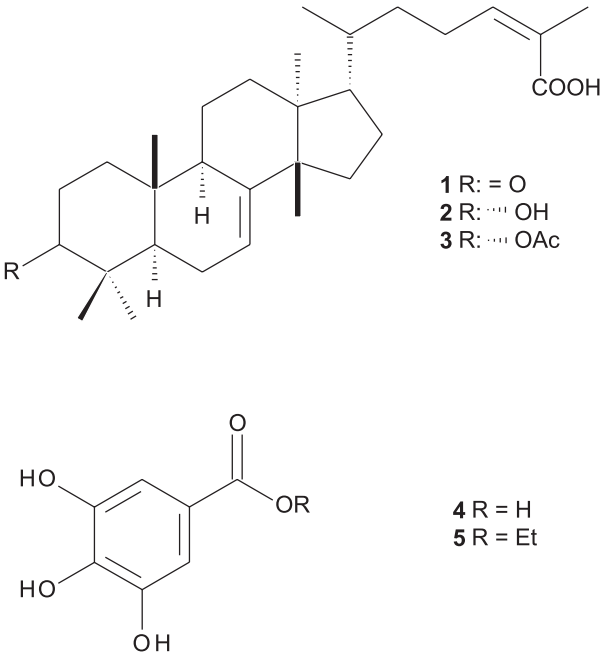

Figure 2. Structures of compounds **1**-**5** isolated from fruits *S. terebinthifolius* by MAE and BMImBr.

those described in the literature.[10] This is the first occurrence of **3** as natural product.

Aiming the discovery of new antitumoral compounds from Brazilian flora, the cytotoxic activity of EtOAc fraction obtained after extraction using ionic liquids from fruits of *S. terebinthifolius* (experiment 1) and compounds **1**-**5** were evaluated against B16F10-Nex2, A2058, HeLa, SiHa, HCT, SKBR-3, and U87 tumor cell lines (Table 1).

According to the colorimetric assay of MTT and light microscopy, all isolated compounds killed 100% of cytotoxic cells at the highest tested concentration, resulting in IC_{50} values in the range of 10.9 ± 1.3 to 94.0 ± 1.5 μg mL^{-1}. Comparison of the IC_{50} values to related tirucallane derivatives **1**-**3** demonstrated that these three derivatives displayed different cytotoxic potentials, suggesting an important effect caused by the substituent in C-3. Compound **1**, which displayed a carbonyl group, showed higher IC_{50} values, ranging from 25.0 ± 1.3 (U87) to 53.0 ± 1.2 μg mL^{-1} (HCT), in comparison to the other tirucallane derivatives **2** and **3**. As could be seen in Table 1, compound **2**, which displayed a hydroxyl group at C-3, was more active than **1**, except to U87, A2058 and B16F10 Nex 2 cell lines, suggesting that the reduction of carbonyl group at C-3 could cause an important effect in the cytotoxicity. Otherwise, the IC_{50} values determined to compound **3** indicated an expressive increase in the cytotoxicity since IC_{50} values were lower than 20 μg mL^{-1} to all tested cell lines, indicating that the presence of acetoxyl group at C-3 play an important role in the cytotoxic effects of tirucallane derivatives. Additionally, it is important to mention that compound **3** displayed higher potential than positive control cisplatin in five cell lines, suggesting that this compound could be used as scaffold to the development of new compounds with promising cytotoxic activity based in natural products.

Compounds **4** and **5** have previously been described as cytotoxic against cell lines HeLa and A2058,[12] although in this study have been tested against other cell lines.

Table 1. Cytotoxic activity of crude EtOAc fraction (experiment 1) and compounds **1**-**5** isolated from fruits of *S. terebinthifolius* after extraction using MAE/BMImBr as well as positive control cisplatin against different tumor cell lines

Tumor cell line	IC_{50} / (μg mL^{-1})						
	EtOAc phase	**1**	**2**	**3**	**4**	**5**	Cisplatin
B16F10-Nex 2	78.3 ± 0.4	33.1 ± 0.3	36.7 ± 1.1	16.3 ± 0.9	15.2 ± 1.6	34.7 ± 1.7	53.1 ± 4.2
A2058	> 200	37.7 ± 0.9	50.2 ± 1.4	12.2 ± 0.3	46.0 ± 1.4	94.0 ± 1.5	43.1 ± 3.6
HeLa	78.5 ± 3.6	52.4 ± 0.3	34.6 ± 2.1	14.0 ± 1.1	13.3 ± 0.8	54.8 ± 1.3	20.0 ± 1.3
SiHa	71.3 ± 3.2	51.3 ± 1.0	32.4 ± 2.8	13.8 ± 0.9	38.2 ± 2.1	49.9 ± 1.6	nd
HCT	68.3 ± 7.4	53.0 ± 1.2	37.4 ± 1.9	10.9 ± 1.3	12.3 ± 0.5	43.6 ± 2.9	67.5 ± 2.1
SKBR-3	> 200	> 200	40.2 ± 2.6	15.6 ± 0.7	23.9 ± 2.3	66.3 ± 2.5	nd
U87	98.3 ± 5.3	25.0 ± 1.3	38.1 ± 1.1	17.3 ± 1.4	21.6 ± 0.7	54.7 ± 3.2	45.2 ± 5.9

nd: not determined.

Thus, these compounds showed cytotoxic activity to all tested cell lines with IC_{50} values ranging from 12.3 ± 0.5 to 94.0 ± 1.5 μg mL^{-1}. As could be seen in Table 1, compound **4** was the more active derivative, especially to B16F10Nex2 (15.2 ± 1.6 μg mL^{-1}), HeLa (13.3 ± 0.8 μg mL^{-1}), HCT (12.3 ± 0.5 μg mL^{-1}), SKBR-3 (23.9 ± 2.3 μg mL^{-1}) and U87 (21.6 ± 0.7 μg mL^{-1}) cells. Similarly to the previous results to antiparasitic activity,[10] the obtained data indicated that the presence of free carboxyl group in the gallic acid is crucial to the cytotoxic potential. However, the use of galloyl derivatives as bioactives must be carefully employed since these compounds could be considered as PAINS (Pan Assay Interference Compounds) since they displayed a catechol unit which can interfere in bioassays via different mechanisms.[36]

Despite the isolated compounds **1**, **2**, **4** and **5** have already been described for the leaves of *S. terebinthifolius*,[10-12] this study demonstrate, at first time, that it is possible to extract these bioactive compounds using a more sustainably method based in the microwave assisted by ionic liquid. Additionally, this work report the cytotoxic effects of compounds **1**-**5**, highlighting the activity observed for compound **3** which displayed lower IC_{50} values of those observed to positive control (cisplatin) to five cell lines.

Conclusion

Ionic liquids have attracted considerable attention as solvents in the extraction and separation of bioactive compounds from plants owing to their excellent properties. In this work, the application of microwave-assisted extraction (MAE) using an ionic liquid aqueous solution (1-butyl-3-methylimidazolium bromide, BMImBr) showed to be an efficient procedure to the extraction of cytotoxic metabolites from fruits of *S. terebinthifolius*. After partition with EtOAc, the extraction yield was approximately 23% in one single step and short time (10 minutes, experiment 1). Comparatively, the use of pure H_2O as solvent extractor (experiment 2) afforded a yield of 0.1%, showing that the presence of BMImBr dissolved in H_2O is fundamental for the success of the extraction. The ionic liquid changes the dissipation factor of solution and the transfer of energy from microwaves to sample, which affected extraction efficiency. The proposed technique was green, simple and rapid. It is strongly believed that an ionic liquid aqueous solution as solvents in the MAE of useful substances in natural sources is a new and interesting approach. Additionally, the EtOAc fraction (experiment 1) displayed cytotoxic activity and was subjected to several chromatographic steps to afford five bioactive compounds, including three tirucallane triterpenoids (**1**-**3**) and two gallate derivatives (**4**-**5**). Among the isolated compounds, 3α-acetoxytirucalla-7,24*Z*-dien-27-oic acid (**3**) displayed higher cytotoxic potential against five cell lines, suggesting that this triterpene could be considered as a lead anti-leukemic agent.

Supplementary Information

Supplementary material is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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