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Original Article

Antioxidant, Antimicrobial and Phytochemical Investigations of Polar Extracts of *Euphorbia resinifera* Beg., Roots, Stems and Flowers

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ABSTRACT

The phytochemical screening of roots, stems and flowers of *Euphorbia resinifera* Beg., revealed the presence of saponins, polyphenols, flavonoids, tannins, terpenoids, coumarins, and cardiac glycosides. Quantitative analysis showed that the methanolic extract of roots is the richest in phenolic compounds with an amount of 131.48 ± 8.5 µg GAE/mg of extract. While the methanol extract of the stems is the richest in flavonoids with an amount of 39.14 ± 0.48 µg QE/mg of extract. The antioxidant activity of polar extracts was studied with the method of DPPHº, the methanol extract of the roots showed a strong antioxidant activity (IC_{50} = 10.01 ± 0.17 µg/ml). The amount of plyphénoles was correlated with the presence of phenolic compounds with (ρ = -0.87, p <0.05). *In vitro* antimicrobial activity of polar extracts was determined against five bacteria and one yeast strain. The results show that extract possessed antimicrobial activity against *S. aureus, B. subtilis, M. luteus*, MBCs and MICs were determined for the first two strains. The extract of ethyl acetate of roots showed the highest antimicrobial activity (MIC and MBC = 0.5 mg/ml) against *B. subtilis*, and (MIC = 0.5 mg/ml and MBC = 1 mg/ml) against *S. aureus*.

Keywords: *Euphorbia resinifera* Beg., Phytochemical screening, Antioxidant activity, DPPHº, IC_{50}, Antimicrobial activity, MIC, MBC.

INTRODUCTION

Euphorbia resinifera Berg., is a species of spurge native to Morocco, where it occurs on the slopes of the Atlas Mountains[1]. The dried latex of this plant has long been an article of commerce under the name of Euphorbium, and is one of the oldest drugs in the Western medicinal tradition. *Euphorbia resinifera* contains a high concentration of the toxin resiniferatoxin (RTX) which is being used as a starting point in the development of a novel class of analgesics[2]. (See image 1.)

RTX was discovered thanks to its extraordinary activity in the mouse-ear erythema assay, in which it is approximately a thousand fold more potent than tetra-decanoylphorbol acetate (TPA), the most powerful tumour-promoting phorbol ester. RTX was later also recognised as an ultrapotent vanilloid, outperforming capsaicin in binding assays by a factor of up to ten thousand[3,4].

We noted that most studies on *Euphorbia resinifera* are focused on the latex of the plant, while it lacks studies on other parties of the plant. In this study the antioxidant, antimicrobial and phytochemical investigations of the polar extracts of *Euphorbia resinifera* Beg., roots, stems and flowers were determined.

The use of synthetic antioxidants in the food industry as additives is being questioned because of the toxicological risks of these compounds[5]. In addition, excessive use of chemical antibacterial agents in human medication led to the emergence of resistance strains bacterial[6]. Hence the need to develop new therapeutic agents to fight against the phenomene of resistance bacterial and oxidation of food. To this aim, the investigation of plants represents a huge potential for the discovery new extracts or compounds with antimicrobial and antioxidant power.

In this context, the objectives of this study were (i) to determine the chemical composition of polar extracts from leaves stems roots of *Euphorbia resinifera*, (ii) to evaluate the antioxidant and antimicrobial power of different polar extracts of *Euphorbia resinifera* especially the roots that have not, to our knowledge, been studied, (iii) the study of possible correlations between phenolic compounds, and antioxidant activities.

MATERIALS AND METHODS

Plant material

The whole plant of *Euphorbia resinifera* was collected from Banimelal region, Morocco in March 2013. Taxonomic identification was performed by Dr. A. Chahed (National Institute of Medicinal and Aromatic Plants, Morocco).whole plant were dried at room temperature and powdered, stored for further analyses.

Extraction

100g of each powdered sample (leaves, stems and roots) was extracted successively with hexane (1L), ethyl acetate (1L), methanol (1L) for 24h with stirring. The obtained extracts were evaporated and stored for further analyses.

Phytochemical screening

The ethyl acetate and methanolic extracts were screened for phytochemical constituents.

Test for phenolic compounds (Ferric chloride test)

A small amount of each extract was dissolved in 5 ml of distilled water and a few drops of 1% ferric chloride have been added. The appearance of a dark green color indicates the presence of phenolic compounds[7].

Test for flavonoïds (Alkaline reagent test)

A few drops of sodium hydroxide were added to extracts dissolved in distilled water, the appearance of an intense yellow color indicates the presence of flavonoïds. Disappearance of the color after the addition of dilute hydrochloric acid confirms the presence of flavonoïds[7].

Test for tannins

Each extract dissolved in distilled water, a solution of 1% ferric chloride was added. The appearance of a green color indicates the presence of tannins[8].

Test for terpenoïds

A dilute solution (2 mL) in chloroform of each extract, 2 mL of H_2SO_4 conc was added. The appearance of a red color at the interface indicates the presence of terpenoïds[9].

Test for coumarins

An amount of each extract was dissolved in distilled water, 3 mL of 10% NaOH was added, and the appearance of a yellow color indicates the presence of coumarins[10].

Test for saponins

A diluted solution of each sample (10 mL) in distilled water was stirred vigorously until the formation of foam. A few drops of olive oil have been added, the mixture was stirred vigorously for a few minutes, the formation of an emulsion was confirmed the presence of saponins[9].

Test for alcaloïds

A quantity of the extract was stirred with a few drops of dilute hydrochloric acid and then filtered. The filtrate was used for following tests[7].

Mayer test

A few ml of filtrate, one or two drops of the Mayer reagent are added. The appearance of a white or creamy precipitate indicates the presence of alkaloids[7].

Wagner Test

A few ml of filtrate, few drops of Wagner reagent are added. The appearance of a reddish brown precipitate confirms the presence of alkaloids[7].

Test for cardiac glycosides (Keller-Kiliani test)

A small quantity of each extract was mixed with 2 ml of glacial acetic acid solution containing one drop of 0.1% ferric chloride. The mixture was then treated with 1 ml of concentrated sulfuric acid. The formation of brown ring at the interface or a greenish ring in the acetic acid layer confirms the presence of cardiac glycosides[9].

Test for amino acids and proteins

An amount of each extract was dissolved in distilled water and then filtered. The filtrate was used for the following tests[7].

Biuret test

A few ml of the filtrate, a drop of copper sulfate solution (2%) to 2 ml of ethanol (95%) was added, followed by the addition of an excess of potassium hydroxide pellets. The appearance of a pink color in the ethanol layer indicates the presence of proteins[7]. A few ml of the filtrate, an equal volume of sodium hydroxide solution (5%) and1% copper sulphate solution was added. The appearance of a violet coloration indicates the presence of the amino acids[11].

Total phenolic content

Total phenol contents of each extract were determined using the Folin-Ciocalteu method[12]. 1ml of dilute solution of each extract was mixed with Folin Ciocalteu

reagent (2, 5ml). After 5 min, sodium carbonate solution (75 g/L in water, 2 mL) was added and the reaction mixture was allowed to stand for 2 hours at room temperature, then the optical density at 765 nm was measured against water blank. Gallic acid was used as a standard calibration curve (0–300 µg/mL), The results were expressed as µg of gallic acid equivalent (GAE)/mg of extract.

Total flavonoids content

Total flavonoid content was determined using the Dowd method as adapted by Arvouret-Grand *et al*[13]. 1ml of dilute solution of each extract was mixed with a 2% solution of aluminium trichloride ($AlCl_3$) in methanol (2 mL). The optical density at 415 nm was measured against blank sample consisting of a methanol (2 mL) and extract (2 mL) without AlCl3. Quercetin was used as a standard calibration curve (0–50 µg/mL), The results were expressed as µg of quercetin equivalent (QE)/mg of Extract.

Antioxidant tests

Reduction of 2, 2-Diphenyl-1-picrylhydrazyl (DPPHº)

The radical scavenging activity, using free-radical DPPH assay, was determined according to the method introduced by Arvouet-Grand *et al*[14]. Briefly 2 ml of each extract (at different concentrations in methanol) was mixed with 2 ml of DPPHº solution (0.004%) in methanol. After an incubation of 30 min the absorbance was measured at 517 nm using MeOH as blank. 2 ml of DPPHº solution (0.004%) mixed with 2 ml of Methanol were used as control. The absorbance (*A*) of the control and samples was measured, and The DPPHº scavenging activity was determined using the following equation:

DPPHº scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}] \times 100$.

The data are presented as mean of triplicate and the concentration required for a 50% reduction (IC_{50}) of DPPHº radical was determined graphically.

Antimicrobial assay

The Antimicrobial assay of each extract was determined by disk diffusion method[15]. All microbial were growen in Muller Hinton (Oxid, UK). Bacterial inoculum (100 µl) was inoculated in Petri dishes containing a sterile Luria-Bertani Agar medium. Sterile filter paper discs (5 mm diameter) were deposited on medium and impregnated with 10 µl of extract solution (500 mg/ml of DMSO to 2%). The control was performed with discs containing 10 µl of DMSO to 2%. Each experiment was performed in duplicate.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The extracts were tested for their antibacterial activity against two reference bacterial strains: Staphylococcus aureus and Bacillus subtilis (Gram-negative). The MICs were determined using the broth microdilution assay as previously described by Bouhdid *et al*[16], with slight modifications, agar at 0.15% (w/v) was used as emulsifier and resazurin was used as bacterial growth indicator. Firstly, 50 µl of Mueller Hinton Broth (Oxoid, UK) supplemented with bacteriological agar (0.15% w/v) were distributed from the second to the 12th well of a 96-well polypropylene Microtiter plate. Essential oil dilution was prepared in Mueller Hinton Broth supplemented with bacteriological agar (0.15% w/v), 100 µl of these suspensions were added to the first test well of each Microtiter line, and then 50 µl of scalar dilution were transferred from the second to the 11th well. The 12th well was considered as growth control. Then, 50 µl of a bacterial suspension were added to each well

at a final concentration of approximately 10^6 CFU/ml. The final concentration of the extract was between 16 and 0, 00391mg/ml. Plates were incubated at 37°C for 20 h. The MIC was determined as the lowest extract concentration that prevented visual growth of bacterial strains. Experiments were conducted in duplicate. The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the extract yielding negative subcultures after incubation at 37°C for 24 h. It is determined by spotting 2 µl from negative wells on LB plates. Experiments were also conducted in triplicate.

Statistical analysis

Data were expressed as the mean ± standard deviation of at least three measurements. Data were analyzed using ANOVA test (Fisher) and determination of the Pearson correlation coefficient (ρ) was used during this work to evaluate and correlate results between them. The data were statistically analysed using IBM SPSS Statistics 21 (statistical software), Pearson correlation coefficient (ρ) was calculated by Excel.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening of the extracts revealed the presence of Phenolic Compounds, flavonoids, tannins, terpenoids, coumarins and cardiac glycosides in all organs of the plant for the two polar solvents. However, saponins are located only in the methanol extract (table 1).

Totals phenolic content and Totals flavonoid content

The methanolic extract of roots showed the highest Totals phenolic content (131.48 ± 8.5 µg GAE/mg of extract). This extract is significantly higher than those obtained from the extracts of leaves, stems, and roots obtained in ethyl acetate ($p < 0.05$) (table 2). Phenolic compounds are known by their effects to scavenge free radicals[17], their presence in large quantities justified a better antioxidant activity. The methanolic extract of stems showed the highest totals flavonoids content (39.14±0, 48 µg QE/mg) of extract, this extract is significantly higher than when compared to the others extract ($p<0.05$) (table 3).

Antioxidant activity

From the values obtained during manipulations performed to evaluate the antioxidant activity, we noticed that the curves have the same shape. The higher the concentration of the extracts is increased, the more anti-radical activity increased until a plateau. Beyond this limit, the activity remains constant (figure1). Furthermore, a lower value of IC_{50} (the concentration of extract which causes 50% inhibition of the activity of DPPHº) indicates a higher antioxidant activity[18]. The antioxidant activity of the methanolic extracts and ethyl acetate obtained with the DPPHº test ranged respectively from 10.01 ± 0.17 to 25.65 ± 1.07 µg/ml and from 18.85 ± 0.12 to 48.28 ± 4.70 µg/ml. The methanolic extract of root showed higher antioxidant activity (10.01 ± 0.17 µg/ml) followed by ethyl acetate extract of roots (18.85 ± 0.12 µg/ml). However, the antioxidant activity of methanolic extract of roots was significantly higher than those obtained from the extracts of leaves and stems ($p < 0.05$). The antioxidant activity of methanolic extracts had been always higher than those of the ethyl acetate extracts for each part of the plant. Numerous studies have shown that the antioxidant activity was correlated with the presence of phenolic compounds[17,19]. Determination of Pearson correlation coefficient between the values obtained with the DPPHº test and Totals phenolic content ($\rho=-0.87$, $p < 0.05$) indicated that antioxidant activity and Totals phenolic content could be correlated.

Antimicrobial activity

The initial screening of antimicrobial activity showed that methanolic extracts and ethyl acetate extracts are active against S. aureus, B. subtilis strains, for all organs of *Euphorbia resinifera*, whereas the only ethyl acetate extract showed activity against strain *M. luteus* (table 5). The negative control used (DMSO 2%) had no inhibitory effect on the tested strains. We noticed that the extracts are only active with Gram-positive strains. Indeed according to the literature, the selectivity of the drug to certain Gram positive bacteria to Gram-negative relative is due to the presence of lipopolysaccharides in outer membrane of Gram-negative bacteria, which acts as a permeability barrier and limits the distribution of active substances[20]. While Gram-positive bacteria allow the direct contact of the extract constutuents with the phospholipid bilayer of the cell membrane, which causes a better permeability[21]. The higher MIC is obtained against S. aureus and B. subtilis strains (0.5 ±0 mg/ml) for the root extract, ethyl acetate always showed strong inhibition (Table 6). The higher MBC is obtained against S. aureus (0.5 ±0 mg/mL) followed by B. subtilis (1±0 mg/ml) for the root extract (Table 7).

CONCLUSION

In conclusion, a high antioxidant activity of the extracts was noted primarily in the methanolic extracts and ethyl acetate of roots. Therefore, the organs can be classified according to their antioxidant capacity by following descending order: root, stem, flowers. The higher antimicrobial activity of the extracts is predominantly found in the ethyl acetate extract of the roots. The good correlation found between antioxidant activity and phytochemical contents indicates that effects observed could be attributed to phenolic compounds. The polar extracts of the roots of *Euphorbia resinifera* potentially can be considered more active than those of the leaves and stems. They showed a strong antioxidant activity and antimicrobial where the possibility of using these extracts in the food industry to remedy the oxidation and in the pharmaceutical industry for the treatment of certain diseases. We can consider these extracts as a new potential source of natural bioactive molecule to be extracted, identify and characterize and finally exploiting them as dietary antioxidants or as drugs.

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Table 1. Phytochemical analysis of *E. resinifera* flowers, stems, roots extracts

Family compounds	Methanolic extract			Ethyl acetate extract		
	flowers	Stems	Roots	flowers	Stems	Roots
Phenolic	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+
Saponins	+	+	+	-	-	-
Alkaloids	-	-	-	-	-	-
Cardiac glycosides	+	+	+	+	+	+
Amino acids	-	-	-	-	-	-
Protein	-	-	-	-	-	-

+ = absence, - = absence

Table 2. Totals phenolic content

Extracts	Totals phenolic content (µg GAE/mg of extract)	
	Flowers	67.55 ± 2.26
Methanol	Stems	94.83 ± 1.27
	Roots	131.48 ± 8.5
	Flowers	50.99 ± 1.39
Ethyl acetate	Stems	51.42 ± 1.86
	Roots	83.70 ± 5.79

Table 3. Totals flavonoid content

Extracts	Totals flavonoid content (µg QE/mg of extract)	
	Flowers	25.90 ± 0.18
Methanol	Stems	39.14 ± 0.48
	Roots	14.48 ± 0.22
	Flowers	8.96± 0.42
Ethyl acetate	Stems	18.75 ± 0.42
	Roots	13.85 ± 0.16

Table 4. Antioxidant activity

Extracts	Totals phenolic content (µg GAE/mg of extract)	
	Flowers	25.65 ± 1.07
Methanol	Stems	22.11 ± 1.11
	Roots	10.01 ± 0.17
	Flowers	48.28 ± 4.70
Ethyl acetate	Stems	33.67 ± 1.14
	Roots	18.85 ± 0.12
Ascorbic acid		3.32 ± 0.03

Table 5. The growth-inhibitory diameters (mm) of polar extract against the tested bacteria

Micro-organisme	Methanolic extract			Ethyl acetate extract		
	Flowers	Stems	Roots	Flowers	Stems	Roots
S. aureus ATCC 29 213	6.67 ±0.33	8.33±0.67	9±0	7±0	10.83±0.17	12.17±0.17
B. subtilis ATCC 3366	10.67±0.33	12±0.29	13.67±0.33	10.67±0.33	16.66±0.33	17.67±0.33
M. luteus ATCC 10240	-	-	-	7±0	10.5±0.10	10±0
P.aeruginosaATCC27853	-	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-	-
C. albicans ATCC 10231	-	-	-	-	-	-

Table 6. Minimum inhibitory concentration of polar extracts of *Euphorbia resinifera* MIC (mg/ml)

Micro-organisme	Methanolic extract			Ethyl acetate extract		
	Flowers	Stems	Roots	Flowers	Stems	Roots
S. aureus ATCC 29 213	2 ±0	> 16±0	> 16±0	4±0	2±0	0.5±0
B. subtilis ATCC 3366	4±0	4±0	2±0	4±0	4±0	0.5±0

Table 7. Minimum bactericidal concentrations of polar extracts of *Euphorbia resinifera*

Micro-organisme	Methanolic extract			Ethyl acetate extract		
	Flowers	Stems	Roots	Flowers	Stems	Roots
S. aureus ATCC 29 213	2 ±0	> 16±0	> 16±0	4±0	2±0	0.5±0
B. subtilis ATCC 3366	4±0	4±0	2±0	4±0	2±0	0.5±0

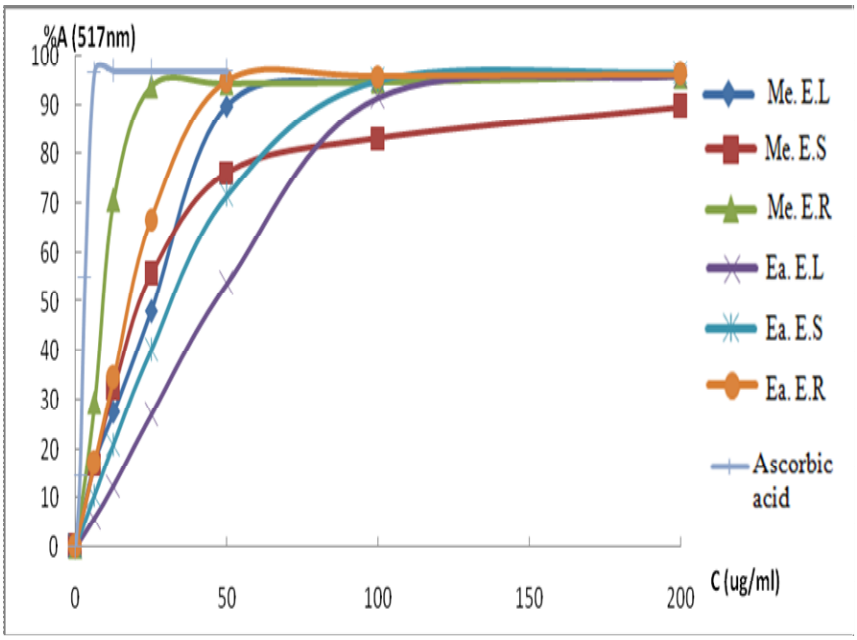

Figure 1. Anti-radical activity of extract of *E. resinifera*, and ascorbic acid in different concentrations. E: Extract; L: flowers; S: Stems R: Roots; Me: Methanolic; Ea: Ethyl acetate

Image 1. *Euphorbia resinifera* Berg