TOXICITY EVALUATION AND POLYPHENOLS ASSESSMENT OF SOME EXTRACTS FROM INDIGENOUS *EUPHORBIA* SPECIES

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Abstract: Euphorbia is a large genus with over 2000 species of terrestrial plants. We assessed the phenolic content of *E. platyphyllos*, *E. stricta* and *E. cyparissias*, in order to establish if these species could be used in phytotherapy as polyphenol sources. We checked for the presence of flavonoids and phenolic acids, glycosides and aglycones by thin layer chromatography and quantified the total phenolic content (TPC). The toxicity of the aqueous extracts was investigated on two invertebrate species: *Artemia salina* and *Daphnia magna*. Caffeic acid, quercetin and hyperoside were identified in all three extracts. Isoquercitrin was identified in *E. platyphyllos* and *E. stricta* extracts and kaempferol in *E. cyparissias* extract. The highest TPC was found in *E. stricta* (206.97 ± 9.8715 µg/mg), followed by *E. platyphyllos* (84.89 ± 1.8529 µg/mg) and *E. cyparissias* (49.33 ± 1.8529 µg/mg). The highest toxicity was induced by *E. cyparissias* on *A. salina*, followed by *E. platyphyllos* and *E. stricta*. On *D. magna*, the highest toxicity was found to be induced by *E. stricta*, followed by *E. platyphyllos* and *E. cyparissias*. The toxic effect of all three extracts is moderate to low thus supporting the use of the three plant species as sources of phenolic compounds.

Key word: Euphorbia, phenolic compounds, thin layer chromatography, *Artemia salina*, *Daphnia magna*.

INTRODUCTION

Euphorbia is a large genus of cosmopolitan terrestrial plants (over 2000 species), most of them native to tropical and subtropical areas on the globe [9, 38].

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The spontaneous Romanian flora includes some *Euphorbia* species. Among these we mention: *E. platyphyllos* (broadleaf spurge), *E. stricta* (upright spurge) and *E. cyparissias* (cypress spurge).

Euphorbia species have different secondary metabolites belonging to the following main classes: triterpenoids, diterpenoids, flavonoids, tannins and lipids [3, 10, 29, 31, 33]. Most of the studies are focused on chemical composition and pharmacological activity of the latex, which is present on all species of this genus. The compounds from the latex are mostly diterpenes – phorbolester, ingenole and euphorbone [3, 10]. Cycloartane triterpenoids are other latex compounds present mainly in tropical species, but they were also found in *E. cyparissias* and *E. broteri* [31]. The compounds from the latex are responsible for the toxicity of these plants and are incriminated for acute dermatitis on local application, poisoning if consumed [1, 12, 27], and even carcinogenic on chronic ingestion [37].

Although latex contains some flavonoids, these are usually located in other tissues [3]. Flavonoids and phenolic acids have been identified in various species of *Euphorbia* genera. Quercetin and kaempferol were identified in *E. helioscopia* and quercetin-3β-D-galactopyranoside gallate has been reported in *E. platyphyllos* [33]. The two main flavonoids isolated from *E. cyparissias* were kaempferol-3-glucuronide and quercetin-3-glucuronide [29]. Soboleva *et al*. (1971) established that quercetin, hyperoside and isomyricetin are common to fifteen species of *Euphorbia*, including *E. stricta* and *E. cyparissias* [33]. The authors also identify myricetin and stepposide, but only in *E. esula* [33]. In *E. lucida* isoquercitrin, avicularoside, hyperoside and rutin were revealed and in *E. maddeni* two glycosides with hypotensive properties – hyperin and kaempferol -4'-O-glucoside. Apigenin glycosides were found in *E. larica* and luteolin glycosides in *E. soongarica* and *E. alatavica* [22]. In *E. hirta*, besides quercetin and kaempferol, there were reported euphorbianin, quercitrin, gallic acid, galloylquinic acid derivatives and euphorbins A-E [4, 8, 28].

In the last two decades, studies on flavonoids from *Euphorbia* genera revealed various pharmacological activities such as anti-inflammatory, antioxidant, anti-malaria, anti-urease, cytotoxicity, DNA damaging, apoptotic promoter and antiproliferative [2, 5, 6, 13, 19, 23]. To the best of our knowledge, the capitalization of the three species as polyphenol sources has not been proposed yet. This is probably due to the toxicity exhibited by latex compounds.

In this research we assessed the phenolic content, in order to establish if these species could be used in phytotherapy as polyphenol sources. Therefore, we determined the presence of flavonoids and phenolic acids glycosides and aglycons by thin layer chromatography (TLC) and the total phenolic content (TPC) by the Folin-Ciocâlteu method. Due to possible toxicity of *Euphorbia* species, the effect of the aqueous extracts on two invertebrate species, *Artemia salina* and *Daphnia magna*, was also investigated.

Artemia salina and *Daphnia magna* bioassays are alternative methods for toxicity testing on invertebrate animals (crustaceans). These methods have a high degree of correlation with the acute toxicity registered in rodents (mice, rats) and can be predictive for the cytotoxicity on human cells cultures [15, 16, 18, 20]. The methods are commonly used to test the toxicity of plant extracts and natural compounds [30, 35].

MATERIAL AND METHODS

PLANT MATERIAL

The plant material was harvested from Nereju, Vrancea County, (*E. cyparissias*) and Buzău (*E. platyphyllos* and *E. stricta*) from the spontaneous flora, during blooming period (June-August, 2013). Voucher specimens are stored at the herbarium of the Department of Pharmaceutical Botany from the Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest. The identification of the three species was conducted both at the site of harvesting and in the laboratory according to the macroscopic characters described in the literature [7, 19, 34].

The aerial parts (stems, leaves, flowers and fruits with seeds) of the fresh plants were dried in the dark, at 25±3 ºC in the laboratory and the dried material was manually cut into small pieces and ground in a mill, then sieved through a 5 mm sieve.

PLANT EXTRACTS

Samples of 1.00 g of each species were extracted for 30 min with 100 mL distillate water by heating under reflux (2 times). The two extractive solutions were combined and the resulting solutions were concentrated at 40 ºC using a rotary evaporator (RVO04, Czech Republic), and lyophilized at –55ºC (ScanVac 55, Denmark).

SAMPLE PREPARATION

For TLC identification of flavonoids and for the quantitative assessment of TPC, 0.1000 g of each dry extract was dissolved in 10 mL ethanol 50%.

For TLC identification of flavonoid aglycones, 0.1000 g of each dry extract was dissolved in 25 mL distilled water. 25 mL 1N HCl were added and the mixture was heated under reflux for at least 45 min. After cooling, the solution was purified

five times with 15 mL diethyl ether and the aqueous solution was discarded. The etheric solutions were combined and reduced to about 3 mL and completed to 5 mL with the same solvent.

CHROMATOGRAPHY CONDITIONS

- Stationary phase: silica gel 60 F254 on Al support ready-to-use plates (Merck, Germany);
- mobile phase 1 (MP 1): ethyl acetate – formic acid – water, 1:1:8 (v/v), for the identification of polyphenols (glycosides);
- mobile phase 2 (MP 2): toluene – ethyl acetate – formic acid, 5:3:1 (v/v) for the identification of phenolic aglycones;
- standard solutions were obtained by dissolving the reference substances in ethanol 96%:
  - 1 mg/mL: caffeic acid, chlorogenic acid, quercetin, kaempferol, myricetin, luteolin, resveratrol, umbelliferone (Sigma-Aldrich, USA), rutin trihydrate (Roth, Germany);
  - 0.2 mg/mL: hyperoside, isoquercitrin (Roth, Germany);
- visualization reagents: Natural Reagent Product (diphenylboric acid aminoethyl ester) (Roth, Germany) and visualization at 366 nm using a UV lamp (Camag, Switzerland) [26, 36].

QUANTITATIVE DETERMINATION OF THE PHENOLIC COMPOUNDS

The assay of TPC was performed according to the Folin Ciocâlteu method described by González *et al*. (2003) with some modifications [14].

The calibration curve was prepared using gallic acid as a standard (Sigma-Aldrich, USA). The standard solution was obtained by dissolving 50 mg gallic acid in 10 mL ethanol 50% and then diluted to 100 mL with the same solvent. Aliquots of different volumes were used to obtain samples of final concentrations of 0.5–10 μg/mL (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5 and 10.0 μg/mL). After the addition of Folin-Ciocâlteu reagent and Na_2CO_3 10% solution, the samples were completed to 10 mL with ethanol 50%. The samples were maintained for 15 min at 50 ºC in the dark using a water bath (Memmert WNB10, Germany). After cooling the absorbance of samples was measured at 740 nm using a UV-VIS spectrophotometer (Halo DB-20-220 Dynamica Precisa, Germany).

The samples were diluted in order to fit the range of linearity and the same procedure was followed as described in the preparation of the calibration curve.

All determinations were performed in triplicate and the results were expressed as mean ± standard deviation.

The limits of detection (*LD*) and quantification (*LQ*) were estimated according to the Q 2(R1) ICH Guidelines of Validation of Analytical Procedures: Text and Methodology (1995) [39], based on the following formulas:

$$LD = 3.3 \times \frac{\sigma}{S} \quad (1)$$

$$LQ = 10 \times \frac{\sigma}{S} \quad (2)$$

where: σ = the standard deviation of the regression line; *S* = the slope of the calibration curve.

BIOLOGICAL ASSAYS

Artemia salina bioassay

A modification to previous protocols was used [24, 25, 17]. 500 mg of brine shrimp cysts were incubated in artificial seawater (40 g/L salinity) at 25±1 ºC for 24 h, with a 16 h photoperiod and 8 h darkness in a plant growth chamber (Sanyo MLR-351 H, USA). Newly hatched nauplii were transferred into another breaker containing fresh artificial seawater, and maintained for another 24 h in the same conditions. After the incubation, 20 µL of seawater with ten 48 h old nauplii were pipetted in 9-well culture plates (Labsystems, Finland) with 80µL of fresh artificial seawater. Five dilutions of the plant extracts were made by dissolving the dry extracts into DMSO and then diluted in order to obtain concentrations in the range of 0.005–2.00 mg/mL, for a final volume of 500 µL and a final concentration of DMSO of 1%. Artificial seawater and 1% DMSO in seawater were used as controls. The tests were performed in quadruplicate. The number of surviving nauplii (the larvae were considered dead only if they did not move their appendages for 30s during observations) was counted at the end of 24 h and mortality was calculated as percentage of death in 40 nauplii.

Daphnia magna bioassay

Daphnia magna Straus. have been maintained parthenogenetically in "Carol Davila" University, Department of Pharmaceutical Botany and Cell Biology, since 2012. 24 h before the assay, several daphnids were selected according to their size and kept in fresh synthetic water under continuous aeration. The bioassay was performed according to the method described in literature [11, 24]. 10 daphnids were inserted in graduated test tubes, added eight dilutions of the same solutions used for the *Artemia* test and completed with synthetic water, in order to obtain concentrations in the 0.005–2.00 mg/mL range, for a final volume of 10 mL and a final 1% DMSO concentration. Synthetic water with and without 1% DMSO were

used as controls. The daphnids were kept in the same conditions as those described for *Artemia salina* bioassay.

The tests were performed in triplicate. The number of surviving daphnids (the daphnids were considered dead only if they did not move their appendages for 30s during observations) was counted at the end of 24 h and mortality was calculated as percentage of death in 30 daphnids.

DATA ANALYSIS

TLC analysis. For each compound, the retention factor (R_f) and the fluorescence ($\lambda = 366$ nm) were compared with those registered for the standards.

Phenolic compounds quantitative determination. The regression equation, standard deviation of the curve and correlation coefficient were calculated.

Biological assays. Five different concentrations for *A. salina* and eight for *D. magna* bioassays were used to determine the concentration of extract that kills 50% of the invertebrate organisms (LC_{50}). LC_{50} and their 95% confidence intervals (*CI*95%) were calculated by means of logarithm of concentration *vs*. lethality curves using the least squares fit method. The goodness of fit was evaluated based on the squared correlation coefficient (r^2).

All statistical interpretation of data was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

RESULTS AND DISCUSSION

TLC ANALYSIS

In all three species of *Euphorbia* the following phenolic compounds were identified: caffeic acid ($R_f = 0.40$), quercetin ($R_f = 0.44$) and hyperoside ($R_f = 0.37$). Caffeic acid was identified only after acid hydrolysis, perhaps due to its presence in the glycoside form. Also, in all extracts, a pale-blue spot ($R_f = 0.51$, MP 2) was observed, corresponding probably to resveratrol or umbelliferone. In both, *E. platyphyllos* and *E. stricta* isoquercitrin ($R_f = 0.44$, MP1) was identified, and in *E. cyparissias* extract kaempferol ($R_f = 0.56$ MP2). Other major spots with positive reaction for phenolic compounds were observed in *E. platyphyllos* (MP 1: $R_f = 0.27$, green color, possibly phenolic acid; MP 2: $R_f = 0.88$, a blue spot, probably phenolic acid), *E. stricta* (MP 1: $R_f = 0.33$, green color, possibly phenolic acid, $R_f = 0.62$ and 0.71 two orange spots, which probably correspond to some flavonoids; MP 2: $R_f = 0.88$, a blue spot, probably phenolic acid) and *E. cyparissias* (MP1: $R_f = 0.33$, green color, possibly phenolic acid and $R_f = 0.42$, orange spot, probably flavonoid; MP 2: $R_f = 0.88$, a blue spot, probably phenolic

acid). The synthesis of the results is presented in Table 1. All identified compounds are mentioned in the literature [4, 8, 28, 29, 33].

Table 1

Qualitative assessment of the phenolic compounds of the three extracts of *Euphorbia*

Standard	***E. platyphyllos***	***E. stricta***	***E. cyparissias***	**Standard R_f**
Flavonoids and phenolic acids glycosides (MP1)				
Hyperoside	x	x	x	0.37
Rutin	–	–	–	0.17
Isoquercitrin	x	x	–	0.44
Chlorogenic acid	–	–	–	0.27
Caffeic acid	x	x	x	0.96
Flavonoids and phenolic acids aglycones (MP2)				
Luteolin	–	–	–	0.35
Chlorogenic acid	–	–	–	0.00
Caffeic acid	x	x	x	0.40
Resveratrol	–	–	–	0.51
Quercetin	x	x	x	0.44
Umbelliferone	–	–	–	0.51
Kaempferol	–	–	x	0.56
Myricetin	–	–	–	0.38

x – present; – – not found.

QUANTITATIVE DETERMINATION OF THE PHENOLIC COMPOUNDS

The regression analysis of gallic acid calibration curve showed a good linearity ($r^2 = 0.9980$) in the 0.5 – 10.0 µg/mL range. Calculated *LD* and *LQ* values were 0.50 µg/mL for *LD* and 1.51 µg/mL for *LQ*.

The results of quantitative analysis are summarized in Table 2.

Table 2

The total content of polyphenols of the aqueous extracts (expressed as gallic acid)

Nr. crt.	**Sample**	***M*±σ (µg/mg)**	***CI*95% (µg/mg)**	***RSD*%**
1.	*E. platyphyllos*	84.89 ± 2.1350	79.58 – 90.20	2.51
2.	*E. stricta*	206.97 ± 9.8715	182.40 – 231.50	4.77
3.	*E. cyparissias*	49.33 ± 1.8529	44.72 – 53.94	3.75

M – average; σ – standard deviation, *CI*95% – confidence interval (α = 0.05); *RSD*% – relative standard deviation.

The highest TPC was found in *E. stricta*. The value is about 2.4 times higher than the one found in *E. platyphyllos* and almost 4.2 times higher than the one found in *E. cyparissias*.

Because of the high values of the TPC found in all three extracts, the plants can be used as sources of phenolic compounds.

BIOLOGICAL ASSAYS

As the extracts presented a convenient amount of polyphenols, we further tried to evaluate the toxicity of the three extracts, in order to find out if the three plants can be reliable sources to be used in phytotherapy.

The results of biological assays are presented in Table 3. The lethality *vs*. concentration logarithms curves are presented in figure 1.

On *Artemia salina* the highest toxicity was induced by the *E. cyparissias* extract, followed by those of *E. platyphyllos* and *E. stricta*. All extracts exhibit low to moderate toxicity on brine shrimps ($0.250 < LC_{50} < \sim 1.500$ µg/mL) according to toxicity scale developed by Meyer *et al*. (1982), where the highest toxicity was induced by podophyllotoxin (0.0024 mg/mL) and the lowest by santonin (>1.0000 mg/mL) [21]. Previous researches on lethality induced by *Euphorbia* species on brine shrimps revealed high toxicity of seeds extract of *E. cyparissias* (LC_{50} = 368 µg/mL). To the best of our knowledge, brine shrimp lethality test was not performed on *E. stricta* and *E. platyphyllos*.

On *D. magna*, the extract from *E. stricta* exhibits toxicity at a much lower dose (about 4.8 times lower), and the extract from *E. cyparissias* at a higher dose (about 3.5 higher) by comparison with brine shrimp assay. The toxic effect of the extract from *E. platyphyllos* is comparable with the effect on *A. salina*. The decreasing order of toxicity of the extracts on *D. magna* is: *E. cyaprissias*, *E. platyphyllos* and *E. stricta*.

Table 3

Toxicity of the aqueous extracts from *E. platyphyllos*, *E. stricta* and *E. cyparissias* on *A. salina* and *D. magna*

Nr. crt.	**Sample**	**LC_{50} (mg/mL)**	***CI*95% of LC_{50} (mg/mL)**	**Goodness of fit (r^2)**
***Artemia salina* bioassay**				
1	*E. platyphyllos*	0.3255	0.0293 – 3.6160	0.8945
2	*E. stricta*	1.5450	0.3160 – 7.5560	0.9656
3	*E. cyparissias*	0.2564	0.0368 – 1.7850	0.9165
***Daphnia magna* bioassay**				
4	*E. platyphyllos*	0.3952	– [a]	– [b]
5	*E. stricta*	0.3218	0.2812 – 0.3682	0.9700
6	*E. cyparissias*	0.9043	0.8342 – 0.9802	0.9404

a – *CI*95% is very wide and could not be calculated; b – r^2 could not be calculated.

Although the two methods are commonly used to assess the toxicity of plant extracts [15], we have not identified in the literature mentions on testing the three *Euphorbia* species by *Daphnia magna* bioassay.

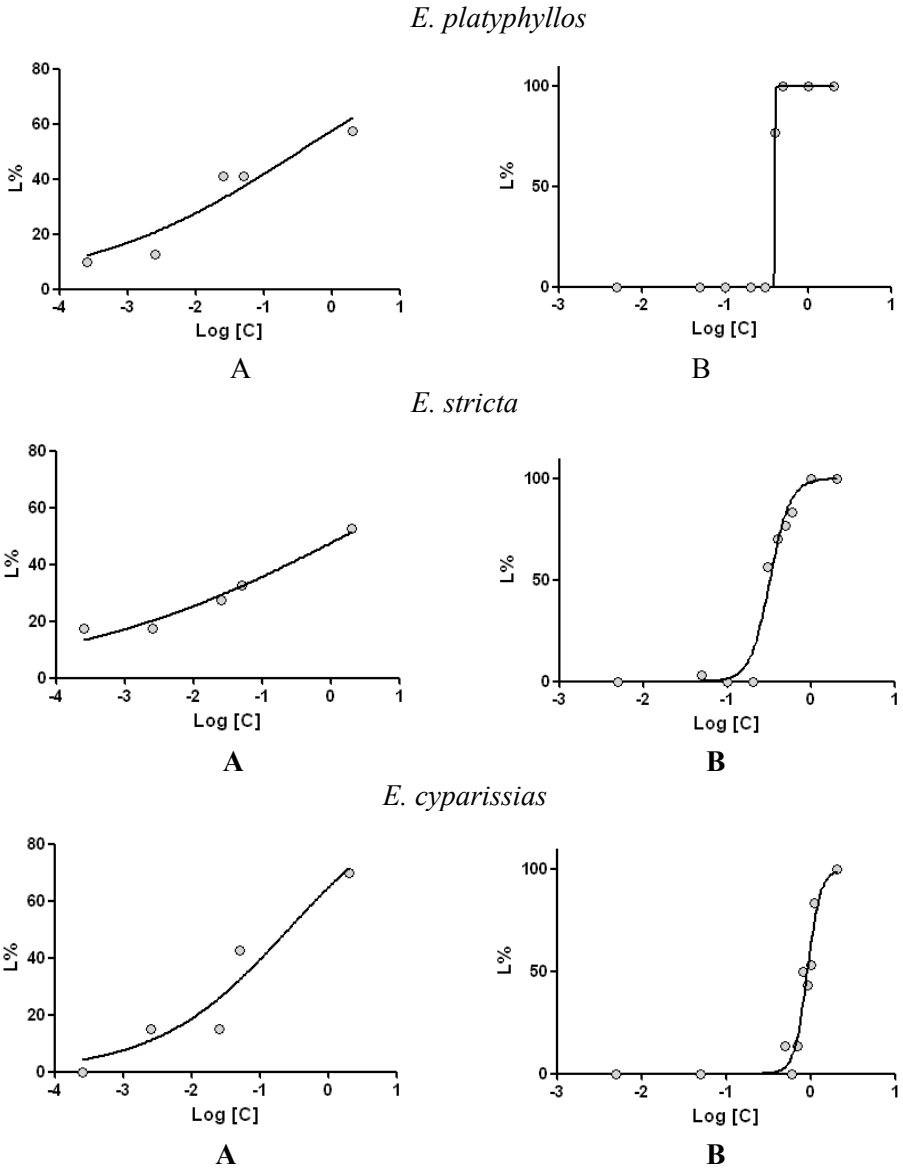

Fig. 1. Dose-lethality curves: A – *Artemia salina* bioassay; B – *Daphnia magna* bioassay.

Although the differences found in the two methods of toxicity assessment are relatively high, the toxic effect of all three extracts is moderate to low (LC_{50} > 250 μg/mL) for both methods, supporting the use of the three plant species as phenolic sources [21].

CONCLUSIONS

Species of *Euphorbia* have an increased content of polyphenolic compounds, but due to their latex toxic properties, their use in therapy is limited. *E. platyphyllos*, *E. stricta* and *E. cyparissias* are three species native to the S-E Europe of this genus. In this study, we have investigated the polyphenolic compounds from the aqueous extracts of these species. We have identified several phenolic compounds, assayed the TPC and their toxicity on invertebrate animals.

Caffeic acid, quercetin and hyperoside were identified in all three extracts. Isoquercitrin was identified in *E. platyphyllos* and *E. stricta* extracts and kaempferol in *E. cyparissias* extract. The highest TPC was found in *E. stricta* (206.97 ± 9.8715 µg/mg), followed by *E. platyphyllos* (84.89 ± 1.8529 µg/mg) and *E. cyparissias* (49.33 ± 1.8529 µg/mg).

The highest toxicity was induced by *E. cyparissias* on *A. salina*, followed by *E. platyphyllos* and *E. stricta*. On *D. magna*, the highest toxicity was found to be induced by *E. stricta*, followed by *E. platyphyllos* and *E. cyparissias*.

Although the differences found in the two methods of toxicity assessment are relatively high, the toxic effect of all three extracts is moderate to low (LC_{50} > 250µg/mL) for both methods. The low toxicity correlated with the high TPC content sustain the use of the three plant species as phenolic compounds sources. Further studies are necessary in order to separate the irritants compounds that are found in latex and capitalize the phenolic compounds from these species.

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