PHYTOCHEMICAL STUDY, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *EUPHORBIA RESINIFERA* L.

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ABSTRACT

The present study was aimed at detecting the phytochemicals and evaluating the antioxidant and antimicrobial activities of *Euphorbia resinifera* known for their medicinal properties in folk medicine. Phytochemical screening was carried out on the aerial part of *Euphorbia resinifera*. The assessment of antifungal activity was performed in terms of percentage of radial growth on solid medium (potatoes dextrose agar PDA) against *Aspergillus flavus* and *Penicillium expansum*. The antibacterial effect was studied by the agar direct contact method using *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strains. Then, the antioxidant evaluation of the extracts of alkaloids, flavonoids and methanolic extract was performed by DPPH[•] free radical scavenging and high performance thin layer chromatography (HPTLC) techniques. The phytochemical estimation revealed the presence of alkaloids, flavonoids and saponosides. These phytochemicals were isolated from the plant with yields of 0.7 %, 0.4 % and 0.35 %. HPTLC screening provided qualitatively the antioxidant effect of extracts under study. Furthermore, it was found that the methanolic, flavonoids and alkaloids extracts had a potent DPPH scavenging potency with IC₅₀ values of 0.0086; 0.378 and 1.171 mg/mL, respectively. Finally, the results of antimicrobial activity of the aqueous extract showed a pronounced antifungal activity against the tested strains. The percentage inhibition values were found to be in the range of 64.14 to 85.51 % against *Aspergillus flavus* and 60.33 to 92.28 % against *Penicillium expansum*. In contrast, the same extract inhibited only the growth of *Escherichia coli* bacteria. Further study is recommended to isolate and elucidate the active compounds and to evaluate *in vivo* their antioxidant and antimicrobial effect.

Keywords: Phytochemicals, extracts, Antioxidant activity, Antimicrobial activity, *Euphorbia resinifera* L.

INTRODUCTION

Nowadays there is a growing demand in natural agents exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as pharmaceutical drugs. The plants have been considered as a major source of natural antioxidants and antimicrobials. However, there is little knowledge about the practical usefulness of most of them. In the variety of secondary plant metabolites, antioxidant phenolic acids, flavonoids and alkaloids are commonly found in various plant organs and they have been shown to provide a defence against cancer and oxidative stress from oxidizing agents and free radicals¹⁻⁵. For thousands of years, natural products have been used as antibiotics and other modern drugs to treat several health problems. The investigation of certain plants for their antimicrobial and antioxidant properties may yield useful results. It has been reported that 10 % of the flowering plants were used by patients to treat various infection, although only 1 % have gained recognition by modern scientists.⁶ The acceptance of folk medicine as an alternative form for health care and the development of microbial resistance to the available antibiotics has led searchers worldwide to investigate the antimicrobial potency of medicinal plants.⁷ Generally, a number of secondary metabolites have been reported from various plants including tannins, alkaloids and flavonoids which showed a large antimicrobial spectrum.⁸ *Euphorbia resinifera* L. is one of 2000 species of Euphorbiaceae family. It is a dense succulent shrub growing to 60 cm tall, forming multi stemmed cushion-shaped clumps up to 2 m wide. Lime to blue green with short brown spines, small leaves that drop early and are rarely seen, new growth takes on a reddish colour. It is four-sided almost square stems that look superficially like a cactus. Besides, it can take

more light and frost than other *Euphorbia*.⁹ It is native to Morocco, where it occurs on the slopes of the Atlas Mountains in the surroundings of Marrakesh.¹⁰ In Algeria, it is widespread throughout the mountains of Naama (South-West of Algeria). *Euphorbia resinifera* L. has been reported to have anticancer activity. In Morocco folk medicine, the patients treat the general cancer by the grind aerial parts of *Euphorbia resinifera* mixed with honey.¹¹ Ethno pharmacological survey carried out in different areas of Algeria Naama department (Mecheria, ain sefra, Djenien Bou Rezg, Tiout and Ain Ben khelil) and performed by means of 150 interviews, revealed that *Euphorbia resinifera* L. is used to treat many diseases such as cancer, rheumatism, cyst and snake bite poisoning). Chemically, *Euphorbia resinifera* is known to contain a high concentration of the toxin resinifera toxin which is being an ultra potent capsaicin analogue present in the latex, interacts at a specific membrane recognition site, expressed by primary sensory neurons mediating pain perception as well as neurogenic inflammation.^{12,13} According to the literature data, antimicrobial activity of crude aqueous extract and antioxidant power of the alkaloids, flavonoids and methanol crude extracts have not been reported. In search of finding new resources and potent antimicrobial and antioxidant, the present study aimed to: 1) carry out a phytochemical study of *Euphorbia resinifera* L.; 2) evaluate the antioxidant power of the alkaloids, flavonoids and methanol crude extracts; 3) to investigate the antibacterial and antifungal activity of aqueous extract of *Euphorbia resinifera* L. against bacteria and fungi strains.

MATERIALS AND METHODS

Plant Material

The plant *Euphorbia resinifera* L. was collected in February 2012 from Naama department, Algeria. A voucher specimen of the plant was identified and authenticated at the laboratory of botany at the biology Institute. The plant was shade dried and ground by an electrical mill mesh and powdered material was defatted with hexane according to the method of Villarreal-Lozoya *et al.*¹⁴ Then, sample was stored in nylon bag in a deep freeze until the time of use.

Preparation of the Extracts

Preparation of Methanol, Diethylether and Aqueous Extracts

About 20 g each of the powdered plant were macerated three times with 60 mL of diethyl ether for ten minutes. The extracts were filtered using Whatman filter paper and concentrated to 25 mL. The filtrates were labelled appropriately as diethyl ether extract. The marc of each part was then macerated in methanol using the same above protocol. The obtained extracts were labelled as methanol extract. Another 5 g of each plant material was extracted by infusion in 50 mL of distilled water. After shaking for 15 minutes, the extract was filtered through Whatman's filter paper and labelled as aqueous extract. The three extracts were used for the phytochemical screening.¹⁵

Preparation of the Flavonoids Extract

A mixture of defatted powder of plant (100 g) in 100 mL of distilled water and 100 mL of acetone was refluxed for 12 h. After filtration of the marc and evaporation of acetone, the remaining aqueous extract was extracted twice with 50 mL of petroleum ether in order to remove chlorophyll and lipophilic compounds. Then, aqueous layer was extracted thrice with 50 mL of ethyl acetate. The organic layer was then dried over Na_2SO_4 , filtered and concentrated to dryness to give crude extract of flavonoids as a brownish solid.¹⁶

Preparation of the Alkaloids Extract

Defatted dry powdered plant (100 g) was alkalinized with 40 mL of ammoniac (0.5 N) for 8 h at room temperature. The alkalinized powder was extracted with dichloromethane (250 mL) in a Soxhlet apparatus for 4 h. The dichloromethane extract was subjected to sulphuric acid (0.5 N) extractions (3 x 150 mL), and then the aqueous layer was adjusted to pH = 9 with concentrated ammoniac. The aqueous basic solution was extracted with chloroform several times. The combined chloroform extract was dried over sodium sulphate and evaporated to give the crude alkaloid mixture.¹⁷

Preparation of the Saponosides Extract

About 100 g powdered plant were dispersed in 200 mL of 20 % ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 mL of 20 % ethanol. The combined extracts were reduced to 40 mL over water bath at about 90°C. The concentrate was transferred into a 250 mL separator funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. About 60 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 10 mL of 5 % aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant

weight. The saponin content as a yellow solid was calculated in percentage.¹⁸

Preparation of the Methanolic Crude Extract

About 20 g of powdered material was macerated with 80 mL of methanol for 24 h. The methanol extract was filtered using Whatman filter paper and then concentrated under vacuum at 40°C using a rotary evaporator. Finally, crude extract was obtained. The crude extract was stored at 4°C prior to antioxidant tests.

Phytochemical Screening

The diethyl ether, methanol and water extracts were screened for phytochemical constituents (flavonoids, alkaloids, saponins, sterols, terpenoids, tannins, anthraquinones, anthracenosides, anthocyanosides, coumarins, reducing sugars, fatty acids and volatile oils) using standard procedures to identify the constituents as described by Sofowora¹⁹ and Harborne.²⁰

Antioxidant activity

Determination of free radical scavenging activity by DPPH method

The antioxidant potential of the alkaloids, flavonoids, saponosides and methanolic crude extracts were determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Briefly, 100 μL of various concentrations of the each extract in methanol was added to 1.9 mL of a methanol solution of DPPH (0.004 %). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 minutes in the dark. The absorbance of the mixture was measured at 517 nm by using a double-beam UV-vis Camspec M550 spectrophotometer. A mixture of 100 μL of methanol and 1.9 mL of DPPH solution was used as the control. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_S)/A_B] \times 100$$

Where A_B is the absorbance of the control reaction (containing all reagents except the test compound), and A_S is the absorbance of the test compound

The commercial known antioxidant, ascorbic acid was used for comparison or as a positive control.²¹ The tests were carried out in triplicate. The extract concentration providing 50 % inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (0.5; 0.25; 0.125; 0.0625; 0.0312; 0.0156; 0.0078 mg/mL).

High performance thin layer chromatography (HPTLC) study of extracts and DPPH

Alkaloids, flavonoids, saponosides and methanolic crude extracts of *Euphorbia resinifera* L. were subjected to High performance thin-layer chromatography (HPTLC) on silica gel plate (20 x 20 cm, Silica gel F254, Merck). The solvent system optimized for crude extracts of *Euphorbia resinifera* was methanol and Chloroform (10:90 v/v). Alkaloids, flavonoids, saponosides and methanolic crude extracts were load on TLC silica gel plate and the plate was developed in a sandwich TLC chamber to a distance of 70 mm. After 15 minutes air-drying, the plate was sprayed with 0.004 % (w/v) DPPH reagent prepared in methanol and 10 % (v/v) sulphuric acid respectively. The spots on the plates were observed after

the plate has been heated at 60°C for 30 minutes exactly after spraying.²²

Antimicrobial activity

Antifungal Activity

The aqueous extract of *Euphorbia resinifera* L. was tested by growth radial technique on solid medium. Two fungi strains were used *Aspergillus flavus* (MTTC 2799) and *Penicillium expansum* (MTTC 1344). Selected concentrations ($V_{\text{extract}}/V_{\text{culture medium}}$, (mL/mL); [1/15], [2/15], [3/15], [4/15] and [5/15]) of *Euphorbia resinifera* L. aqueous extract were prepared. After agitation, the selected solutions were transferred into a Petri plates which were inoculated with sporal solution (spores of each fungi + 0.2 % agar solution + 5 % tween 80) and incubated for 7 days at $25 \pm 2^\circ\text{C}$. Mycelial radial growth was measured from the third day of incubation.^{23,24} The inhibition percentage of mycelial growth of each concentration was calculated using the following formula ($\text{PIg} = ((\text{DT} - \text{D})/\text{DT}) \times 100$) where DT is mean diameter of mycelial growth in control and D is mean diameter of mycelial growth in treatment.²⁵

Antibacterial Activity

The antibacterial activity was studied by the agar direct contact method.^{26,27} Three bacteria strains were used *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). Petri dishes (90 cm in diameter) were prepared with 15 ml of a base layer of Müeller-Hinton gelose medium (MHG) and the test organisms were inoculated on nutrient agar plates and spread uniformly using a sterile glass spreader. Various concentrations ($V_{\text{extract}}/V_{\text{culture medium}}$, (mL/mL); [1/15], [2/15], [3/15], [4/15] and [5/15]) of the extract were tested. Control experiments comprising inoculums without plant extract were set up. The plates were allowed to stand for one hour at room temperature ($25 \pm 2^\circ\text{C}$) for diffusion of the substances to proceed before the growth of organisms commenced. The plates were incubated at 37°C for 24 h. All tests were performed in triplicate and the antibacterial activity was expressed by the presence or absence of inhibition.

Statistical Analysis

All values are expressed as Mean $\pm$ standard deviation. The data of inhibition percentage were analyzed using One-way Analysis of Variance (ANOVA). The p-value < 0.05 was considered as significant.

Table 1: Phytochemicals Detected in Extracts of *Euphorbia Resinifera* L.

Extracts	Phytochemicals	Results
Aqueous	saponins	+
	flavonoids	+
	Reducing sugars	-
	Starch	+
	Anthocyanosides	-
Methanolic	Tannins	+
Ethanollic	Anthracenosides	+
	Alcaloids	+
Chloroformic	Anthraquinones	-
	Steroles	+
Diethyl ether	Terpenoids	+
	Coumarines	+
	Essential oils and fatty acids	+

Key: + = present; - = absent

Table 2: Extractions screening of alkaloids, flavonoids and saponosides

Phytochemicals	Mass of plant material (g)	Mass of phytochemicals extract (g)	Yield (%)
alkaloids	100	0.70	0.70
flavonoids	100	0.3508	0.35
saponosides	100	0.412	0.41

Table 3- Antifungal screening of aqueous extract against *A. flavus* and *P. expansum*

Concentration (v/v)	Percentage of inhibition $\pm$ SD	
	<i>A. flavus</i>	<i>P. expansum</i>
1/15	64.14 $\pm$ 0.33	60.33 $\pm$ 0.27
2/15	70.25 $\pm$ 0.29	82.60 $\pm$ 0.18
3/15	81.97 $\pm$ 0.18	83.34 $\pm$ 0.19
4/15	82.37 $\pm$ 0.22	88.65 $\pm$ 0.11
5/15	85.51 $\pm$ 0.14	92.28 $\pm$ 0.08

Values are Mean $\pm$ SD, the sample was run in triplicate (n = 3), p<0.05, evaluated by One-way analysis of variance (ANOVA).

Table 4: The antibacterial activity of aqueous extract of *E. resinifera* against some bacterial strains

Bacteria	Extract concentrations (V/V)				
	1/15	2/15	3/15	4/15	5/15
<i>S. aureus</i>	+	+	+	+	+
<i>P. aeruginosa</i>	+	+	+	+	+
<i>E. coli</i>	+	-	+	+	-

- = no growth of bacteria (inhibition); + = Resistance (growth of bacteria)

Figure 1: Antioxidant activity of vitamin C (a) and isolated extracts (methanol (b), flavonoids (c) and alkaloids (d)).

Figure 2: Diameter (cm) of zone inhibition of *E. Resinifera* aqueous extract against *A. Flavus* and *P. Expansum*

RESULTS AND DISCUSSION

Qualitative phytochemical screening of the aerial part were performed on its diethyl ether, methanolic, ethanolic, chloroformic and water extracts to identify its phytochemicals by using suitable chemicals and reagents (Table 1). In this experience, five solvents with different polarity have been used starting with idea that the solvent polarity is an essential parameter of the solubility of the constituents in the used solvent. The study indicated that alkaloids, saponins, flavonoids, sterols, terpenoids, tannins, anthracenosides, coumarines, reducing sugars, starch, fatty acids and volatile oils were present in the extracts under study. In contrast, the other secondary metabolites like anthraquinones and anthocyanosides were not detected.

Alkaloids, flavonoids and saponosides are the natural compounds which can influence the nutritive value of different food stuffs utilized by human and other animals. These phytoconstituents are known also for their medicinal properties such as antioxidant and antimicrobial activities.²⁸⁻³² In fact, in this part of the present work, these compounds were extracted and the results are given in the Table 2.

The reading of the results revealed that *Euphorbia resinifera* L. contained alkaloids, flavonoids and saponosides with variable yields. So, from 100 g of plant material, the content of isolated extracts were observed in this order; alkaloids (0.70 g) > (0.41 g) flavonoids > saponosides (0.35 g). The findings showed clearly that the studied plant contains more alkaloids than flavonoids and saponosides. On the basis of the fact that the phenolic compounds and alkaloids are known for their properties to trap the free radicals³³, it appeared important to us to make a study of the antioxidant activity of flavonoids, alkaloids and crude methanolic extracts. Using HPTLC bio autography technique, we observe on the TLC plate, the appearance of zones of anti radicalaire activity of yellow colour pale on purple bottom for the extracts under study as for the ascorbic acid.³⁴ Purple colour spots were visualized after spraying the plates with sulphuric acid demonstrated the presence of several bioactive compounds in the methanolic crude extract. Furthermore, yellow spots were observed also after spraying the plates with DPPH solution indicating, the presence of antioxidant compounds in the extracts under study. In conclusion, the bio autography

HPTLC method revealed that flavonoids and alkaloids possessed antioxidant effect. Besides, the antioxidant activity observed in the crude methanolic extract probably contributes by phenolic substances it contained. The evaluation of the oxidizing activity with the DPPH by spectrophotometric measurement gave the following results illustrated in Figure 1 (a-d).

The model of scavenging the stable DPPH[•] radical is a widely used method to evaluate the free radical scavenging ability of various samples.³⁵ DPPH is a stable nitrogen-centred free radical, the colour of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers.³⁶ The reading of Figure 1a showed a potent antioxidant effect of ascorbic acid with IC₅₀ value of 0.0331 mg/mL. The scavenging reaction carried out in short time and attains the steady state (100 % of inhibition). These results are in agreement with the findings of Hasan *et al.* (2009) who reported that vitamin C has antioxidant activity evaluated by the DPPH assay.³⁷ Regarding antioxidant activity of *Euphorbia resinifera* crude methanolic extract (Figure 1b), it seems interesting that it showed a higher antioxidant activity compared to acid ascorbique with IC₅₀ value of 0.0086 mg/ml. The antioxidant effectiveness of this extract is mainly attributed to the active compounds present in it such as phenolic compounds, alkaloids and others. Moreover, this effect can be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them. As shown in fig.1b, the scavenging potency of extract increased with the concentration which correlated well with the findings of Popovici *et al.*³⁸ Prabu *et al.*³⁹ and Liu *et al.*⁴⁰ As seen in Figure 1c, the flavonoids extract exhibited an antioxidant effect better than alkaloids extract and lower than methanolic one. So, the half maximum inhibitory concentration IC₅₀ was estimated to 0.378 mg/ml. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability⁴¹. From the results it showed clearly that the flavonoids extract had an acceptable antioxidant power as ascorbic acid. It's important to underline that our findings are in harmony with several

reports describing the antioxidant activity of flavonoids.⁴²⁻⁴⁵ DPPH radical scavenging activity of alkaloids extract of *Euphorbia resinifera* was presented in Figure 1d. Results showed that the radical scavenging activity of alkaloids extract of *Euphorbia resinifera* increased with increasing concentration. Isolated alkaloids extract of *Euphorbia resinifera* shows IC_{50} 1.171 mg/mL and R^2 was found to be 0.8294. However Ascorbic acid is used as a standard and its radical scavenging activity was found to be more potent. From the results it seems that alkaloids are the capability to scavenge the free radical DPPH. Several reports confirmed these data.^{28,46,47} On the other hand, The antifungal activity of *Euphorbia resinifera* specie was tested to prevent the mycelial growth of two fungal organisms *Aspergillus flavus* and *Penicillium expansum* that are harmful to human beings and that are known for their production of aflatoxin and ochratoxin. Different concentrations of aqueous extract of aerial parts were tested (Table 3). The antifungal activity was determined by measuring the diameter of inhibition zone recorded. As seen in Figure 2, the diameter of inhibition zone is enhanced by increase in the concentration of the *Euphorbia resinifera* aqueous extract. From the obtained results it showed clearly that the extract under study has a pronounced antifungal activity against *Aspergillus flavus* and *Penicillium expansum*.

Using the formula described in material and method section, the percentage of mycelial growth inhibition of aqueous extract at different concentrations are given in Table 3.

As illustrated in Table 3, the inhibitory plant extract showed capabilities to suppress *Aspergillus flavus* and *Penicillium expansum* grown on broth medium. Although, the inhibitory effect of the plant extract increased in proportion to their concentrations. The reading of Table 3 revealed that the aqueous extract of *Euphorbia resinifera* exhibited a pronounced antifungal activity at all tested concentrations. The findings showed significant percentages of mycelial growth inhibition in the range of 64.14 to 85.51 % against *Aspergillus flavus* and 60.33 to 92.28 % against *Penicillium expansum*. The results of the present study authenticated the effective *in vitro* activity of aqueous extract from *Euphorbia resinifera* specie and encourage further studies for its application in antibiotic therapy of fungal diseases. The antibacterial activity of *Euphorbia resinifera* aqueous extract was assayed *in vitro* by agar diffusion method against *Staphylococcus aureus* Gram-positive bacteria and *Pseudomonas aeruginosa* and *Escherichia coli* gram-negative bacteria. Among the extract concentrations tested, [2/15] and [5/15] showed at least the growth inhibition of *Escherichia coli* strain; while the remaining concentrations were not active against any of the tested organisms (Table 4). This means that the plant part used and the type of extraction might have resulted in nil activity in the test performed for this study. The differences in potency may be due to locality of the plant specie, time of collection of the plant sample, storage conditions, different sensitivity of the test strains, and method of extraction.⁴⁸

It's important to underline that our results agree with those of Malika et al. who found that honey sample from *Euphorbia resinifera* has antibacterial activity.⁴⁹ The dilutions of honey ranging from 1/2, 1/4, 1/8 and 1/16 were tested by the agar well diffusion method on various strains of bacteria including *E. coli*, *Staphylococcus aureus*, *Bacillus* and *P. aeruginosa*. Results revealed that most of strains were inhibited by the dilution 1/2 and 1/4. The Gram negative strains were found to be more sensitive than Gram positive ones. But the same

sensitivity among the Gram negative was observed for all the strains of *P. aeruginosa* and *E. coli*.

CONCLUSION

This is the first report of antioxidant activity of alkaloids, flavonoids and methanolic extracts from *Euphorbia resinifera* L. Besides, this is the first evaluation of antibacterial and antifungal activities of the aqueous extract from the same plant. The results revealed that methanolic extract has a high antioxidant activity followed by flavonoids and alkaloids extracts suggesting the potential of this plant as a natural source of strongly antioxidant substances that can be use as a natural additive in food and pharmaceutical industries. Furthermore, the findings from the present study revealed that the aqueous extract showed a good antifungal activity against all tested microorganisms. In contrast, antibacterial effect was observed only against *E. coli* strain.

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