RESEARCH ARTICLE

# Isolation and Identification of Terpenes in *Euphorbia tirucalli* Cultivated in Iraq

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**ABSTRACT**

The plant *Euphorbia tirucalli*, which belongs to the family Euphorbiaceae has many medicinal uses. It is used in folk medicine in East Africa, Malaysia, India, and Brazil to treat different diseases. It has many pharmacological activities such as anticancer activity, antioxidant, antimicrobial, antiviral, hepatoprotective and central nervous system (CNS) depressant. This project provides the first comprehensive research done in Iraq to study the phytochemicals and the extraction and isolation methods from *E. tirucalli* cultivated in Iraq.

**Objective:** The study aims to investigate terpenes of the leaves and the root of *E. tirucalli* since no phytochemical investigation had been done for the plant previously in Iraq. Furthermore, to investigate if the Iraqi soil and weather might affect the existence of the terpenes in this plant.

**Method:** The leaves and the root of the plant were defatted separately in n. hexane for 24 hours. The defatted materials were extracted in 80% methanol using the hot method (soxhlet) and the cold method (maceration), then the crude extracts were fractionated using different solvents according to the increasing in polarity petroleum ether, chloroform, ethyl acetate and n-butanol. The hexane fraction for the leaves and the root, chloroform fraction for the leaves and the ethyl acetate of the leaves were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Furthermore, gas chromatography-mass spectrometry (GCMS) was used for further analysis for the hexane fractions. Preparative high-performance liquid chromatography was used to isolate the terpenes from the hexane fraction of the leaves. The isolated compounds were identified by using analytical TLC, analytical HPLC, Fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV), high-performance compact mass spectrometer (CMS).

**Results:** The different chromatographic and spectroscopic results revealed the presence of beta-sitosterol, euphol and stigmasterol in the hexane fraction of the leaves and the plant's root.

**Keywords:** *Euphorbia tirucalli,* Euphol*,* Beta-sitosterol, Stigmasterol, Terpenes.

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**Conflict of interest:** None

## INTRODUCTION

The spurge family is a large family of flowering plants with approximately 300 genera and about 8,000 species. This family occurs mainly in the tropics, with most of the species in the Indo-Malayan region and tropical America. A large variety occurs in tropical Africa, but they are not as abundant or varied as in these two other tropical regions, but also there are species in non-tropical areas like the Mediterranean Basin, the Middle East, South Africa, and the southern USA. The family contains many phytotoxins, mainly diterpene esters, alkaloids, glycosides, and ricin-type toxins. One of the most characteristic of the subfamilies Euphorbioideae and Crotonoideae is the milky latex, a white poisonous material that cause blistering on contact, as well as, temporary blindness if it contacts eyes. This latex has been used as a laxative.

In medicine, some species of Euphorbiaceae have proved effective against genital herpes. There are many traditional uses five of this family such as for asthma, abscess, anthelmintic, astringent, bronchitis, bedsores, cancer, cough, diuretic, diarrhoea, dysentery, eczema, earache, headache, inflammations, jaundice, kidney disease, leprosy, paralysis, skin diseases, scabies, toothache, ulcers, ringworm and others.[1-3] Euphorbia is one of the major genera of the family Euphorbiaceae, which is considered the second largest genus of flowering plants with 2150 species. Linnaeus was the first who describe the genus Euphorbia. He mentioned 56 species, while Boissier was the first who put the foundations of the gender classification system, he counted 723 species.The vegetative forms of this genus range from flat plants to tall ones. Many succulents also appear in this genus with two

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types spiny succulent and non-spiny succulent. Succulents of this genus are similar in appearance to cacti but can be distinguished by the presence of milky latex in their stems and the lack of their flowers of calyx and corolla, unlike cactuses. This genus is distinguished by the special characteristics of all races in the plant kingdom: the syphilis system, Systems of photosynthesis and chromosomal system.[4] The diversity within this genus is wonderful; from low-growing gardens, weeds called spurges to giant, cactus-like succulents that competitor in size as shown in Figure 1. South African euphorbias have developed to succulent, spine covered stems that are greatly similar to North American cacti, a biological phenomenon known as convergent evolution.[5]

### Taxonomic Hierarchy of Euphorbia Tirucalli[6]

Kingdom: Plantae.
Division: Magnoliophyta.
Class: Magnoliopsida.
Order: Malpighiales.
Family: Euphorbiaceae.
Genus: Euphorbia.
Species: *E. tirucalli*.
Binomial name: *Euphorbia tirucalli* L.

### Phytochemical Constituents of *E. tirucalli*

Phytosterols are steroid alcohol compounds found in plants that have the same function and structure as cholesterol except that they always contain some substitutions at the C24 position on the sterol side chain.[7,8]

Phytosterols as shown in Figure 2 are fateful steroid molecules that stabilize the phospholipid bilayers of cellular membranes in plants.[9]

Euphol is tetracyclic triterpene alcohol as shown in Figure 3 and the main constituent found in *E. tirucalli*, which had been shown anticancer effect (basal cell carcinomas, leukaemia, and lung, prostate and breast cancers),[10] anti-inflammatory, antiviral activities, analgesic effect as well as antinociceptive properties.[11]

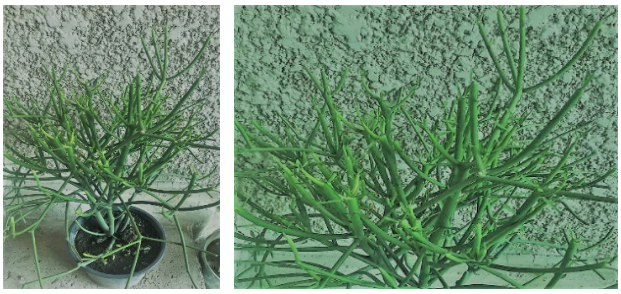


**Figure 1:** *E. tirucalli* plant.

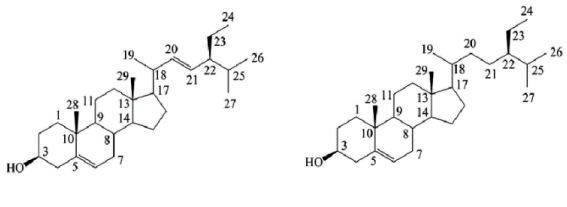



**Figure 2:** Chemical structure of beta and stigmasterol.

## MATERIALS AND METHOD

### Collection of Plant Materials

The whole plant of *E. Tirucalli* was collected from Baghdad in April 2020. The plant was authenticated by Assistant Professor Dr. Khansaa R. Al-Joboury, Iraqi Natural History Museum Herbarium. The plant was cleaned, dried in shade at room temperature, pulverized by electrical milled and then weighed.[12]

### Extraction

Firstly, the pulverized plant material (about 120 gm of leaves and 20 gm of root were defatted separately with a sufficient amount of hexane solvent) for 24 hours to remove chlorophyll and the hexane soluble compounds like waxy material by filtration then evaporation.[13]

### Preliminary Qualitative Phytochemical Analysis for Crude Extract

*Steroid detection (Libermann-Burchard's test)*

1 mLof methanolic extract + 1-mL chloroform+2–3 mL acetic anhydride +1- 2 drops of sulphuric acid were added carefully, an array of a colour change indicates the presence of phytosterol.[14-16]

*Terpenoid Detection (Salkowski's test)*

2 mL of alcoholic extract + 2 mL chloroform +2 mL concentrated sulphuric acid was then added and heated for about 2 min, a grey colour solution indicates the presence of terpenoids while a golden yellow layer at the bottom indicates the presence of triterpenoids.[14-16]

### Thin-layer Chromatography Examination for Hexane Fraction

1 mg of hexane fraction was suspended in about 1 mL of chloroform, applied on a readymade analytical TLC plate precoated with silica gel $GF_{254}$, and developed in different mobile phases to reach the best separation, the best ones were:

- S1: Hexane: methanol (70:30) (hexane layer was taken) (modified)[17]
- S2: Hexane: ethyl acetate (60:40)(modified)[17]
- S3: Toluene: ethyl acetate (90:10)(modified)[18]

After drying the plates, they were sprayed with Vanillin-sulphuric acid reagent (VS) (1% ethanolic vanillin (solution 1), 10% ethanolic sulphuric acid (solution 20)). The plate is sprayed with solution 1, followed immediately by solution 2. Then heating at 110ºC for 5 to 10 minutes under observation, the spots appeared in different colours.[19]

### Analysis and Isolation of Different Constituents from Hexane Fraction by HPLC

Qualitative and quantitative estimations were carried out for identification and isolation of terpenes from the hexane fraction, the retention time of the analyzed samples was compared with the retention time of the standards under the same conditions of the HPLC.[19]

For quantification measurements, the calibration curve was plotted using the area under the curve AUC referred to

by the y-axis versus four concentration levels of the standards referred to by the x-axis. A straight line equation (y = mx + c) was obtained from which the concentration of the analyte was calculated, where m: is the gradient of the line (the slope) while c: is its intercept with the y-axis.[20,21]

*Sample:* Hexane fraction (0.1 mg in 45 mL)

*Standards:* Beta-sitosterol, euphol, stigmasterol by using four concentrations (10, 20, 30, 40 µg/mL).

*HPLC condition:* Model SYKAMN (Germany)

*Mobile phase = acetonitrile:* DW: acetic acid (60: 25: 5) isocratic elution.

Column = C18-ODS (25 cm * 4.6 mm)

Detector = UV- 280 nm (S 2340)

Column oven model = S 4115

Fraction collector model = FOXY R1

Pump model = S 2100 quaternary gradient pump

Autosampler model = S 5200

Flow rate = 1 mL/min

Injection volume = 100 µL for analysis and 200 µL for isolation.

10 mg solvent-free dried extract was dissolved in 5 mL methanol to prepare a concentration of 2 mg\mL (stock solution). The aliquot was then filtered through a 0.45 µm membrane filter before injection.[22]

Under these same conditions, the calibration curve analysis, isolation, and calculation were performed for the three isolated compounds referred to by A, B, and C.

### Identification of the Isolated Compounds (A, B, C) from Hexane Fraction

*Compound A was identified by*

- High-Performance Liquid Chromatography (HPLC) with the standard
- Spiking by taking part of the isolated A sample which mixed with a small amount of the standard and then reanalyzed by HPLC[24]
- High-performance compact mass spectrometer (CMS).

*Compound B was identified by*

- Fourier-transform infrared spectroscopy (FTIR).
- Analytical thin-layer chromatography (TLC).
- CMS.
- Ultraviolet (UV-1900) spectra.

*Compound C was identified by*

- Analytical HPLC with the standard.
- UV.
- CMS.

## RESULTS AND DISCUSSION

*Extraction method:* As shown in Table 1.

### Phytochemical Investigation of the Fractionated Extract-

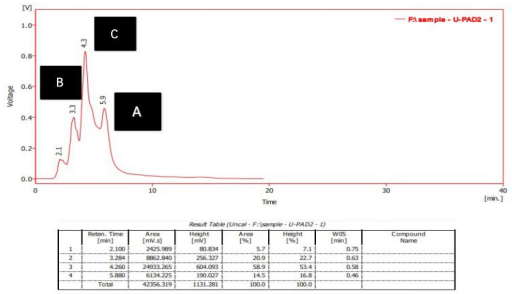
Phytochemical tests screening were done on hexane fraction to confirm the presence of the active constituents to choose the suitable mobile phase for further identification and isolation. The results are presented in Table 2.

The results of the phytochemical screening for each fraction confirm the presence of steroids, terpenoids and also they gave more specific information about the location of each active constituent.

### Thin-layer Chromatography (TLC) for the Hexane Fraction

By using different mobile phases and standards (beta-sitosterol, euphol, and stigmasterol), the spots of these compounds appeared in the fraction after spraying with vanillin-sulphuric acid reagent (VS) and then heating at 110°C for 5 minutes in both the leaves and the root of *E. tirucalli*. As shown in Figure 3.

### Analysis and Isolation of Active Constituents (A, B, C) from Hexane Fraction by HPLC




The sample

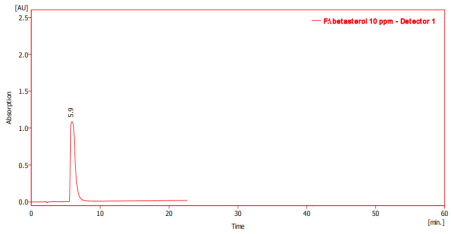



**Table 1:**- The weight and the percentage of hexane fraction of the leaves and root of *E. tirucalli*

| *The part of the plant* | *The fraction* | *The weight* | *The percentage in plant* |
|---|---|---|---|
| Leaves (total weight 120 gm) | hexan | 2 gm | 1.6% |
| Root (20 gm of powder) | hexan | 0.7 gm | 3.5% |

**Table 2:** Phytochemical screening of the fractionated extract.

| *The fraction* | *Steroid test* | *Terpenoid test* |
|---|---|---|
| Hexane f. | + | + |
| Observation | An array of colours changes. | A grey colour solution indicates the presence of terpenoids while a golden yellow layer at the bottom indicates triterpenoids presence. |

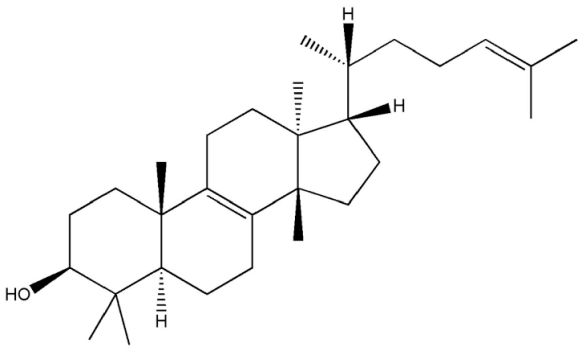



**Figure 3:** Chemical structure of Euphol (C30H50O).

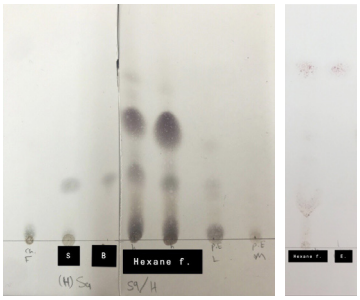
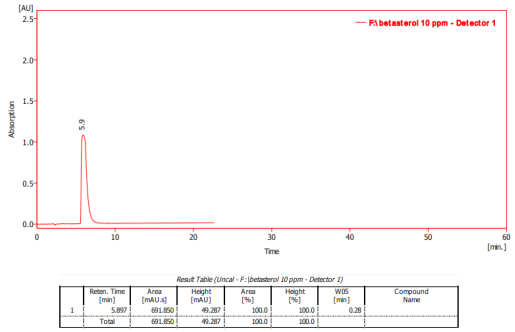



**Figure 4:** Thin layer chromatography for hexane fraction with beta-sitosterol std. (B), stigmasterol std. (S) and euphol std. (E) developed in S1: Hexane: methanol (70:30), spraying with vanillin-sulphuric acid reagent (VS).

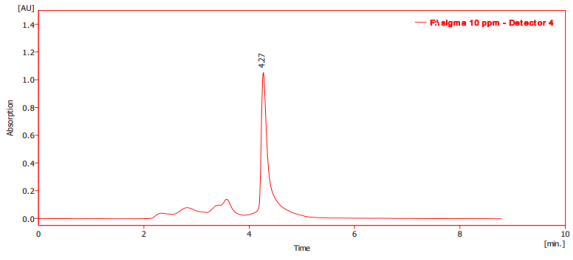
## Betasitosterol Standard




Result Table (Uncal - F:\betasterol 10 ppm - Detector 1)

| | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 5.897 | 691.850 | 49.287 | 100.0 | 100.0 | 0.28 | |
| | Total | 691.850 | 49.287 | 100.0 | 100.0 | | |

## Euphol Standard




Result Table (Uncal - F:\sigma 10 ppm - Detector 4)

| | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.267 | 159.494 | 100.344 | 100.0 | 100.0 | 0.03 | |
| | Total | 159.494 | 100.344 | 100.0 | 100.0 | | |

**Figure 5:** The isolation of the active constituent (A, B, C) by HPLC from hexane fraction of the leaves.

## Stigmasterol Standard

The Figure 5 above showed that the isolated compound A with the same retention time with beta-sitosterol std. (5.9 minutes) while the isolated compound B with the same retention time with euphol std. (3.3 minutes) and the isolated compound C with the same retention time with stigmasterol (4.3 minutes). Quantitative identification was also carried out by using a calibration curve for the isolated compounds A, B, C.
Correlation factor = 0.9996687

Using the linear equations from the standards and the area under the curves of the isolated compounds A, B, C as shown in Figure 6, the latter's concentration can be calculated.

Identification of the Isolated Compound (A):

**By HPLC:** by comparing with beta-sitosterol standard and spiking.

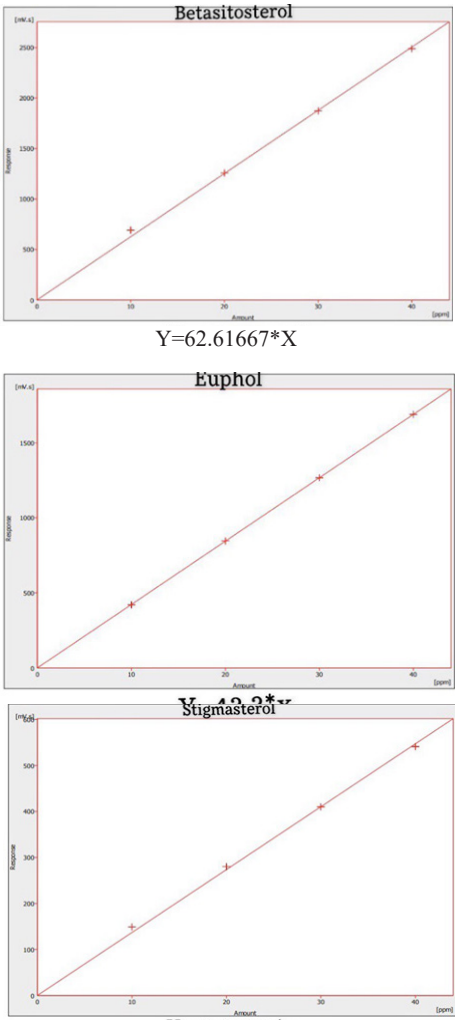



**Figure 6:** The calibration curves for the isolated compounds A, B, C.

**Table 3:** The concentration of the isolated compound from the hexane fraction of the leaves in the plant

| *The compounds* | *The concentration in 120 gm plant leaves in gm/mL* |
|---|---|
| A | 0.000036 |
| B | 0.000078 |
| C | 0.00068 |

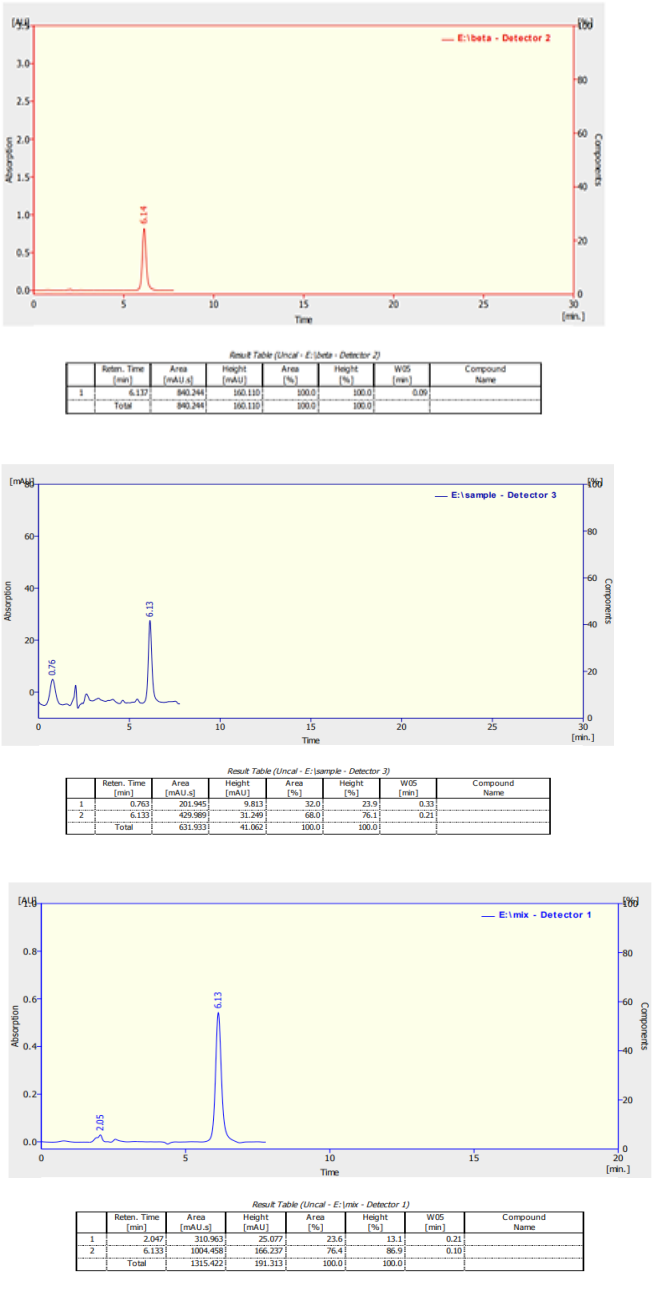



**Figure 7:** Identification of the isolated compound by HPLC

## Beta-sitosterol

Isolated compound A

Mix (compoundA+beta std).

From the Figure 7 above, the results showed that the retention time for the isolated compound (A) is the same as the retention time for the beta-sitosterol std. (6.1 min). Furthermore, the spiking by mixing compound A with the std. showed an increase in %area from 68% to 76%.

- *By CMS:* Run time 3 minutes, temperature 300°C.

From the Figure 8 above, the results showed that molecular ion peak at m/z 397 that corresponds to $[M\text{-}H2O+H]^+$, m/z 396 corresponds to $[M\text{-}H2O]^+$, m/z 255 corresponds to [M-side chain $-H_2O]^{+}$[25]

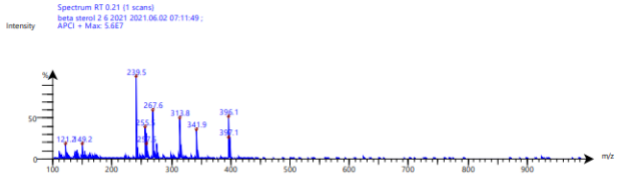



**Figure 8:** The mass spectrum of the isolated compound (A) from hexane fraction of the leaves

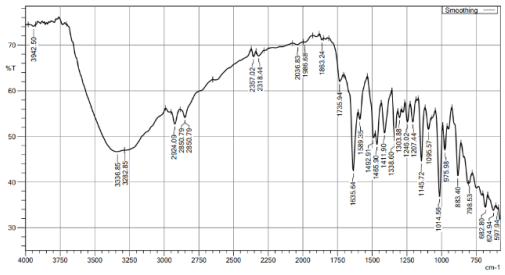



**Figure 9:** FTIR for the isolated compound (B) from hexane fraction of the leaves.

We can conclude from all the results mentioned above that the isolated compound (A) might be beta-sitosterol.

## Identification of the Isolated Compound (B)

- By FTIR

From the Figure 9 above, the results showed that 3400–3300 OH Stretching, 2950 & 2800 CH asymmetric and symmetric stretching,1690-1610 Alkenyl C=C stretch,1430-1290 methylene CH plan, and 990-910 Methylene CH bending vibration.[26]

- By TLC

The Figure 10 showed that the three spots (euphol std., isolated B compound and the latter one was a mix of the standard and the isolated B compound) have the same $R_f$ value (0.42 cm).

- By CMS

From the Figure 11, the result showed that the molecular ion peak at m/z 411 that corresponds to $[M\text{-}CH3]^+$, m/z 393 corresponds to $[M\text{-}CH_3\text{-}H_2O]^+$, m/z 383 corresponds to [M-side chain-2H]$^+$ m/z 370 corresponds to [M-side chain -CH3] $^+$ m/z 329 corresponds to [M-side chain-ring E clevage-$CH_3$]$^+$.[25]

- By UV

From the Figure 12, the result showed that the euphol std. and the isolated compound (B) had the same absorbance spectrum at the same wavelength.

We can conclude from all the aforementioned results that the isolated compound (B) might be euphol.

## Identification of Compound C

- By HPLC

From the Figures 13 and 14, the result showed that the stigmasterol std. and the isolated compound (C) had the same retention time (4.3 minutes).

- By UV

From the Figure 15, the result showed that the stigmasterol std. and the isolated compound (C) had the same absorbance spectrum at the same wavelength.

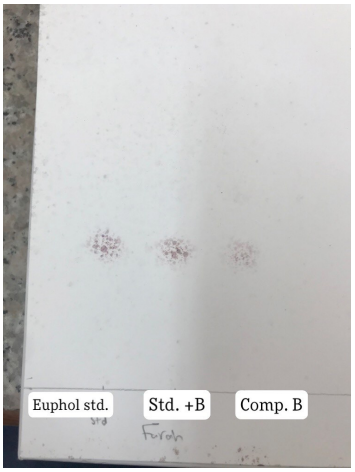



**Figure 10:** TLC analysis for the isolated compound (B) from hexane fraction of the leaves after spraying with VS reagent.

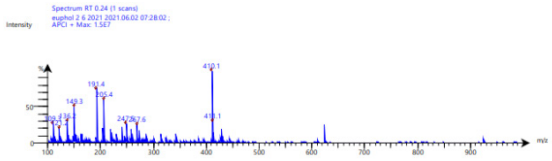


**Figure 11:** The mass spectrum of the isolated compound (B) from hexane fraction of the leaves

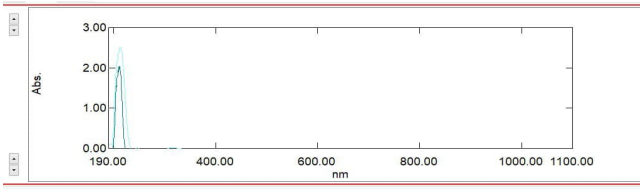
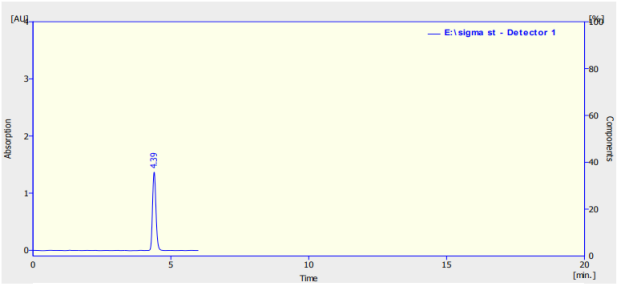



**Figure 12:** UV spectrum of the euphol std. and the isolated compound (B) from the hexane fraction of the leaves.




Result Table (Uncal - E:\sigma st - Detector 1)

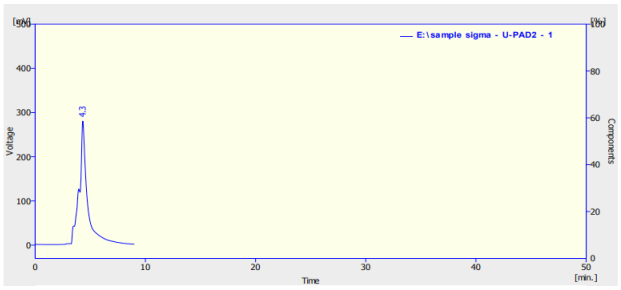
| | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.393 | 461.882 | 148.483 | 100.0 | 100.0 | 0.04 | |
| | Total | 461.882 | 148.483 | 100.0 | 100.0 | | |

**Figure 13:** HPLC analysis for stigmasterol std.

- By CMS

From the Figure 16, the result showed that molecular ion peak at m/z 395 corresponds to $[M\text{-}H2O+H]^+$, m/z 271 corresponds to $[M\text{-side chain -2H}]^+$, m/z 255 corresponds to$[M\text{-side chain-}H_2O]^+$ [25]

We can conclude from all the results mentioned above that the isolated compound (C) might be stigmasterol.




Result Table (Uncal - E:\sample sigma - U-PAD2 - 1)

| | Reten. Time [min] | Area [mV.s] | Height [mV] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.320 | 8199.379 | 241.797 | 100.0 | 100.0 | 0.41 | |
| | Total | 8199.379 | 241.797 | 100.0 | 100.0 | | |

**Figure 14:** HPLC analysis for the isolated compound (C) from the hexane fraction of the leaves.

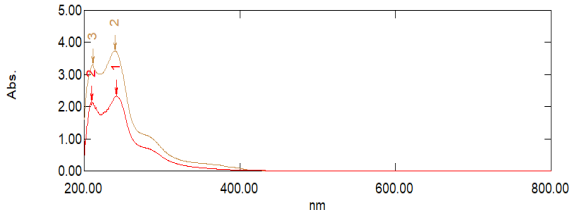



**Figure 15:** UV spectrum of the stigmasterol std. and the isolated compound (C) from the hexane fraction of the leaves.

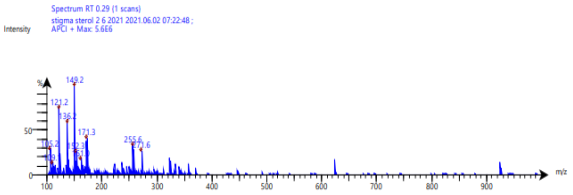


**Figure 16:** The mass spectrum of the isolated compound (C) from hexane fraction of the leaves.

## CONCLUSION

Three compounds were isolated from the hexane fraction of the leaves of *E. tirucalli*: beta-sitosterol, euphol, and stigmasterol.

## ACKNOWLEDGMENT

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