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ORIGINAL ARTICLE

Antilymphoma Potential of the Ethanol Extract and Rutin Obtained of the Leaves from *Schinus molle* Linn.

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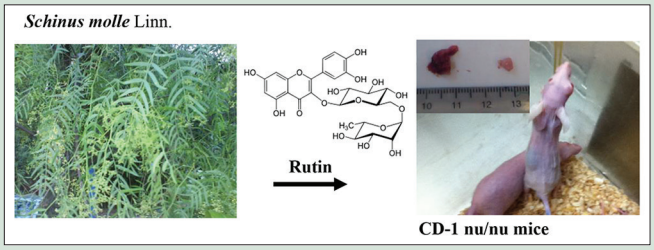
ABSTRACT

Background: *Schinus molle* Linn. (*Anacardiaceae*) is a medicinal plant used by traditional healers in Mexican traditional medicine as antitumoral. **Objective:** This study was undertaken to obtain information that support the traditional use of the leaves from *S. molle* as antitumoral. **Material and Methods:** Antilymphoma properties of the ethanol extract of the leaves from *S. molle* (EEL*Sm*) and rutin were made on athymic CD-1 nu/nu and CD-1 mice inoculated with U-937 cell line (human leukemic monocyte lymphoma [HLML]), and for their antiproliferative effects on U-937 cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Furthermore, the extract and rutin were tested for brine shrimp (BS) toxicity test. In addition, HPLC analysis was realized to known the content of rutin in the leaves from *S. molle*. **Results:** An EEL*Sm* and rutin exhibited important cytotoxic effects on U-937 cells line (IC_{50} from 172.0 μg/mL and 9.6 μg/mL, respectively) and showed *in vivo* antitumoral properties on HLML in two murine models (EC_{50} from 52.2 and 9.5 mg/kg to CD-1 nu/nu mice; EC_{50} from 99.4 mg/kg and 6.8 mg/kg to CD-1 mice, respectively). In addition, both showed strong lethality on BS larvae ($LC_{50} \leq 22.2$ μg/mL). The result of HPLC showed that rutin was the major constituent of EEL*Sm*. **Conclusions:** These test results support traditional medicinal use of *S. molle* as antitumoral and also suggest that both rutin and EEL*Sm* possess antitumor effect on HLML in murine models. Finally, rutin may play an important role in anticancer properties of *S. molle*.

Key words: *Anacardiaceae*, antilymphoma properties, rutin, *Schinus molle*

SUMMARY

- Antilymphoma properties of the ethanol extract of the leaves from *S. molle* (EEL*Sm*) and rutin were made on athymic CD-1 nu/nu and CD-1 mice inoculated with U-937 cell line (human leukemic monocyte lymphoma/HLML), and for their antiproliferative effects on U-937 cell line by MTT assay. Also, the extract and rutin were tested for brine shrimp toxicity test. In addition, HPLC analysis was realized to known the content of rutin in the leaves from *S. molle*. The results suggest that both rutin and EEL*Sm* possess antitumor effect on HLML in murine models.



Abbreviations used: EEL*Sm*: Ethanol extract of the leaves from *S. molle*, HLML: Human leukemic monocyte lymphoma, HPLC: High pressure liquid chromatography, BS: Brine shrimp, BSLT: BS lethality test, DMSO: Dimethyl sulfoxide, MTT: 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide.

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INTRODUCTION

Schinus molle Linn.(*Anacardiaceae*) is an evergreen tree with leaves imparipinnate and a winged rachis and 20–40 leaflets; leaflets linear-lanceolate, margins entire or dentate, 2–5 cm × 4–8 mm and found in many parts worldwide and it has different uses.[1] In Mexico, the plant grows in Guerrero, Morelos, Oaxaca, Puebla, and Cd de México. It has different vernacular names in different places such as "pirú, pirul, preconcuahuitl, copalquahuitl, yag lachi (zapoteco), and ntaka (popoloca)." In Mexico, the leaves are used by traditional healers to treat stomach ache, colic, heartburn, diarrhea, and inflammatory disorders such as rheumatism and asthma and also are used to treat urogenital infections, endometritis, and leukorrhea. In addition, the leaves are used to treat malaria and as antitumoral.[2] *S. molle* possess terpenoids, flavonoids, and other phenolic compounds.[2-7] Biological studies carried out with alcoholic extracts of the leaves of *S. molle* show that this plant exerts several pharmacological effects such as antioxidant, trypanocidal, insecticidal, repellent, antidepressant, and cytotoxic.[6,8-12] Furthermore, the acute and subchronic exposure caused an increase in locomotor activity in rodents.[9,13,14] In addition, it is important to point out that histopathological studies in mice showed that extract is safe.[14]

Flavonoids occur in a variety of medicinal plants and display a broad variety of biochemical and pharmacological activities.[15] In fact,

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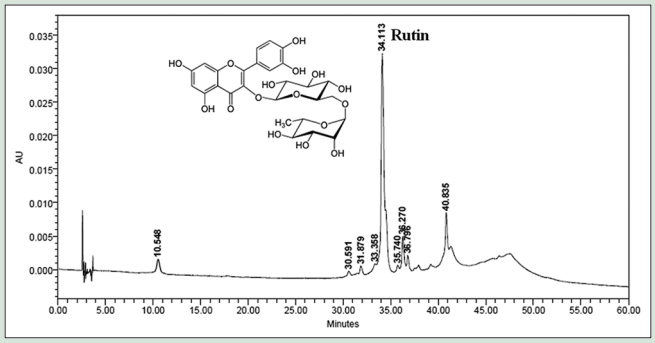

Figure 1: Chromatographic profile of ethanol extract of the leaves from *Schinus molle*

flavonoids have been reported to have antimicrobial, antiallergenic, antithrombotic, anti-inflammatory, antiproliferative, antiprotozoal, and antihyperglycemic activities.[15-19] In the case of rutin is extensively found in many plants, including *S. molle*, *Azadirachta indica*, *Tribulus terrestris*, *Olea europaea*, *Annona cherimola*, *Nelumbo nucifera*, *Croton lechleri*, and *Phoradendron serotinum*.[5,20-24] This flavonoid glycoside has a wide range of pharmacological activities such as anti-inflammatory, neuroprotective, antiulcerogenic, antimicrobial, antiviral, antiparasitic, antiurolithiatic, antihyperglycemic, antitumoral, and antioxidant.[5,18,20-26] Although rutin has shown antitumor properties in many murine models, its anticancer properties on human leukemic monocyte lymphoma remain to be researched. Therefore, the present work aimed to evaluate the antilymphoma potential of the ethanol extracts and rutin obtained from the leaves of *S. molle* using two induced lymphoma mice model systems; their cytotoxic activity on U-937 cells and lethality on brine shrimp (BS) larvae. Furthermore, HPLC analysis was realized to known the content of rutin in the leaves from *S. molle*.

MATERIAL AND METHODS

Plant material

S. molle leaves were collected by Dr. Fernando Calzada in May 2010 in the garden at National Medical Center Siglo XXI, IMSS, Cd Mexico, Mexico. Plant material was identified by M. en C. Abigail Aguilar Contreras of the Herbarium IMSSM of the Instituto Mexicano del Seguro Social (IMSS) where the voucher specimen is conserved under reference number No: 15670. The fresh plant material (600 g) was ground and extracted with EtOH (4 L) by maceration for 1 week (twice) at room temperature. Then, the macerate was filtered and evaporated to dryness under reduced pressure at 40°C. The EtOH extract (67 g; 11.1% w/w yield) was stored at −20°C for further analysis.

Chemicals

Rutin, isoquercitrin, quercitrin, quercetin, methotrexate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), L-glutamine, penicillin/streptomycin, RMPI 1640 medium, acetonitrile HPLC grade, acetic acid HPLC grade were purchased from Sigma-Aldrich, USA. EtOH and MeOH AR grade were purchased from JT Baker, Mexico. Fetal bovine serum was purchased from Gibco, Mexico.

Animals

Male CD-1 nu/nu nude and CD-1 mice (25–30 g) were obtained from the animal house of the IMSS. These studies were conducted with the approval of the Speciality Hospital Bio-Ethical Committee of the National Medical Center "Siglo XXI" from IMSS (Approval No: R-2014-3601-217, R-2012-3601-207, R-2014-785-015, and R 2014 3601 217). Investigation using experimental animals was conducted in accordance with the official Mexican norm NOM 0062-ZOO-1999 entitled technical specifications for the production, care, and use of laboratory animals.[27] To CD-1 mice, the animals were maintained with a 12 h light-dark cycle at 22°C ± 2°C at the controlled condition. They were fasted overnight, but tap water was available *ad libitum* until the start of the experiments. In the case of CD-1 nu/nu mice were housed under pathogen-free conditions with a 12 h light/12 h dark schedule and fed with an autoclaved diet and water *ad libitum*.

Cell culture

The human leukemic monocyte lymphoma U-937 cell line (ATCC: CRL 1593.2, Middlesex, UK) was used. Cell culture was tested for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza Walkersville, Inc.).

Culture conditions

Two million cells per milliliter were seeded in T75 cm^2 flasks (Invitrogen, Paisley, UK) in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum, 1.5 mM L-Glutamine, and 100 μg/ml penicillin/streptomycin (complete RPMI) and incubated at 37°C with 5% CO_2.[28]

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

This assay was used to determine the effect of the extract of the leaves from *S. molle* (EEL*Sm*) and rutin on cellular viability of U-937 cells. The EEL*Sm* and rutin were dissolved in DMSO, and the final concentration of DMSO used was 0.1% (v/v) for each treatment. Cells were seeded into white 96-well plates (Fisher Scientific, Loughborough, UK) at a density of 5.0×10^3 cells per well in 100 μL and treated with rutin or EEL*Sm* at five serial concentrations between 2 and 500 μg/mL for 24 h in 5% CO_2 at 37°C. Control cells treated with 0.1% DMSO served as the vehicle group. All treatments were performed in triplicate, in three independent experiments. After incubation for specified times, MTT reagent (10 μl, 5 mg dissolved in 1 mL of PBS) was added to each well and incubated for 4 h. The plates were centrifuged (10 min at 350 × g) and the purple formazan crystals of metabolized yellow tetrazolium salt by viable cells were dissolved in 150 μl of DMSO. Absorbance was quantified at 570 nm using the ELISA plate reader. Results were expressed as a percentage of viability, with 100% representing control cells treated with 0.1% DMSO alone. Then, the IC_{50} was determined. This was defined as the treatment concentration at which 50% reduction in cellular proliferation was observed. This was calculated from a linear regression equation; the regression coefficient, its level of significance, and correlation coefficient were calculated.[28]

Determination of the effect of ethanol extract of the leaves from *Schinus molle* and rutin on induced lymphoma mice model systems

Eleven groups (6 animals per group) of CD-1 nu/nu male nude mice or CD-1 mice (G1, G2, G3 [G3a, G3b, and G3c], G4 [G4a, G4b, and G4c], and G5 [G5a, G5b, and G5c]). For comparison, G1 was neither inoculated with cancer cells nor treated with EEL*Sm* or rutin. Groups G2 to G5 were injected intraperitoneally with 1×10^6 U-937 cells. As the group G2 was reserved as cancer control, it was not treated with EEL*Sm* or rutin but only with saline. Twenty-four hours after, the animals of G3, G4, and G5 were treated with EEL*Sm* (150, 100, and 50 mg/kg), rutin (15, 10, and 5 mg/kg), and methotrexate (1.5, 1.25, and 0.5 mg/

kg) orally, respectively. The treatment was continued for 9 days. Animals were maintained under observation for 30 days recording the daily survival.[29] After completion of the experiment, all animals were sacrificed, and axillary and inguinal lymph nodes were removed and weighted. Antilymphoma activity of the samples was measured as the total of weight of lymph nodes and expressed in percent of inhibition. After the plot of percentage of inhibition against concentration was made, the best straight line was determined by regression analysis and the 50% effective inhibitory concentration (EC_{50}) values were calculated. The regression equation and the coefficient of correlation were then derived from the curve.

Brine shrimp lethality test

The extract and pure compounds were routinely tested for BS lethality test (BSLT) using procedures and analysis described previously.[30,31] Samples of the EEL*Sm* (20 mg) and rutin (2 mg) for BSLT were dissolved in 2 mL of EtOH from this solution transfer 500, 50, and 5 µL to vials of 10 mL to obtain the final concentrations of 1000, 100, and 10 µg/mL (or 10, 1, and 0.1 in the case of rutin and methotrexate), respectively. Then, the EtOH was evaporated and was obtained six replicates in each concentration. Methotrexate (2 mg) was used a standard anticancer test drug and a control test (vehicle, EtOH) was also prepared. After, 4 mL of the artificial seawater was added to each test tube and 10 BSs were introduced into each tube. Thus, there were a total of sixty shrimps per dilution. Then, the volume was adjusted with artificial seawater up to 5 mL per test tube. The number of surviving shrimps was counted and recorded after 24 h. LC_{50} values were obtained from the best-fit line plotted concentration versus percentage lethality. The regression equation and the coefficient of correlation were then derived from the curve. LC_{50} of <100 ppm was considered as active, whereas LC_{50} value of >1000 µg/mL is nontoxic.[30,31]

High-pressure liquid chromatography analysis

High-pressure liquid chromatography (HPLC) was performed on a Waters 2795 instrument equipped with binary LC pump, a Water 996 Photodiode Array detector, and a Spherisorb S5 ODS2, analytical column (Waters, 250 mm × 4.6 mm × 5 µm); solvent A: acetonitrile, solvent B: 2% acetic acid as eluent gradient: (acetonitrile – 2% acetic acid [4: 96, v/v, 0 min; 12:88, v/v, 20 min; 20:80, v/v, 30 min; 50:50, v/v, 45 min; and 4: 96, v/v, 60 min; flow rate of 1.0 mL/min, λ 260 nm]). 20 mg of the EEL*Sm* was dissolved with MeOH (10 mL) and a sample of 20 µL was injected. Rutin was identified by comparison of its retention time (34.1 min; 72.1% of rutin/mg of extract) using commercial rutin as standard. The calibration curve of rutin was constructed by injecting, in triplicates, five concentrations of stock solutions (0.01, 0.03, 0.06, 0.09, and 0.12 mg/mL). The regression equation and the coefficient of correlation were then derived from the curve.

In addition, the flavonoids, quercitrin, isoquercitrin, and quercetin were identified but were not tested.

Statistical analysis

The plot of percentage of inhibition against concentration was made; the best straight line was determined by regression analysis, and the EC_{50} or IC_{50} or LC_{50} was calculated. All data were expressed as mean ± standard deviation of six measurements. Statistical analysis of data was performed using ANOVA one-way. A probability value of $P < 0.05$ was considered statistically significant. Differences between groups were analyzed by Bonferroni and Dunnett *post hoc* test. Analyses were performed using GraphPad Prism Version 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

Table 1: Antilymphoma, cytotoxic, and toxic properties of the ethanol extract of the leaves from *Schinus molle* and rutin

Sample	Antilymphoma activity (EC_{50} mg/kg)*		Cytotoxic activity (IC_{50} µg/mL)*	BS lethality test (LC_{50} µg/mL)*
	Mice		Cells (U-937)	
	Nu/nu	CD-1		
EELSm[a]	52.2±0.92	99.4±0.50	172.0±0.017	22.2±0.201
Rutin	9.5±0.18	6.8±0.13	9.6±0.067	5.8±0.311
Methotrexate,	0[b]	0.86±0.02	ND	72.4±0.269

[a]Collected in May 2010, [b]10 mg/kg, 7.5 mg/kg, 5 mg/kg, 2.5 mg/kg, and 1.25 mg/kg caused 100% of mortality in nu/nu mice, ND: Not determined, correlation coefficient >* $P<0.05$. BS: Brine shrimp; EEL*Sm*: Ethanol extract of the leaves from *Schinus molle*

Table 2: Flavonoids by high-pressure liquid chromatography analysis of ethanol extract of the leaves from *Schinus molle*

Flavonoids	Retention time (min)	Percentage/mg of EEL*Sm*
Unknown	10.5	3.33
Unknown	30.5	0.92
Unknown	31.8	2.27
Unknown	33.3	1.14
Rutin	34.1	72.44
Quercitrin	35.7	1.16
Isoquercitrin	36.2	7.86
Unknown	36.7	2.75
Quercetin	40.8	8.13

EELSm: Ethanol extract of the leaves from *Schinus molle*

RESULTS AND DISCUSSION

Antilymphoma, cytotoxic, and toxic properties [Table 1] of the ethanol extract of leaves from *S. molle* and its major flavonoid, rutin, were studied on induced lymphoma mice model systems. According to the results, the EEL*Sm* showed strong antilymphoma activity on athymic CD-1 nu/nu mice inoculated with U-937 cell line with EC_{50} from 52.2 mg/kg on the growth of the solid tumors. In contrast, the antilymphoma effect on CD-1 mice was moderate with EC_{50} from 99.4 mg/kg. In the case of major flavonoid rutin, it showed strong antilymphoma properties in both mice with EC_{50} values of 9.5 mg/kg for nu/nu mice and 6.8 mg/kg for CD-1 mice. In CD-1 mice model, rutin was less active than methotrexate (EC_{50} 0.86 mg/kg). In these contexts, it is important to point out that at rutin showed important antilymphoma activity on nu/nu mice and methotrexate caused 100% of mortality at doses tested.

The EEL*Sm* and rutin showed cytotoxicity on U-937 cells with IC_{50} values of 172.0 and 9.6 µg/mL, respectively. In relation with the BSLT, it was used to predict of cytotoxic activity.[30] In these sense, the extract and rutin showed strong lethality on BS larvae with LC_{50} values of 22.2 and 5.8 µg/mL, respectively. The cytotoxic activity on BSs of rutin and the EEL*Sm* was major than of methotrexate a standard anticancer drug, used as positive control. This significant lethality of the EEL*Sm* and rutin to BS is an indicative of their potential cytotoxic effects.[30,31]

It is important to point out that, previously, of *S. molle* leaf and fruit essential oil, several terpenoids had been reported such as preisocalamendiol, shyobunol, lupenone, β-myrcene, limonene, α-pinene, β-phellandrene, and α-phellandrene. These compounds have been associated with its antioxidant, antimicrobial, tranquilizing, insecticidal, and anticancer properties.[3,32-38] In this sense, the anticancer properties have been associated with the presence of the cyclic monoterpene, α-phellandrene. It monoterpene-induced DNA damage and affect DNA repair protein expression in WEHI-3 murine leukemia cells.[3,32-37] In the case of

ethanolic and methanolic extracts from the aerial parts from *S. molle,* several flavonoids had been isolated such as 2"-*O*-α-L-rhamnopyranosyl-hyperin6"-*O*-gallate,2"-*O*-alpha-L-rhamnopyranosyl-hyperin,quercetin 3-*O*-β-D-neohesperidoside, quercetin 3-*O*-β-D-galacturonopyranoside, isoquercitrin, hyperin, isoquercitrin 6"-gallate, hyperin 6"-O-gallate, (+)-catechin, and rutin.[6,11] These flavonoids have been associated with its antioxidant, antidepressant-like, and cytotoxic effects.[6,11,12,22] In the case of rutin (3′ ,4′ ,5,7-tetrahydroxy-flavone-3-rutinoside), it is a glycosyl flavone that is widely consumed from plant-derived beverages and foods as traditional medicine worldwide. Till date, over 130 registered therapeutic medicinal preparations are containing rutin in their formulations. To date, it is reported that more than seventy plant species contain rutin. The major commercial sources of rutin include *Ruta graveolens* L. (*Rutaceae*), *Sophora japonica* L. (*Fabaceae*), *Maranta leuconeura* E. Morren (*Marantaceae*), *Orchidantha maxillarioides* (Ridl.) Schum (*Lowiaceae*), *Strelitzia reginae* Banks ex Aiton (*Strelitziaceae*), *Eucalyptus* spp. (*Myrtaceae*), *Canna indica* L. (*Cannaceae*), *Canna edulis* Ker Gawl. (*Cannaceae*), and *Labisia pumila* (*Blume*) Mez (*Primulaceae*); these species contain up to 1.5% of rutin. Rutin has a wide range of pharmacological activities such as anti-inflammatory, neuroprotective, antiulcerogenic, antimicrobial, antiviral, antiparasitic, antiurolithiatic, antihyperglycemic, antitumoral, and antioxidant.[39-44] Antitumor effects have been demonstrated on nude mice bearing SW 480 tumors, skin carcinogenesis in Swiss albino mice, murine leukemia (WEHI-3), and colonic neoplasia.[3,24,25,36] Furthermore, it has shown antitumor effects in models such as NK/Ly ascites and B16F10 cells.[24] To our knowledge, this is the first report of antilymphoma activity of the EELS*m* and rutin. Since that the EELS*m* and rutin tested in this study exhibited antilymphoma properties in two induced lymphoma mice model systems and cytotoxic activity against U-937 cells. The results presented here confirm the antitumor properties of the EELS*m* and rutin and extend these studies. Rutin may be useful in cancer prevention as chemopreventive agent that can inhibit initiation and also to act as blocking and suppressing agent.[24,37] In addition, it can help attenuated effects caused by antitumor agents as cisplatin.[39]

On the other hand, the results obtained in BSLT to EELS*m* and rutin showed a good correlation with antilymphoma and cytotoxic properties using U-937 cells. EELS*m* showed strong lethality on BS larvae with LC_{50} value of 22.2 μg/mL [Table 1], it is in agreement with the previously reported to essential oils obtained from the leaves of *S. molle* (LC_{50} of 47.4 μg/mL).[40,41] In these sense, BSLT may be a biological model for the preliminary selection of antilymphoma components from medicinal plants. *Artemia salina* has been suggested for use as a model for several preliminary evaluations of pharmacological activities such as insecticidal, antioxidant, cytotoxic, antimutagenic, antimicrobial, and antitumor activity.[30,31,40,41]

Ethanol extract of *S. molle* leaves collected in Mexico was obtained by maceration and characterized by HPLC analysis [Figure 1 and Table 2] allowing the identification of rutin as major active constituent. The ethanol extract was chromatographed and resolved by HPLC, using a Spherisorb S5 ODS2 analytical column. Rutin was identified by comparison with authentic sample (Sigma) available in our laboratory. Considering the major constituent, the literature reveals that the ethanol extract of the aerial parts from *S. molle* had high concentrations of rutin.[5] In agreement with this result, the HPLC analysis revealed that rutin is the major constituent of the EELS*m*. These results support traditional medicinal use of *S. molle* as antitumoral[2] and also suggest that rutin may play an important role in anticancer properties of *S. molle*. Additional studies to evaluate antilymphoma properties of flavonoids such as quercitrin, isoquercitrin, quercetin, and other compounds will be carried out to explain the antilymphoma effects of the EELS*m*. Furthermore, of the EELS*m* and rutin will be studied in cellular proliferation, viability, apoptosis, proteomic, and genomic approach to confirm their potential uses as therapeutic agents.

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Conflicts of interest

There are no conflicts of interest.

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