Chemical Profile and Hepatoprotective Activity of Ethyl Acetate Extracts of Euphorbia paralias and Euphorbia geniculata (Euphorbiaceae) from Egypt

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ABSTRACT

Background: Plants belonging to the genus Euphorbia were used traditionally to treat several health disorders and diseases. Objective: the aim of this study is evaluation of secondary metabolites and hepatoprotective activity of the ethyl acetate fractions of the aerial parts of Euphorbia paralias (Ep) and Euphorbia geniculata (Eg). Materials and Methods: UPLC-ESI-MS/MS technique was used for identification of the secondary metabolites. The hepatoprotective potential of the two plants was evaluated for the first time in male rats with thioacetamide induced liver injury. Results: A total of 32 secondary metabolites were identified in the ethyl acetate fractions of the aerial parts of both species. Ellagitannins such as tetragalloyl hexoside, ellagic acid, gallic acid, and flavonoids such as kaempferol-3-O-β-D-β-D-galloyl-O-glucopyranoside, quercetin glycosides (glucoside and arabinoside) were found to be the major components in Ep whereas flavonoid glycosides including quercetin rutinoside, quercetin glycosides (glucoside, arabinoside and rhamnoside) and kaempferol glycoside derivatives were highly abundant in Eg. Administration of thioacetamide resulted in marked elevation in liver enzymes, elevation of lipid profile and alteration in oxidative stress parameters. While pretreatment of rats with Ep and Eg ethyl acetate fractions significantly attenuated the hepatic toxicity through reduction of liver biomarkers, improving the redox status of the tissue and so brought down the serum biochemical parameters and lipid profile nearly toward the normal levels. Conclusion: The studied fractions show hepatoprotective potential with promising value as hepatoprotective drugs of natural origin in comparison with silymarin as the standard hepatoprotective drug.

Key words: Euphorbia, UPLC-ESI-MS/MS, Polyphenolics, Hepatoprotective.

INTRODUCTION

Plants have used as an essential source of drugs and remedies on treatment of diseases and health disorders since ancient times¹. Flavonoids and phenolic compounds containing plants which are common among medicinal plants were reported for its various health benefits and applications. They have a wide spectrum of pharmacological activities including anti-inflammatory, antioxidant, hepatoprotective, anticancer and antimicrobial activities. They also decrease the risk of cardiovascular diseases, enhance regeneration of the liver and increase life expectancy²,³. As liver damage can be life threatening and its damage is caused by several factors such as alcohol, viruses, organic chemicals, metabolic and genetic abnormalities¹. Liver transplantation was improved survival rate of patients in some cases only and is limited to a small number of patients due to non-availability of suitable donors. And so, finding new drugs that are able to enhance liver regeneration and prevent liver failure is a very important need. Natural products as plant extracts exhibiting antioxidant and hepatoprotective activities can be useful in these needs². The aim of the current study was to identify the polyphenolics and flavonoids in the ethyl acetate (EA) fractions of the aerial parts of Ep and Eg using UPLC-ESI-MS/MS and to investigate the possible hepatoprotective activities of the studied fractions.

MATERIALS AND METHODS

Plant material and extraction

Aerial parts of E. paralias L. and E. geniculata Ortega were collected in the flowering stage on May and August 2015, respectively. E. paralias was collected from the North beach of Alexandria, Egypt. While E. geniculata was collected from roadsides in the vicinity of Banha, Qalubya, Egypt. The identification was kindly verified by Dr. Ahmed Abd El-Razik Lecturer of Plant Taxonomy, Department of Botany, Faculty of Science, Banha University, Egypt. The vouchers specimens (no. 3303 and 3304) were deposited in National Research Centre, Dokki, Cairo, Egypt. The air-dried powdered plant materials Ep and Eg (500 g of each plant) were extracted by cold maceration with 70 % methanol until complete exhaustion. The methanolic extracts were evaporated under reduced pressure at 45°C. The greenish brown viscous residues (105.0 and 100.5 gm respectively) were separately dissolved in MeOH-H₂O mixture.
(500 ml, 1:9 v/v) and subjected to fractionation with dichloromethane then by ethyl acetate to afford ethyl acetate fractions (18.5 and 9.2 gm) of Ep and Eg, respectively.

**UPLC-ESI-MS/MS**

The sample (100 μg/ml) solution of each fraction (ethyl acetate fractions of E. p. and E. g.) were prepared using high performance liquid chromatography (HPLC) analytical grade solvent of MeOH, filtered using a membrane disc filter (0.2 μm) then subjected to LC-ESI-MS analysis. Samples injection volumes (10 μl) were injected into the UPLC instrument equipped with reverse phase C-18 column (ACQUITY UPLC-BEH C18 1.7 μm particle size- 2.1×50 mm column). Mobile phase elution was made with the flow rate of 0.2 ml/min using gradient mobile phase comprising two eluents: eluent A is H2O acidified with 0.1 % formic acid and eluent B is MeOH acidified with 0.1 % formic acid. Elution was performed using the following gradient: 20 % B, 0-1 min; 20-90 % B, 1-18 min; 20 % B, 18-20 min. Mass spectra were detected in the ESI negative ion mode between 50-900 at 30 ev capillary conc. and capillary voltage 3 KV. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing the retention time (Rt) and mass spectrum with reported data. For fragmentation collision energy 40 eV was used.

**Hepatoprotective materials**

**Animals**

Male Sprague Dawley rats weighting (220±5 g) and Balb C mice (20-25 g) were purchased from Theodor Bilharz Institute (Giza, Egypt). The animals were housed in the animal facility of Faculty of Pharmacy (Boys), Al-Azhar University, Cairo, Egypt. They were fed with standard diet pellets (El-Nasr Company, Abou-Zaabal, Cairo, Egypt), and allowed free access to water. The animals were kept at room temperature (25°C±2.0) and natural humidity (it was 55%) with 12 h-light/12 h dark cycle. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and comply with the guidelines for the care and use of laboratory animals. The approval committee was given by ZU-IACUC committee with approval number ZU-IACUC/3/F/145/2019.

**Chemicals**

Silymarin (Sil): it was purchased as yellow fine powder and dissolved in normal saline, from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Thioacetamide (TAA): was purchased as powder purity (98.1 %) then dissolved in normal saline, from Sigma-Aldrich Chemical Company, St. Louis, MO, USA.

**Acute toxicity or lethality (LD50) test**

To assess for Ep and Eg extracts safety margin, the lethality test (LD_{50}) was carried out by estimation of acute toxicity monitored in animals. Ethyl acetate fractions of Ep and Eg were dissolved in dist. water at the mentioned doses below. The (LD_{50}) was estimated in mice by oral intake following the procedure of (Boys), Al-Azhar University, Cairo, Egypt. They were fed with standard diet pellets (El-Nasr Company, Abou-Zaabal, Cairo, Egypt), and allowed free access to water. The animals were housed in the animal facility of Faculty of Pharmacy (Boys), Al-Azhar University, Cairo, Egypt. They were fed with standard diet pellets (El-Nasr Company, Abou-Zaabal, Cairo, Egypt), and allowed free access to water. The animals were kept at room temperature (25°C±2.0) and natural humidity (it was 55%) with 12 h-light/12 h dark cycle. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and comply with the guidelines for the care and use of laboratory animals. The approval committee was given by ZU-IACUC committee with approval number ZU-IACUC/3/F/145/2019.

**Histopathological examination**

Autopsy samples were taken from the rats livers in the different groups and fixed in 10 % neutral buffered formalin for 24 h. Then serial dilutions of alcohol (ethyl, absolute ethyl and methyl) were used in specimens' dehydration. The specimens were cleaned by xylene and embedded in paraffin in a hot air oven at 56°C for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at microtome. The obtained sections of tissue were embedded between glass slides, deparaffinized, stained by hematoxylin and eosin stain, and another slides from the same samples stained with a specific stain (Masson's trichrom) then all were examined using the light electrical microscope.

**Statistical analysis of data**

All data are presented as mean±SEM. Statistical analysis was performed using GraphPad prism version 7 (GraphPad, San Diego, CA). Group differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer for multiple comparison tests. The difference was considered significant at P ≤ 0.05.

**RESULTS**

UPLC-ESI-MS/MS identification of secondary metabolites

Structural analysis of different compounds found in the aerial parts of Ep and Eg ethyl acetate fractions resulted in the separation and tentative
identification of 32 compounds using ULPC-ESI-MS/MS. Identification of compounds was performed using (M-1)/MS² and comparison with reported data. Ellagitannins and phenolic acids (22.00 and 11.48 %), flavonoids such as quercetin glycosides (35.00 and 39.97 %) and kaempferol glycosides (20.00 and 3.44 %) were found to be the major components in Ep and Eg, respectively; as ellagitannins (tetragalloyl hexoside, ellagic acid, gallic acid) and flavonoids (kaempferol-3-O-β-(6''-galloyl-O-glucopyranoside, quercetin glycosides (glucose and arabinose)) were found to be the major components in Ep while quercetin rutinoside and other quercetin glycosides (glucose, arabinose and rhamnoside) were highly abundant in Eg. As observed; molecular and fragment ions were listed in Table 1. LC-MS/MS profiles for Ep and Eg ethyl acetate fractions in the negative ion mode are shown in (Figure 1).

### Identification of Tannins

Peak 1 with deprotonated molecular ion peak at m/z 787 [M-H] and MS² at m/z 635, 483, 331, 169, so it was tentatively identified as tetragalloyl hexoside. While peak 2 was tentatively identified as gallic acid as its deprotonated molecular ion peak at m/z 169 [M-H] and MS² at m/z 125 (M−H−COOH). Peak 5 with molecular ion peak at m/z 183 [M-H] while MS² at m/z 125, so it was tentatively identified as methyl gallate. Peak 24 showed a molecular ion peak [M-H] at m/z 301, and MS² fragmentations at m/z 151, 211 so it was identified as ellagic acid. Peaks 8 and 10 with deprotonated molecular ion peak at m/z 635 [M-H] and MS² at 483 [M−H–galloyl–H₂O], 331 (M−H–2 (galloyl-H₂O)), 169 (M−H–hexoside-2 (galloyl-H₂O)), so they were tentatively identified as trigalloyl hexoside and its isomer respectively.

### Table 1: Compounds identified in the ethyl acetate fractions of E. paralias and E. geniculata by using UPLC ESI-MS/MS in negative ionization mode.

| No | RT min | (M-H) M/z | MS²/MS fragment ions | Expected compounds | Ep % | Eg % | Ref |
|----|--------|-----------|----------------------|--------------------|------|------|-----|
| 1  | 0.8    | 787       | 635, 483, 331, 183, 168.9 | Tetragalloyl-O-hexoside | 6    | 1.52 | 10  |
| 2  | 1.5    | 169       | 125                  | Gallic acid         | 6    | 1.07 | 15  |
| 3  | 3.5    | 585       | 271(100 %)           | Nargarin-galloyl-hexoside | +   | -   | 12  |
| 4  | 4.4    | 463       | 301(100 %)           | Quercetin-3-g-glucopyranoside | +   | +   |     |
| 5  | 5.2    | 183       | 151, 125             | Methyl gallate      | 6    | 2.6  | 10  |
| 6  | 6.2    | 353       | 191, 179             | Chlorogenic acid    | 1.07 | 1.07 | 12  |
| 7  | 6.3    | 615       | 463, 301(100 %), 152, 125 | Quercetin-galloyl-hexoside | +   | -   | 10  |
| 8  | 6.5    | 635       | 465, 331, 169        | Tri-galloyl-hexoside | 0.46 | 0.46 | 10  |
| 9  | 6.9    | 635       | 635, 431, 285        | Kaemferol-rhamn.-acetyl-glu isomer. | 1.44 | 16  |
| 10 | 7.1    | 635       | 331, 169             | Trigalloyl hexoside isomer | -   | 1.75 | 10  |
| 11 | 7.5    | 461       | 299                  | Diosmetin hexoside  | +    | -   | 12  |
| 12 | 7.7    | 433       | 301(100 %)           | Quercetin xyloside  | +    | -   |     |
| 13 | 8.3    | 325       | 193                  | Feric acid pentoisde | -    | +   |     |
| 14 | 8.4    | 461       | 285                  | Kaemferol-gluconoride | +    | -   | 15  |
| 15 | 8.7    | 599       | 447, 285, 169        | Kaemperol-3-O-β-(6''-galloyl-0- glu) | 15  | +   |     |
| 16 | 9.1    | 431       | 285 (100 %)          | Kaemferol rhamnosides | +   | -   |     |
| 17 | 9.2    | 651       | 609, 447, 301(100 %) | Quercetin-cetyl-hexoside- rhamnoside | -   | +   | 13  |
| 18 | 9.3    | 942       | 927, 463, 301, 151   | Quercetin-3-O-glucopyranoside | 28  | 6.25 | 14  |
| 19 | 9.8    | 433       | 433, 301, 151        | Quercetin-3-O-arabinoside | 7   | 8.58 | 10  |
| 20 | 9.9    | 615       | 463, 301 (100 %)     | Quercetin-galloyl glucopyranoside | -   | +   | 16  |
| 21 | 10.0   | 609       | 301, 447(100 %),     | Quercetin-rutinoside (rutin) | -   | 8.74 | 10  |
| 22 | 10.2   | 447       | 301, 447, 609        | Quercetin-3-rhamnosides | -   | 16.4 | 13  |
| 23 | 10.4   | 447       | 447, 285             | Kaemperol-3-O-glucopyranoside | 3   | +   | 10  |
| 24 | 11.8   | 301       | 211, 151             | Ellagic acid        | 4    | 2   | 11  |
| 25 | 11.9   | 431       | 285, 255             | Kaemperol rhamnosides | -   | +   | 10  |
| 26 | 13.4   | 609       | 299 (100 %)          | Diosmetin derivative | -   | +   | 13  |
| 27 | 13.6   | 651       | 547 [M-104], 427 [M-104-120], 301 [M-350] | Quercetin-3-rhamnosides-acetyl hexoside | -   | +   |     |
| 28 | 18.4   | 635       | 285 (100 %), 227, 241 | Kaemperol-rhamnosyl-acetyl glucoside | -   | +   | 10  |
| 29 | 23.7   | 301       | 151                  | Quercetin           | +    | +   |     |
| 30 | 23.4   | 285       | 151, 125             | Kaemperol           | +    | -   |     |
| 31 | 12.8   | 329       | 269                  | Dimethoxy apigenin  | +    | -   | 13  |
| 32 | 26.4   | 329       | 341 [M-CH₃], 299 [M-2CH₃], 271 [M-2CH₃-CO] | Diosmetin dimethyl ether | +   | -   |     |
Identification of phenolic acids and their derivatives

Peak 6 with deprotonated molecular ion peak at \( m/z \) 353 \([\text{M−H}]\) and \( MS_2\) at \( m/z \) 191 (M−H−caffeoyl, 161) and 179 (M−H−quincic) so it was tentatively identified as chlorogenic acid\(^{12}\). Peak 13 with deprotonated molecular ion peak at \( m/z \) 325 (M−H), and \( MS_2\) at \( m/z \) 193 (M−H−arabinose) so it was tentatively identified as ferulic acid pentoside\(^{10}\).

Identification of flavonoid compounds

Many flavonoid glycosides were identified in the ethyl acetate fractions of \( E. \) paralias and \( E. \) geniculata where peak 3 detected at \( m/z \) 585 [M−H] with daughter ions at \( m/z \) 271, 100 % so it was attributed to naringenin–galloylglucoside\(^{13}\). Peaks 4, 7, 12, 17-22, 28 and 31 all identified as quercetin derivatives as they have quercetin moiety (301 amu) at all, they are different in types of sugar moieties according to the \( m/z \) lost during fragmentation process as (-glucose or galactose, -162), (-rhamnose, -146), (-xylene or arabinose, -132) and (-acetyl glucose, -206) and some of which identified as galloyl glycoside due to lose of galloyl moiety (-152) as shown in table (1)\(^{10,13,14}\). As the same were peaks 9, 14-16, 23, 25, 29 and 30 were identified as kaempferol derivatives due to presence of kaempferol moiety (285 amu); In addition to sugars moieties and galloyl moiety as shown in table (1)\(^{10,13,14}\). Peak 11 identified as diosmetin–hexoside to presence of molecular ion peak at \( m/z \) 461, [M−H] and \( MS_2\) fragments at 299 amu corresponding to diosmetin moiety (M−H−162)\(^{13}\). Peak 32 identified as dimethoxy apigenin due to molecular ion peak at 329 m/z and daughter ion at 269 amu\(^{13}\).

Hepatoprotective

Thioacetamide is hepatotoxic agent known to induce acute or chronic liver disease (fibrosis and cirrhosis) in the experimental animal model\(^{17}\). In the present work, TAA is used as potent hepatotoxic agent in rats. A dose of 200 mg/kg ip TAA administration is reported to be the cause of hepatic toxicity. Its effect is due to increased oxidative stress\(^{18}\).

Acute toxicity or lethality (LD\(_{50}\)) test

The results showed that the animals survived during the 24 h observation and no visible signs of toxicity were observed. According to Hodge and Sterner toxicity scale\(^{19}\), the LD\(_{50}\) values of the two fractions were in the practically non-toxic categories.

Antifibrotic effects

Evaluation of liver biochemical parameters

Exposure of animals with the hepatotoxic agent, TAA resulted in significant (\( p \leq 0.05 \)) increase in the liver enzymes (ALT and AST) and total bilirubin in serum, lipid profiles (CH, TG and LDL) and lipid peroxidation (MDA) while significant decrease in HDL, GSH,
Figure 3: The effect of E. paralias, and E. geniculata on thioacetamide-treated rats on A: Cholesterol, B: triglycerides C: low density lipoprotein D: High density lipoprotein mg/dl in thioacetamide-treated rats on comparison with Silymarin.

Figure 4: The effects E. paralias, and E. geniculata A: on malondialdehyde, B: On reduced glutathione C: On catalase activity and D: On superoxide dismutase in thioacetamide-treated rats on comparison with Silymarin.

Discussion

The liver is the largest gland in the human body and susceptible to almost many different diseases including hepatitis, cirrhosis, alcohol related disorders and liver cancer. A major cause of these disorders is due to exposure to different environmental pollutants and xenobiotics. Also, the exposure to a lot of chemicals, such as carbon tetrachloride, bromobenzene, ethanol, thioacetamide and polycyclic aromatic hydrocarbons have been implicated in the etiology of liver diseases. It is fundamentally known that the regulation of apoptosis is a potential mechanism through which many agents such as polyphenolic compounds; can prevent toxicity and carcinogenesis. Silymarin is a mixture of natural flavanolignans contains at least seven compounds. The hepatoprotective and antioxidant activities of silymarin were attributed to control free radicals (FR), produced by the hepatic metabolism of toxic substances. The present study revealed that both Ep and Eg contain several types of gallotannins, phenolic

Histopathological results

Histopathological examinations of the sections of rat liver exposed to TAA showed (in H and E staining) severe tissue damage and hepatocytes degeneration. Pretreatment of experimental animals with (Ep and Eg) EA fractions reversed the TAA-induced hepatotoxicity and restored the elevated levels of liver biomarkers toward normality in comparison with silymarin treated group as follow; Ep, Eg and silymarin caused significant decrease in the liver enzymes (ALT, AST and total bilirubin in serum) (Figure 2), lipid profiles (CH, TG and LDL) (Figure 3) and lipid peroxidation (MDA) while significant increase in HDL, GSH, CAT and SOD (Figure 4).
acids and flavonoids which tentatively identified through UPLC-ESI-MS/MS analysis of ethyl acetate fractions of \textit{Ep} and \textit{Eg}. Polyphenolic compounds including flavonoids constitute 77.10 and 51.51\% of the ethyl acetate fractions of \textit{Ep} and \textit{Eg}, respectively. Quercetin and its derivatives in \textit{Eg} (39.97\%) represent about twice the kaempferol compounds in \textit{Ep} (20.00\%). These compounds were known to have high antioxidant activity that attributed to their ability to control FR demonstrated by high hepatoprotective activity in comparison with standard drug silymarin\textsuperscript{25,26}. That was evidenced by the significant improvement of liver enzymes (ALT and AST), total bilirubin, lipid peroxidation (MDA), oxidative stress related parameters (CAT, GSH and SOD) and lipid profile (CH, LDL, TG and HDL). According to the study, the potency strength of the fractions and silymarin on the liver enzymes differs from one to other; \textit{Ep+TAA gp} show more improvement in ALT and CH, than \textit{Eg+TAA gp} but nearly equal to Sil+TAA gp, while \textit{Eg+TAA gp} show more improvement in GSH and CAT in comparison with Sil-gp but the both plant fractions were equally in improvement degree in the rest of liver enzymes. Histopathologically, parallel structural improvement was elicited by either plants extracts as compared to silymarin. This is evidenced by the views of rat’s liver tissues treated with both plants as showing no collagen aggregation around central veins, no inflammatory infiltrate and no fibrosis. The results of the study on liver were indicating the promising values of these plants as hepatoprotective herbs in the future with more flow up.

CONCLUSIONS

This study revealed the identification of 32 polyphenolic compounds in the ethyl acetate fractions of the \textit{Ep} and \textit{Eg} using UPLC-ESI-MS analysis; mainly tannins and flavonoid glycosides. Hepatoprotective activity exhibited by the studied extracts might be attributed to the high

**Figure 5:** Light photomicrograph of H & E stain of liver sections tissues as: A: (Cont gp): showing average central vein (CV), and hepatocytes arranged in single cell cords (black arrow) with intervening blood sinusoids. B: (TAA-gp): mildly expanded portal tract with mild portal and peri-portal inflammatory infiltrate (black arrows) and scattered apoptotic hepatocytes in peri-portal area (yellow arrow). C: (Sil+TAA gp): average central vein with apoptotic hepatocytes in peri-venular area (black arrows) D: (Ep+TAA gp): average portal tract with average portal vein (PV) and average hepatocytes in peri-portal area (black arrow) E: (Eg+TAA gp): dilated central veins, and scattered apoptotic hepatocytes in peri-venular area (black arrow) (×400 except D×200).

**Figure 6:** Light photomicrograph Masson’s trichrome as: A: (Cont gp): liver section showing average collagen distribution in portal tract (black arrows). B: (TAA gp): excess collagen bundles with complete nodule formation (black arrow). C: (Sil+TAA gp): high power view showing excess collagen in portal tracts with fibrous septa extending into hepatic lobule (yellow arrow). D: (Ep+TAA gp): average collagen around central vein (black arrow). E: (Eg+TAA gp): average collagen in portal tracts (black arrow) (×400).
content of these compounds. These findings need more explored and investigated through further set of experiments to recommend the ethyl acetate extracts of the two plants as hepatoprotective drugs of natural origin.

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GRAPHICAL ABSTRACT

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Prof. Dr. Afaf El-sayed Ali Abdel Ghani graduated in pharmaceutical sciences in 1981 and subsequently completed her MSc (1985) at University of Zagazig and PhD (1990) degree in Doctorate of philosophy through a channel system mission with the institute of pharmaceutical chemistry, Bonn University, Germany. Assistant Prof. (1998) in Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt. Professional in May 2005 as Prof. of pharmacognosy at Faculty of Pharmacy Zagazig University.

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