Phytochemical and Biological Study of Certain *Euphorbia* Species Cultivated in Egypt.

A thesis submitted

By

**Shaimaa Rashad Ahmed Morsi**

(Assistant lecturer, Pharmacognosy Department, Faculty of Pharmacy, Cairo University)

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Doctor of Philosophy in Pharmaceutical Sciences (Pharmacognosy)

Under the supervision of

Prof. Dr. Moshera Mohamed EL-Sherei

Prof. of Pharmacognosy

Faculty of Pharmacy

Cairo University

Prof. Dr. Wafaa Tawfik Islam

Prof. of Pharmacognosy

Faculty of Pharmacy

Cairo University

Prof. Dr. Sayed Abdelhamed El-Toumy

Prof. of Chemistry of Plant Phenolics

and their applications

National Research Center

Prof. Dr. Riham Salah El-Dine

Assistant Prof. of Pharmacognosy

Faculty of Pharmacy

Cairo University

Pharmacognosy Department

Faculty of Pharmacy

Cairo University

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Abstract

The chloroform fraction of the latex and methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br., its chloroform and *n*-butanol fractions were tested for their cytotoxic activity on breast cancer (MCF7), hepatocellular carcinoma (HepG2), and cervix cancer (HELA) cells. The highest effect of all tested extracts was observed against the breast cancer (MCF7) while the least against the cervix cell line (HELA). Moderate activity against hepatocellular carcinoma (HepG2) was shown by the latex and *n*-butanol extracts. *In vitro* antioxidant effect of the methanol extract, chloroform and *n*-butanol fractions of the aerial parts was determined using DPPH scavenging method. The *n*-butanol fraction showed the highest antioxidant activity followed by the methanolic extract. The chloroform fraction showed the lowest antioxidant activity. Pretreatment with the methanolic extract and the *n*-butanol fraction of the aerial parts significantly showed an inhibition in the paracetamol-induced hepatotoxicity while pretreatment with the chloroform fraction of the aerial parts showed no significant effect which confirmed by histopathological studies. Three compounds were isolated from the chloroform fraction of the latex (euphol, obtusifoliol and 12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate) and three compounds from the chloroform fraction of the methanolic extract of the aerial parts identified as (12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate, 3β-O-acetyl-urs-12-en-oic acid methyl ester and lup-20(29)-en-3β, 23-diol) while eight compounds isolated from the *n*-butanol fraction of the methanolic extract of the aerial parts (gallic acid, bervifolin carboxylic acid, kampferol-3-O-β-D-rutinoside, corilagin, 3, 3′-dimethoxy ellagic acid, 3, 4, 4′-trimethoxyellagic acid, ellagic acid and kaempferol.

The total phenolic and total flavonoid contents in the methanolic extract of the aerial parts of the plant were determined. The cytotoxic activity of isolated compounds was studied.

**Key Words:** *Euphorbia cooperi*, cytotoxic, antioxidant, paracetamol-induced hepatotoxicity, total phenolic, total flavonoid.
Introduction

Euphorbiaceae (Spurge family) is a large family of the flowering plants, including 300 genera and over 5000 species. The genus *Euphorbia* is the largest in the family, comprising about 2000 species, a significant percentage mostly those originating in Africa and Madagascar, are succulent.

Some species of the genus *Euphorbia* have been used as medicinal plants in folk medicine for the treatment of skin diseases, gonorrhea, migraine, intestinal parasites and as wart cures.

Members of the genus *Euphorbia* are known for their rich content in secondary metabolites and interesting biological activities. The Euphorbias are characterized by the production of milky irritant latex with a very diverse composition, including toxic compounds as well as other interesting and potentially bioactive molecules such as diterpenes and triterpenes.

Latex has been used to treat cancers, tumors, and warts, from the time of Hippocrates. Modern studies have highlighted the wide spread use of several of these plants to treat cancerous conditions in the traditional medicine of many areas of the world.

Diterpenes occurring in plants of the genus *Euphorbia* are the focus of natural product discovery because of the wide range of their therapeutically relevant biological activities (e.g. antitumor, cytotoxic, multi-drug resistance reversing, anti-viral, and anti-inflammatory).

Additionally, flavonoids and tannins from Euphorbiaceae are well documented for their anti-tumor, hepatoprotective and antioxidant activities.

Plants of genus *Euphorbia* are known for their rich content in secondary metabolites including triterpenes, diterpenes, steroids, flavonoids, tannins and several bioactive constituents.

Diterpenes occurring in members of this genus are characterized by their great structural diversity with many different core frameworks such as jatrophanes, lathyranes, tiglianes, ingenananes, myrsinols, etc.

To our knowledge, over 650 diterpenoids, incorporating more than 20 skeletal types, have been isolated from *Euphorbia* plants.

Diterpenes are considered to be important taxonomic markers due to their limited occurrence in Thymelaeaceae and Euphorbiaceae families.
Reviewing the current literature, **only one report** was traced dealing with the isolation of a skin irritant diterpene ester named 12-deoxyphorbol-16-isobutyrate-13-tiglate from the latex of *Euphorbia cooperi* N. E. Br.

This literature review was performed on *Euphoria* species as an attempt to shed light on the data reported concerning the chemical composition and biological activities. This survey will, moreover, serve as a helpful guide during the foregoing research.

Reviewing the current literature, nothing was traced concerning the study of *Euphorbia cooperi* N. E. Br. except only one report dealt with the isolation of a diterpenoid compound.

**Therefore,** our aim is searching for novel secondary metabolites with cytotoxic, antioxidant and hepatoprotective activities from the plant to find new sources of bioactive compounds.

### 1- Plan of work

1- Literature review for all data related to the subject of the study.

2- Collection and taxonomical authentification of *Euphorbia cooperi* N. E. Br.

3-Preliminary phytochemical screening of the latex and aerial parts of *Euphorbia cooperi* N. E. Br.

4-Biologically guided study which includes study of the cytotoxic, antioxidant and hepatoprotective activities of *Euphorbia cooperi* N. E. Br.

5-Phytochemical study of the biologically active fractions which includes:

   A. Phytochemical investigation of the active chloroform fraction of the latex of *Euphorbia cooperi* N. E. Br.

   B. Phytochemical investigation of the active chloroform fraction of the methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br.

   C. Phytochemical investigation of the active n-butanol fraction of the methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br.

6- Quantitative determination of total phenolics and total flavonoids in *Euphorbia cooperi* N. E. Br.

7- Cytotoxic activity of isolated compounds
2- Methodology

I-Plant material

Samples of *Euphorbia cooperi* N. E. Br. used in this study were collected from Ahmed Alaa botanical garden, Toukh, Qalubia, Egypt in (March 2012, 2013). Identification of the plant material was kindly verified by Prof. Dr. Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University. The voucher specimen (No. 432015) is kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

**Plant extracts for phytochemical and biological studies**

a) The latex (1 litre) was collected from the aerial parts (10 kg) by incision and was suspended in methanol (200 ml) then freeze-dried. Evaporation of the solvent in vacuum gave a whitish, oily material (70 g). The residue was suspended in distilled water (200 ml) and extracted with chloroform (4 x 200 ml) yielding a chloroform fraction (22 g) residue.

b) Fresh aerial parts (25 kg) after removal of latex were extracted with 70% methanol (4 times) at room temperature. Evaporation of the solvent in vacuum gave brown residue (200 g). A portion of the residue (150 g) was suspended in distilled water (500 ml) and partitioned successively with non-polar solvent (chloroform, 4 x 200 ml) then polar solvent (n-butanol, 6 x 200 ml) yielding chloroform (40 g) and n-butanol (50 g) fraction residues.

II-Authentic Reference Material

1. **Phenolics:**
   
   Kaempferol obtained from E-Merck Co., Darmstadt, Germany.
   
   Quercetin and Gallic acid used as references in quantitative assays were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. **Sugars:**
   
   Glucose, rhamnose, galactose were obtained from E-Merck Co., Darmstadt, Germany.

III-Material for chromatographic study

1. Precoated TLC plates, silica gel 60 GF<sub>254</sub> (E-Merck, Darmstadt, Germany), (20x20 cm).
2. Silica gel 60 (Fluka, Sigma-Aldrich Chemicals, Germany) for normal phase column chromatography.
3. Silica gel H 60 without gypsum (E-Merck, Darmstadt, Germany) for vacuum liquid chromatography.

IV-Reagents

1. Spray reagents

a. $P$-anisaldehyde–sulphuric acid spray reagent (Stahl, 1969)

For sterols and triterpenes: 0.5 ml $P$-anisaldehyde mixed with 10 ml glacial acetic acid, 85 ml methanol and 5 ml of concentrated sulphuric acid were added cautiously.

b. Aluminium chloride spray reagent (Stahl, 1969)

For flavonoids: aluminium chloride (2 g) was dissolved in 100 ml methanol and filtered.

c. Aniline phthalate spray reagent (Mabery et al., 1970)

For PC of sugars: O-phthalic acid (1.66 g) and aniline (0.93 g) were dissolved in 100 ml n-butanol saturated with water. The mixture was shaken until completely dissolved and the solution kept in dark coloured container.

2. Shift reagents for UV spectroscopic analysis of flavonoids (Mabery et al., 1970)

a. Sodium methoxide solution: freshly cut metallic sodium (2.5 g) was added cautiously in small pieces to cooled spectroscopic grade methanol (100 ml).

b. Aluminium chloride solution: anhydrous aluminium chloride (5 g) is cautiously dissolved in 100 ml spectroscopic grade methanol.

c. Hydrochloric acid: 50 ml concentrated hydrochloric acid was mixed with 100 ml distilled water.

d. Sodium acetate: anhydrous powder, reagent grade.

e. Boric acid: anhydrous powder, reagent grade.

• Reagents for spectrophotometric determination (Mabery et al., 1970)

a. Aluminium chloride solution: For colorimetric assay of flavonoids, (0.1 M) AlCl$_3$: anhydrous AlCl$_3$ (13.3 g) dissolved in one liter distilled water.

b. Folin-Ciocalteu: used for determination of total polyphenolic content obtained from Loba-Chemie (Mumbai, India)

V-Test solutions (Egyptian Pharmacopoeia, 1984)
Dilute acids (hydrochloric and sulphuric acids), alkalies (postassium hydroxide) T.S., alcoholic α-naphthol, Fehling's solution, ferric chloride (T.S.) and ruthenium red were prepared according to E.P. (1984) monographs.

VI-Solvents
- The following solvents were used in this work: n-hexane, chloroform, ethyl acetate and methanol were purified adopting the procedure described by Vogel (1967). Ethanol 95% and n-butanol were also used.
- Methanol used for HPLC was supplied by E-Merck Co., Darmstadt, Germany.
- Solvent systems:
  \[ S_1: \text{n-hexane- ethyl acetate (90:10 v/v)} \]
  \[ S_2: \text{n-hexane- ethyl acetate (70:30 v/v)} \]
  \[ S_3: \text{Chloroform- methanol (90:10 v/v)} \]
  \[ S_4: \text{15\% acetic in water} \]
  \[ S_5: \text{Butanol - acetic acid- water (BAW) (4:1:5 v/v/v) upper phase.} \]

VII-Material for biological study
A. Experimental models
   1. **For in vitro screening of the cytotoxic activity**
      Human tumor cell lines (MCF7, HepG-2, HELA) and normal melanocyte (HBF4) used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). The tumor cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial sub-culturing.
   2. **For toxicity study**
      Male albino mice of Sprague Dawely strain (25-30 g) obtained from the animal house colony, National Research Centre, Giza, Egypt.
   3. **For in vivo assessment of the hepatoprotective activity**
      Male albino rats of Sprague Dawely strain weighing 130-150 g obtained from the animal house colony, National Research Centre, Giza, Egypt.

B. Culture media and laboratory diet
   1. Cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% CO\(_2\) atmosphere at 37°C.
2. Experimental animals were kept on standard laboratory diet composed of vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), casein 95% pure (10.5%) and starch (54.3%) provided with water ad libitum.

C. Drugs, chemicals and biochemical kits

1) Dulbecco’s modified Eagle’s medium, fetal bovine serum, trypsin, penicillin G, streptomycin and phosphate-buffered saline were obtained from Gibco Invitrogen (Carlsbad, CA, USA).

2) Doxorubicin: Sigma-Aldrich chemicals Co., St. Louis, MO, USA) as reference cytotoxic drug.

3) DPPH: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St Louis, MO, USA) for anti-oxidant activity.

4) Ascorbic acid (Sigma-Aldrich, St Louis, MO, USA) as reference antioxidant drug.

5) Paracetamol: paracetamol, Misr Co., Egypt to induce hepatic damage.

6) Silymarin: Sigma-Aldrich chemicals Co., St. Louis, MO, USA) as reference hepatoprotective drug.

7) Biochemical kits: Biodiagnostic kits (Biodiagnostic Co., Dokki, Giza, Egypt) for measurement of AST, ALT, ALP, bilirubin, MDA and GSH levels.

Apparatus

- Equipments and apparatus for phytochemical study

1. Sensitive electric balance (Sartorius, Germany).

2. Rotatory evaporator (Buchi, G. Switzerland).


4. Atomizer for spraying.

5. Graduated glass pipettes.

6. Ultrasonic agitator.

7. Glass columns for separation by column chromatography.

8. Chromatographic equipment and apparatus:

- Glass jars of different dimensions, for development of TLC and PC chromatograms.

- Micropipettes (0.1 ml) for spotting.

- UV lamp portable ultraviolet, a product of Hanovia lamps, for localization of spots on chromatograms.
- **HPLC apparatus**, Agilent 1200 Series HPLC coupled with a photodiode array detector (DAD). The column was an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm x 150 mm, 5 µm). (National Research Center, Dokki, Giza, Egypt).

- **UV-visible spectrophotometer**, Shimadzu UV-1650 PC was used for recording UV spectra of the isolated compounds, determination of total phenolic and flavonoid contents and *in vitro* antioxidant activity.

- **Bruker High Performance Digital FT-NMR-spectrophotometer (Avance III HD)**: $^1$H-NMR (400 MHz), $^{13}$C-NMR (100 MHz), Bremen, Germany, Faculty of Pharmacy, Cairo University and Varian VNMRS ($^1$H-NMR, 600 MHz, $^{13}$C-, 150 MHz, Germany).

The NMR spectra were recorded in CD$_3$OD, CDCl$_3$ or DMSO using TMS as internal standard and chemical shift values were recorded in δ ppm.

- **Mass spectrometer**: Varian Mat 711 (USA), Finnigan SSQ 7000 was used for EI/MS. Micro Analytical Center, Faculty of Science, Cairo University.

**Techniques**

I. Chromatographic Techniques

a. **Thin layer chromatography** (TLC): Ascending technique was followed (**Stahl, 1969**).

b. **Paper chromatography** (PC): Descending technique was used (**Heftmann, 1967**).

c. **Column chromatography** (CC): The wet method was followed for packing columns.

II. Acid hydrolysis of glycosides and investigation of hydrolysates (**Barbera et al., 1986**).

About (3mg) of each isolated glycoside were mixed with 5 ml hydrochloric acid (20%) in methanol (50% aqueous). The mixture was refluxed for 3 hours. The resulting hydrolysate was diluted with distilled water (10ml) and extracted with successive portions of ethyl acetate. The solvent was then evaporated under vacuum to dryness. The obtained residue (containing the aglycone) was dissolved in the suitable solvent and subjected to TLC analysis. The remaining aqueous solution after separation of the aglycone was neutralized with sodium carbonate, then evaporated to dryness under vacuum. The residue (sugar moiety) was dissolved in pyridine, then filtered.
The latter solvent was again evaporated and the sugar residue was dissolved in isopropanol (10%) for the identification of the sugar moiety.

a. Identification of the aglycone moiety

TLC chromatographic investigation of the aglycones was carried out alongside with authentic references using S₁ as a solvent system for flavonoidal aglycones. The chromatograms were visualized in visible and UV (at 365 nm) lights before and after exposure to ammonia vapours and spraying with AlCl₃ reagent.

b. Identification of the sugar components

The sugars separated were identified by paper chromatography (Whatmann no.1) alongside with several authentic sugars using S₃ as solvent system. The separated spots were visualized by aniline phthalate spray reagent followed by heating in an oven at 110°C for 5 min.

III. Estimation of total phenolic content

Spectrophotometric determination of the total phenolic content was carried out using the Folin-Ciocalteu colorimetric method.

IV. Estimation of total flavonoid content

Spectrophotometric determination of the total flavonoid content was carried out using the aluminium chloride colorimetric method.

V. Techniques for biological studies

A. Study of the cytotoxic activity of Euphorbia cooperi N. E. Br.

The chloroform fraction of the latex and methanolic extract of the aerial parts of Euphorbia cooperi N. E. Br., its chloroform and n-butanol fractions were tested in vitro for their cytotoxic activity on different human cancer cell lines, breast cancer (MCF7), hepatocellular carcinoma (HepG2), and cervix cancer (HELA) cells by Sulphorhodamine-B colorimetric assay (SRB) using doxorubicin as a positive control. In addition, human normal melanocytes (HFB4) were used as normal nonmalignant cells.

- The chloroform fraction of the latex possessed potent cytotoxic activity against MCF7, moderate against HepG2 and weak against HELA cell lines.
- The methanolic extract of the aerial parts showed potent cytotoxic activity against MCF7 and weak against HepG2 and HELA cell lines.
- The chloroform fraction of the aerial parts showed potent cytotoxic activity against MCF7, weak activity against HepG2 and HELA cell lines.
The \textit{n-butanol} fraction of the aerial parts showed moderate cytotoxic activity against MCF7 and HepG2 and weak against HELA cell lines. The highest effect of all tested extracts was observed against the breast cancer cell line (MCF7) while the least against the cervix cell line (HELA). Moderate activity against hepatocellular carcinoma (HepG2) was shown by the latex and \textit{n-butanol} extracts.

In the present study, \textit{doxorubicin}, used for the treatment of a great variety of cancer diseases, was the standard. However, this drug not only demonstrated cytotoxicity against all tested cancer cell lines, but also on the human normal one. Due to its high toxicity on human normal cells, the continuous use of doxorubicin can cause major adverse effects such as cardiotoxicity, neutropenia and gastrointestinal toxicity.

The extracts under investigation viz: chloroform fraction of the latex, the methanolic extract, chloroform and \textit{n-butanol} fractions of the aerial parts indicated their possible selectivity against the cancer cells rather than the normal cells compared to doxorubicin.

\textbf{B. Study of the antioxidant activity of }\textit{Euphorbia cooperi} \textit{N.E. Br.}

\textit{In vitro} antioxidant effect of the methanol extract, chloroform and \textit{n-butanol} fractions of the aerial parts of \textit{Euphorbia cooperi} was determined using DPPH scavenging method and ascorbic acid as reference drug.

The \textit{n-butanol} fraction showed the highest antioxidant activity followed by the methanolic extract. The chloroform fraction showed the lowest antioxidant activity. It is worthy to mention that the \textit{n-butanol} fraction of the aerial parts was found to be much more potent than ascorbic acid, the standard antioxidant drug, followed by the \textit{methanolic} extract of the aerial part which was also more potent than ascorbic acid.

\textbf{C. Study of the hepatoprotective activity of }\textit{Euphorbia cooperi} \textit{N. E. Br.}

- **Determination of median lethal dose (Toxicity study)**
  
  Methanolic extract of \textit{Euphorbia cooperi} N. E. Br. showed LD\textsubscript{50} = 3.8 g/kg b.wt. and from this result it could be concluded that the methanolic extract was safe and non-toxic

- **Hepatoprotective activity**
  
  1. Administration of paracetamol at a dose (600 mg/kg) significantly increased serum alanine amino transferase (ALT), aspartate amino transferase (AST), serum bilirubin, the level of hepatic malondialdehyde (MDA) and significantly depleted the hepatic glutathione (GSH) when compared with control rats, however, serum alkaline phosphatase (ALP) levels not significantly changed.
2. Pretreatment with the methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br. at a dose of (380 mg/kg) significantly showed an inhibition in the paracetamol-induced elevated levels of serum ALT, AST, total bilirubin. Additionally, it significantly decreased the formation of lipid peroxidation product (MDA) and restored the level of hepatic GSH.

3. Pretreatment with the chloroform fraction of the aerial parts of *Euphorbia cooperi* N. E. Br. at a dose of (100 mg/kg) showed no significant effect on the paracetamol-induced elevated levels of serum ALT, AST and total bilirubin as well as the depleted GSH. Only, it is significantly decreased the formation of MDA.

4. Pretreatment with the *n*-butanol fraction of the aerial parts of *Euphorbia cooperi* N. E. Br. at a dose of (100 mg/kg) showed significant inhibition on the paracetamol-induced elevated levels of serum ALT, AST and total bilirubin. It is significantly decreased the formation of hepatic (MDA) and restored the level of GSH.

The highest activity was observed by the *n*-butanol fraction followed by the methanol extract while the chloroform fraction showed the least activity.

All compared to the standard drug, *silymarin* which significantly inhibited the paracetamol-induced elevated levels of serum ALT, AST, total bilirubin and MDA and recovered the level of GSH.

Additionally, the *n*-butanol fraction of the aerial parts showed more potent hepatoprotective activity than the reference drug silymarin on the elevated levels of ALT, AST and MDA and a comparable to its potency of decreasing the level of total bilirubin and restoring the GSH level.

**Histopathological studies**

The histological features of hepatocellular damage manifested by degeneration (fatty change) in hepatocyte, severe dilatation and congestion of the central and portal veins as well as dilatation of bile duct with periductal inflammatory cells infiltration in paracetamol treated group, were markedly ameliorated on pretreatment with methanolic extract of aerial parts of *Euphorbia cooperi* N. E. Br. and its *n*-butanol fraction.

**Phytochemical study**

From the previous preliminary biological screening, the chloroform fraction of the latex, the chloroform and *n*-butanol fractions of the aerial parts were subjected to
phytochemical investigation aiming to localizing and isolating the constituents responsible for their cytotoxic, antioxidant and hepatoprotective effects.

A- Phytochemical investigation of the active chloroform fraction of the latex of *Euphorbia cooperi* N. E. Br.

The chloroform fraction of the latex was subjected to flash silica gel column chromatography. Elution was started with *n*-hexane and the polarity of the eluent further increased with ethyl acetate. The collected fractions were evaporated under reduced pressure and monitored by TLC.

The chromatograms were examined under visible and UV (at 365 nm) lights then visualized by spraying with *p*-anisaldehyde reagent. Similar fractions were combined together according to their chromatographic patterns yielding two crude fractions A and B. The fractions were subjected to further purification for isolation of their components.

Three compounds (*E*₁, *E*₂ and *E*₃) were isolated and identified based on physichochemical examinations, spectral analyses and comparison with published data.

**Compound E₁**: Euphol.

**Compound E₂**: Obtusifoliol (*4α, 14α*-dimethylergosta-8, 24(28)-dien-3β-ol).

**Compound E₃**: 12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate Or 4β, 9α-dihydroxy-13α-isobutyrate-16-angelate-20-acetate-1,6-tigliadien-3-one.

B- Phytochemical investigation of the active chloroform fraction of the methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br.

The chloroform fraction of the methanolic extract of the aerial parts was subjected to VLC chromatography eluted with *n*-hexane (100%), *n*-hexane-chloroform mixtures to chloroform (100%) then chloroform-ethyl acetate mixtures to ethyl acetate (100%). The collected fractions were evaporated under reduced pressure and monitored by TLC.

The chromatograms were examined under visible and UV (at 365 nm) lights then visualized by spraying with *p*-anisaldehyde reagent. Similar fractions were combined together according to their chromatographic patterns yielding two crude fractions A and B. The fractions were subjected to further purification for isolation of their components.

Three compounds (*E*₃, *E*₄ and *E*₅) were isolated and identified based on physichochemical examinations, spectral analyses and comparison with published data.

**Compound E₃** (previously isolated from the latex).
Compound E₄: 3β-O-acetyl-urs-12-en-oic acid methyl ester.

Compound F₄: Lup-20(29)-en-3β, 23-diol.

C- Phytochemical investigation of the active n-butanol fraction of the methanolic extract of the aerial parts of *Euphorbia cooperi* N. E. Br.

The n-butanol fraction of the methanolic extract of the aerial parts was subjected to polyamide column chromatography. Gradient elution was carried out using water/methanol mixtures. The collected fractions were evaporated under reduced pressure and monitored by PC.

The chromatograms were examined under visible and UV lights before and after exposure to ammonia vapours and spraying with AlCl₃ reagent. Similar fractions were pooled together yielding, four collective fractions. The fractions were evaporated under reduced pressure and subjected to further purification for isolation of their components.

Eight compounds (E₆ – E₁₃) were isolated and identified based on physichochemical examinations, spectral analyses and comparison with published data.

**Compound E₆:** Gallic acid.

**Compound E₇:** Bervifolin carboxylic acid.

**Compound E₈:** Kampferol-3-O-β-D-rutinoside.

**Compound E₉:** 1-O-galloyl-3,6-hexahydroxydiphenyl-β-D-glucopyranoside (Corilagin).

**Compound E₁₀:** 3, 3'-dimethoxy ellagic acid.

**Compound E₁₁:** 3, 4, 4'-trimethoxyellagic acid.

**Compound E₁₂:** Ellagic acid.

**Compound E₁₃:** Kaempferol.

D. Quantitative determination of total phenolics and total flavonoids in *Euphorbia cooperi* N. E. Br.

I. Estimation of Total Phenolic Content

Total phenolic content (TPC) was measured using Folin-Ciocalteu colorimetric assay. The phenolic content was expressed as mg gallic acid equivalent (GAE)/g plant extract with reference to pre-established standard curve of gallic acid.

The total phenolic content in the methanolic extract of the aerial parts of *Euphorbia cooperi* calculated as gallic acid equivalent (GAE)) was 44.3 ± 0.58 mg GAE per gram of extract.
II. Estimation of Total Flavonoid Content

Total flavonoids content (TFC) was measured using AlCl$_3$ colorimetric assay. Total flavonoid content was expressed as mg quercetin equivalent (QE)/g plant extract with reference to pre-established standard curve of quercetin. The total flavonoid content in the methanolic extract of the aerial parts of *Euphorbia cooperi* calculated as quercetin equivalent (QE) was 23.14 mg QE per gram of extract.

The high total contents of phenolics and flavonoids in the methanolic extract of the aerial parts of the plant under investigation explains the strong promising hepatoprotective and free radical scavenging effects (DPPH) demonstrated in the tested extract. These results are in agreement with many published data referring these activities to the presence of phenolics and flavonoids.

**Part IV: Cytotoxic activity of isolated compounds**

The pronounced cytotoxic activity observed for different tested extracts of the plant on certain cancer cell lines (Part II) encouraged the authors to investigate this activity on the compounds isolated from those studied fractions.

Compounds $E_1$ (euphol), $E_3$ (12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate) and $E_9$ (corilagin) displayed a remarkable cytotoxic activity while the other compounds showed moderate activity on MCF7 cell line.

On HepG2 cell line, compounds $E_1$ (euphol), $E_5$ (Lup-20(29)-en-3β, 23-diol), $E_9$ (corilagin), $E_{10}$ (3, 3′-dimethoxy ellagic acid) and $E_{11}$ (3, 4, 4′-trimethoxy ellagic acid) demonstrated moderate activity with but other compounds showed weak activity.

*Correlation between biological activities and chemical constituents*

- The potent cytotoxic activity of the chloroform fraction of the latex could be attributed to the presence of tetracyclic triterpene (euphol) and tigliane diterpene (12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate).

- The potent cytotoxic activity of the chloroform fraction of the aerial parts could be attributed to the presence of tigliane diterpene (12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate).

This is in agreement with the reported data as:

- Certain tigliane diterpene esters have been shown to possess anti-tumor activity against leukemia in mice as well as cytotoxic activity against certain cancer cell lines.
Certain 12-deoxyphorbol esters isolated from *Euphorbia cornigera* showed antitumor activity against KB human leukemic cells.

Previous reports concerning the cytotoxic activity of euphol against different cell lines as CS12 gastric cancer and T47D human breast cancer.

The *n*-butanol fraction of the aerial parts showed significant hepatoprotective and high antioxidant as well as cytotoxic activities due to the presence of:

- Flavonoids (*kaempferol and kaempferol-3-O-β-rutinoside*).
- Phenolic acids (*gallic acid*).
- Tannins (*corilagin, bervifolin carboxylic acid, ellagic acid, dimethoxy and trimethoxy ellagic acid*).

This is in agreement with literature which reported that:

- Flavonoids from Euphorbiaceae are well documented for their anti-tumor, hepatoprotective and antioxidant activities.
- Ellagic acid is known to possess antiproliferative, hepatoprotective and antioxidant properties.
- Tannins are strong antioxidant and can inhibit carcinogenesis.

3- Studying period

تاريخ التسجيل: 19/11/2012
تاريخ التشكيل: 26/7/2015
فترة الدراسة: سنتان و 8 شهور.

- Plant samples were collected from Ahmed Alaa botanical garden, Toukh, Qalubia, Egypt in (March-May 2012)

- Biological activity study: 5 months

- Phytochemical study including: extraction, isolation and identification of chemical constituents: 2 years

4-Date of research: 2012-2015