

New Bioactive Molecules Isolated for the First Time from *Hyoscyamus albus* L. and their Mechanisms Underlying the Anticancer Effects

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ABSTRACT

Background: *Hyoscyamus albus* L is a small genus from Solanaceae family known by its use in old traditional medicine in the east of Algeria. **Aim:** This study aimed to characterize new bioactive molecules from *H. albus*, evaluate their anticancer activity in several cancer cells and investigate their possible molecular mechanism. **Materials and Methods:** New compounds (Peak h of fraction F), (Peak 3 of Fraction F), (Peak 1 of fraction C) were isolated from *H. albus* L. by using high-performance chromatography (HPLC), mass spectrometry (MS) and proton NMR (NMR H¹). All isolated compounds were subjected to cytotoxicity and antiproliferative assays against a panel of the four cell lines: DU-145, U-2 OS, U-87 MG and LN-229 cell lines and was determined using MTT assay, Annexin V and propidium iodide was used to evaluate apoptosis. **Results:** The phytochemical study of *H. albus* Fractions led to the isolation of quercetin-3-O-β-d-glucopyranosyl-(1 → 6)-β-d-glucopyranosid, N-trans-feruloyltyramine, Hydrocaffeoyl-N8-caffeoylspermidine. The biological results indicated that all cell lines were consistently sensitive to P1 FC in a dose-dependent manner. This difference in cytotoxic sensitivity was more pronounced in osteosarcoma cell line, U-2 OS when compared to prostate cancer and U-87 MG. Cell viability data also demonstrated that only U-87 MG cells were responsive to treatment with Ph FF. compounds P1 FC and Ph FF have induced necrosis and apoptosis in large part of LN-229 cells. **Conclusion:** The overall results of the present study provided evidence that isolated compounds are potential therapeutic entities against cancer.

Key words: *Hyoscyamus albus* L., Solanaceae, HPLC, Cytotoxic activity, HAMEOH, NMR H¹, Annexin V, Apoptosis.

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INTRODUCTION

Cancer is a group of diseases involving out-of-control of cell growth due to the accumulation of defects, or mutations, in their DNA and with an impendence to invade or spread to other parts of the body¹ cancers progress, treatment typically include radiation, chemotherapy, in hormone-regulated tumors and hormone ablation therapy.⁷ despite the use of the chemical's medication, the use of botanicals-plants, herbs, fungi, as medicine's predates recorded history and represent the most

significant direct antecedent to modern medicine in cancer therapy^{21,23}

H. albus L. also called the white henbane, is a small genus belongs to Solanaceae family which is classed among the important plants family due to its richness of secondary metabolites *H. albus* have been used in traditional medicine from a long time ago as a nervous sedative and para sympatholytic. During time, researchers have isolated scopolamine, hyoscyamine and 2,3-dimethyl nonacosane from this plant.⁵



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Recently *H. albus* have shown an antidiabetic effect and new group of polyhydroxylated nortropane alkaloids called calystegines have been isolated from its seeds²⁴. The aim of the present study is to screen the different methanolic fractions of *H. albus*, identify some bioactive compounds on the plant and study their anticancer activity in different cancer cell lines using MTT assay, annexin V/PI, HPLC, LC mass and NMR H.

MATERIALS AND METHODS

Plant material

H. albus were collected from IGHZAR N'AITH ABDI region, Batna City, Algeria in period of Mai 2015. The plant was identified by Doctor OUDJHIH, Laboratory of Botanic, Department of Agronomy, Batna Algeria. Plant leaves were dried for 40 days at an ambient temperature under shade, after; the leaves were crushed to obtain a fine and homogeneous powder and kept in dry place.

Extraction

The vegetal materials were powdered (1Kg) and extracted with 3 different solvents separately: ether of petrol, chloroform and methanol at room temperature. The solvents were removed in a rotary evaporator at 30°C for ether of petrol and chloroform and 40°C for methanol.

The extracts were stored in sterile bottles and conserved in refrigerator at 4°C until use.

Purification on Sephadex gel

The methanolic extract of *H. albus*'s leaves (HAMEOH) was submitted to column chromatography over Sephadex LH-20 (D. Farmacia. Italy), using methanol as eluent (mobile phase). The obtained fractions (A,B,C,D,E,F) (Merck). Were analysed by Thin Layer Chromatography (TLC) on Selica gel 60 F₂₅₇ plate (Merck) precoated aluminum plates (thickness = 200 µm) using butanol-glacial acetic acid-water system and anisaldehyde sulfuric and FeCl₃ reagents as a spray reagent, finally the similar profiles were combined. All the reagents and solvents used were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany). We obtained four fractions FA (0.147 g), FB (1.35 g), FC (4.08 g), FD (4.22 g), FE (0.71 g), FF (0.65 g), FG (1.76 g).

Preparative HPLC

We have used the reverse phase high performance liquid chromatography (HPLC) to analyze the present compounds in the fractions of *H. albus*. This HPLC

equipped with a C18 column (kintex 5UXB- C18) and UV-photodiode array detection (DAD) was performed at 220 nm and method file 10-60 in 20 min. Pump. With gradient system consisting of solvent A (Acetonitrile) and solvent B (water) with a flow rate 500 µl/min and the volume of injection was 20 (µl) and the temperature of 25°C. Analytical RP-HPLC indicated >95% purity.

Analysis with Mass Spectrum (MS)

Electrospray ionization mass spectroscopic (ESI-MS) of compounds in fractions was performed using an applied Bio systems (LC/MSD TRAP × CT) agilent 6110. Mass spectra were achieved by electrospray ionization in positive mode. We adjusted the prob-flow to 1 ml/min. The continuous mass spectra were obtained by scanning from 100 to 1000 m/z.

Analysis with NMR

NMR was recorded on a Bruker Avance DPX 400 equipment (Germany) operation 400 MHz for NMR H¹ and using methanol deuterium (MeOH-d₄) as a solvent to solubilize compounds Ph FF, P3 FF, P1FC.

Cell Cultures

Human prostate adenocarcinoma cell lines (DU-145), glioblastoma cell lines (LN-229, U-87 MG), osteosarcoma cell lines (U-2 OS) were provided by ATCC. All cell lines were grown in medium, RPMI-1640 or DMEM (Gibco®, Life Technologies, Carlsbad, CA) containing L-glutamine, supplemented with heat-inactivated 10% FBS (Gibco®, Life Technologies, Carlsbad, CA), 100 (U/ml) penicillin and 100 mg/ml streptomycin (Lonza Group Ltd., Switzerland) (Gibco®, Life Technologies, Carlsbad, CA) in a humidified incubator containing 5% CO₂ at 37°C.

MTT Cell Proliferation Assay

The anticancer activity of isolated compounds P1 FC, Ph FF, P3 FF was evaluated by MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide^{18,17} Sigma-Aldrich, Merck, Germany). Our compounds P1 FC, Ph FF, P3 FF were solubilized in Dimethyl sulfoxide (DMSO).

After trypsinization, all cell lines were seeded in 100 µl of medium 96-well plates. One day later, the cells were treated with compounds at a large concentration range, from 200 (µg/mL) to 1.6 (µg/mL), with the fractions C and F at concentration ranging from 500 µM with the fractions C and F a concentration from 50 (ng/mL) to 0.39 (ng/mL). The same protocol was used for the treatment with DMSO, used as control. After 72 h of incubation, the cells were exposed to a 10% MTT solution for 3-4h at 37°C. After the incubation period

the MTT solution was removed and formazan crystals were solubilized with isopropanol/HCl 1N solution for 20 min under constant stirring.

The absorbance was measured at 570 nm with Bio-Rad microplate reader using Microplate Manager Software (Bio-Rad, California, USA).

The cellular viability was calculated as follows:

$$\% \text{ Viability} = (\text{Abs treated} / \text{Abs control}) \times 100.^{18}$$

The half maximal inhibitory concentration (IC_{50}) values were calculated as the concentrations that show 50% of proliferation inhibition on any tested cell line.

Experiments were performed in triplicate.

Annexin V/propidium iodide (PI) staining

Apoptosis analysis was performed by flow cytometry (CytoFLEX flow cytometer, Beckman Coulter, California, USA) and according to the manufacturer's instructions using EzWay™ Annexin V-FITC apoptosis detection kit (KOMA Biotech, Seoul, Korea) Annexin V-FITC (fluorescein isothiocyanate) was used in conjunction with a vital dye, propidium Iodide (PI), to distinguish apoptotic from necrotic cells. Briefly, cells were incubated with Annexin V-FITC and propidium iodide in a binding buffer (eBioscience, Thermo Fisher Scientific, USA) for 30 min at room temperature, washed and resuspended in the same buffer. Analysis of apoptotic cells was performed by CytExpert Software for CytoFLEX (Beckman Coulter, California, USA). For each sample, 1×10^4 events were acquired.⁹

RESULTS AND DISCUSSION

1-Identification of Ph of fraction F quercetin-3-O-β-d-glucopyranosyl-(1→6)-β-d-glucopyranosid

Yellow amorphous powder; $^1\text{H-NMR}$ (500 MHz, CD_3OD) δ : 7.91 (^1H , d, $J = 1.8$ Hz, H-20), 7.62 (^1H , dd, $J = 8.4, 1.8$ Hz, H-6'), 6.98 (^1H , d, $J = 8.4$ Hz, H-5'), 6.53 (^1H , d,

$J = 1.8$ Hz, H-8), 6.29 (^1H , d, $J = 1.8$ Hz, H-6), 5.22 (^1H , d, $J = 8.4$ Hz, H-1''), 3.93 (^1H , d, $J = 7.2$ Hz, H-1''); (Figure 1)

EIMS m/z : 649 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{27}\text{H}_{30}\text{O}_{17}$; 626) (Figure 1).

The structure of compound Ph of fraction F (Figure 2)

2- Identification of P3 of fraction FN-trans feruloyltyramine: (Figure 3)

The structure of compound P3 of fraction F: (Figure 4)

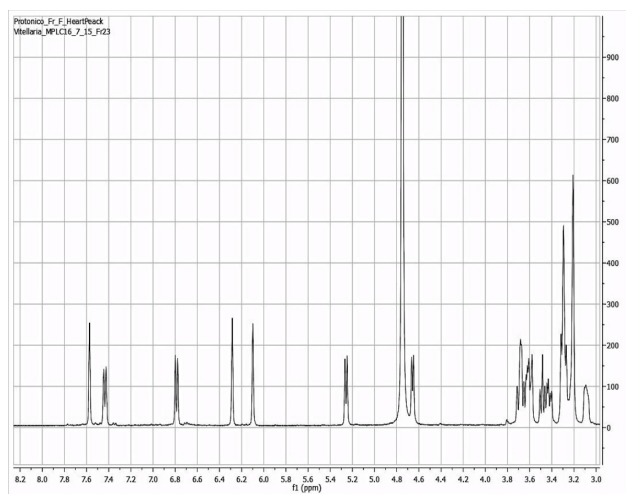


Figure 1: NMR ^1H Profile of P5 fraction F.

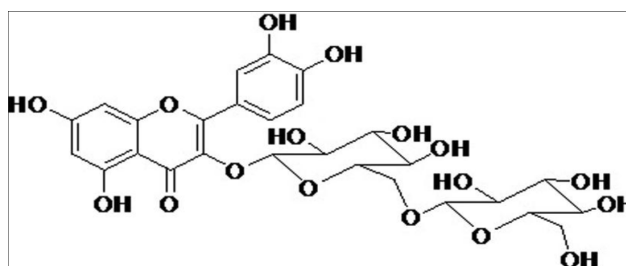


Figure 2: quercetin-3-O-β-d-glucopyranosyl-(1 → 6)-β-d-glucopyranosid.

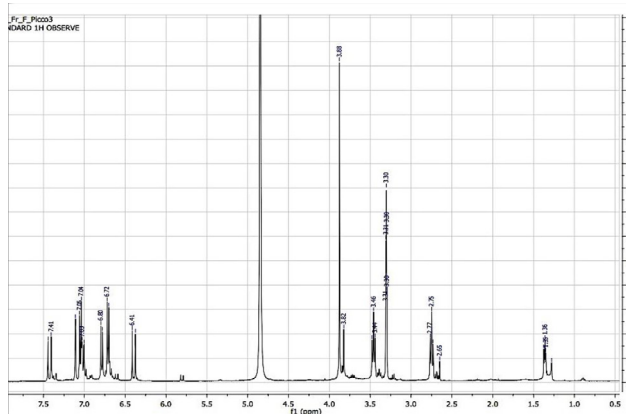


Figure 3: NMR ^1H Profile of P3 fraction F.

The mass spectrum of molecular ion $\text{C}_{18}\text{H}_{19}\text{NO}_4$ of m/z 336 $[\text{M} + \text{Na}]^+$, 192 $[\text{M-ethylbenzene}]$, 177 $[\text{M-ethylaminobenzene}]$. This compound has been reported previously for *Smilax aristolochiifolia*.⁶

3-Identification of P1 of fraction C, N1-(hydro) caffeoyl-N8-(hydro) caffeoylspermidine (Figure 5)
The structure of compound P1 of fraction C: (Figure 6)

This compound has been reported previously and compared to literature [Sun et al. 2015]

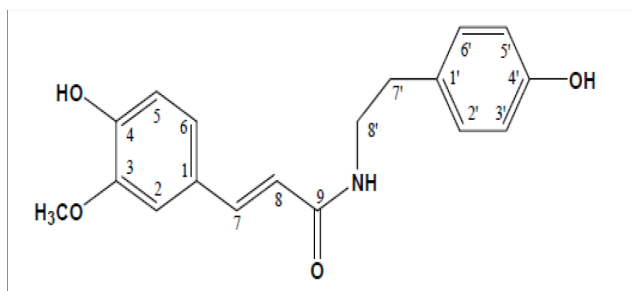


Figure 4: Chemical structure of N-trans-feruloyltyramine.

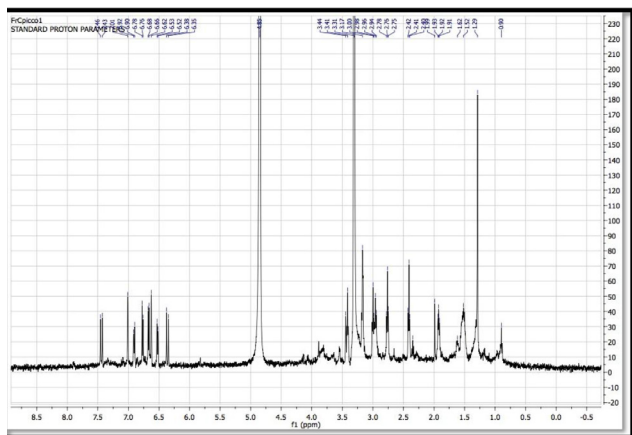


Figure 5: NMR spectrum Profile of P1 fraction C.

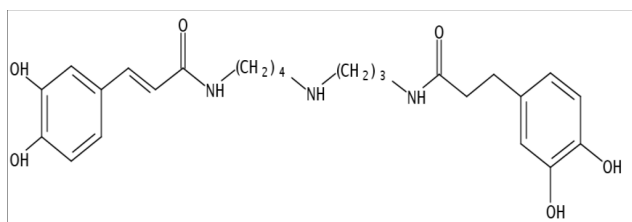


Figure 6: Chemical structure of N¹-(hydro)caffeoyl-N⁸-(hydro)caffeoylspermidine.

3-Cytotoxic activity of compounds

Subsequently, the different compounds were separated from the more active extracts C and F: P1 FC (Peak 1 Fraction 1), Ph FF (Peak h Fraction F), P3 FF (Peak 3 Fraction F). In order to investigate the effect of the purified compounds, we treated the prostate cancer cell line more responsive DU-145, the osteosarcoma cell line U-2 OS and the glioblastoma cells (LN-229, U-87 MG) for 72 hr. IC₅₀ data indicated that all cell lines were consistently sensitive to P1 FC in a dose-dependent manner. This difference in cytotoxic sensitivity was more pronounced in osteosarcoma cell line, U-2 OS (146 mM) when comparing to prostate cancer and glioblastoma cell lines. Cell viability data

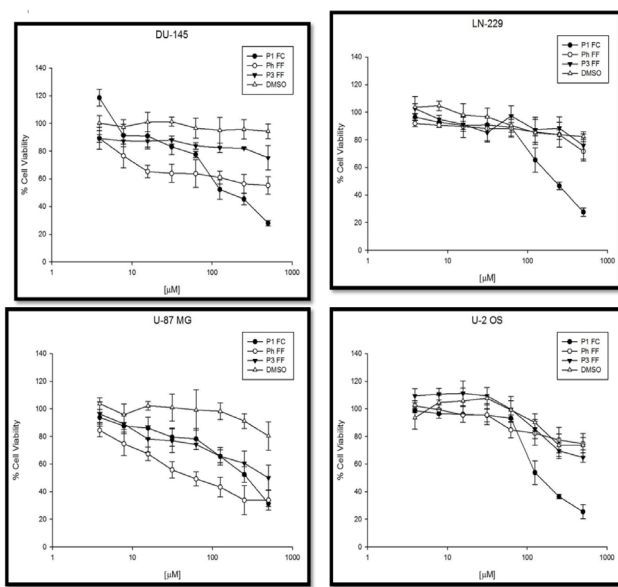


Figure 7: Percentage of viability of cell lines (DU-145, LN-229, U-87 MG, U-2 OS) after treatment with P1 FC, Ph FF, P3 FF.

also demonstrated that only U-87 MG cells were responsive to treatment with Ph FF, with a significantly reduced IC₅₀ (58 mM). The same cell line was also responsive to the treatment with P3 FF but at the highest concentration tested. (Figure 7).

The five tested compounds occurring an anticancer activity against all cell line used, Statistical analyses revealed FC P02 and Ph FF exhibited significantly higher ($P < 0.05$) cytotoxicity in LN-229 and DU-145 cells. FC P01, FC P02 results demonstrated that these compounds induced significant ($P < 0.001$) cell death against DU-145, PC3, Ln-229 cell lines, further FC P03 and FF P03 induced significant ($P < 0.001$) cell death against all the cell lines used.

4- Flow cytometric estimation of induced apoptosis

In order to investigate the biochemical mechanisms on the basis of the growth inhibition, we evaluated the effects of P1 FC, Ph FF on apoptosis by double labelling of LN-229 cells with Annexin V and propidium iodide at FACS. On (Figure 8) the lower left quadrants of each panel show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper left quadrants contain the non-viable, necrotic cells, negative for FITC-Annexin V binding and positive for PI uptake. The lower right quadrants represent cells in early apoptosis that are FITC-Annexin V positive and PI negative. The upper right quadrants represent the cells in late apoptosis, positive for both FITC-Annexin V binding and for PI uptake. After 48 hr of treatment, we found

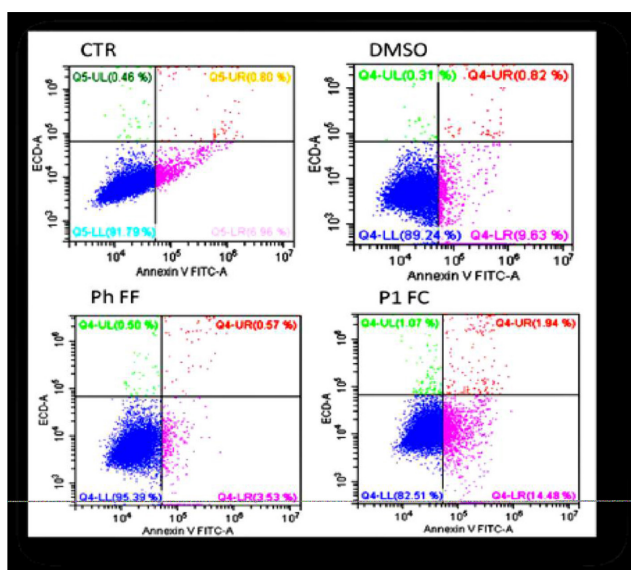


Figure 8: Apoptosis evaluation in LN-229 cells after 48h treatment

that the different treatments induce late apoptosis or necrosis in LN-229 cells. On the contrary, P1 FC induced early apoptosis in 54, 80% of cells as compared with the DMSO, Ph FF (35.35%) and untreated control (6.96%).

Annexin V staining test showed that Ph FF, P01 FC induce cell death by apoptosis, statistical analyses revealed that FC P01 induced significant ($P < 0.001$) cell death by early apoptosis and FF Ph induced significant ($P < 0.001$) cell death by late apoptosis.

As a result to discover novel natural compounds with low toxicity and high selectivity of destroying cancer cells is an important cancer research sector. Due to the wide range of biological activities and low toxicity in animal models, some natural products have been used as alternative treatments for many cancer types. *H. albus* is Mediterranean small plant belong to solanaceae family in which many bioactive compounds have been isolated as atropine, scopolamine, hyoscyamine and 2, 3 – dimethyl nonacosane.²³ Many natural compounds isolated from plants, marine flora and fauna, micro-organisms have been used for cancer treatment or prevention whereby their therapeutic potential is improved by molecular modification.⁹ From plant sources we can find many molecules with an important antitumoral effect as Flavopiridol which is a synthetic flavone derived from the plant alkaloid rohitukine and was isolated from *Amoora rohituka* leaves and stems.²³ Homoharringtonine is an alkaloid isolated from the Chinese tree *Cephalotaxus harringtonia* and have a strong activity against various leukaemias,²⁷ lapachone is a quinone obtained from the bark of the lapacho tree

(*Tabebuia avellanedae*) which induce apoptosis and necrosis on several human carcinoma cells, prostate, colon, lung and breast.²⁸

The phytochemical study of the aerial part of *Hyoscyamus albus* L., Solanaceae, led to the isolation of quercetin-3-O- β -d-glucopyranosyl-(1 \rightarrow 6)- β -d-glucopyranosid (QCGG), of the alkaloid *N-trans*-feruloyltyramin and N^1 -(hydro)caffeyl- N^8 -(hydro)caffeylspermidine, using NMR H, LC mass and HPLC.

The results indicated that compounds isolated from HAMEOH possessed a significant activity against cells lines showed a marked anti-cancer activity and induce different apoptosis and necrosis which can be initiated via two alternative signaling pathways: the death receptor-mediated extrinsic apoptotic pathway and the mitochondrion-mediated intrinsic apoptotic pathway.²⁹

Many researches proved that flavonoids are the best candidates with protective effects against the different kinds of cancer.¹⁹

In our study all cell lines used were consistently sensitive to N^1 -(hydro)caffeyl- N^8 -(hydro)caffeylspermidine in a dose-dependent manner and induce 14, 9 % of an early apoptosis and 1, 7% necrosis on Ln-229 cells. This difference in cytotoxic sensitivity was more pronounced in osteosarcoma cell line, U-2 OS (146 mM) when comparing to prostate cancer and glioblastoma cell lines. Cell viability data also demonstrated that only U-87 MG cells were responsive to treatment with Quercetin 3-O- β -D glucopyranosyl-(1--6)- β -D-glucopyranoside], with a significantly reduced IC_{50} (58 mM). The same cell line was also responsive to the treatment with *N-trans* feruloyltyramine we found that the fraction F contains quercetin-3-O- β -d-glucopyranosyl-(1 \rightarrow 6)- β -d-glucopyranosid and in previous studies they proved that Quercetin has to prevent against prostate cancer especially.²⁰ Also, they mentioned that Quercetin has a capacity to inhibit the development of breast cancer (MCF-7 and MDA-MB231).¹⁶ The quercetin is known Haut du formulaire by its antioxidant activity against oxidative stress. Also, the quercetin protects the cells against the damages caused by free radicals by antioxidant effect.

The previous studies showed that the quercetin induce the apoptosis of cancer cells and inhibit the protein kinase C¹⁴ and modulate the oxido reduction processes.¹³ The derivative of quercetin which is the quercetin-3-O-b-D-glucopyranosyl-(16)-b-D-glucopyranoside (QCGG), was isolated for the first time from traditional Korean medicinal herb, Persimmon (*Diospyros kaki*). Also, they proved that the QCGG has a effect on melanin synthesis and its underlying signaling pathways in alpha-melano-

cyte- stimulating hormone (α -MSH)-stimulated B16F10 cell the apoptosis by prooxidant activity and inhibit tumorigenesis.¹²

N-trans-feruloyltyramine active phenylpropanoid were also isolated from the leaves of *Solanum sordidum* and showed an antioxidant activity and have a protective effect against $A\beta$ (1-42)-induced neuronal death.³⁰

CONCLUSION

H. albus showed an anticancer activity against several cells line due to its richness of many bioactive molecules which have been isolated in our study or the perviousness one.

All compounds were characterized from fraction F and C. after HPLC, MS and NMR H¹ analysis, so this compounds were tested separately to improve their efficacy on inhibiting the cells growth.

The isolated compounds are potential candidates for drug development based on their effective cytotoxic and antiproliferative activities.

The perspectives for future research is to isolate more bioactive compounds from *H. albus*, to test their anti-cancer potential and to investigate more molecular mechanisms underlying its proprieties.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

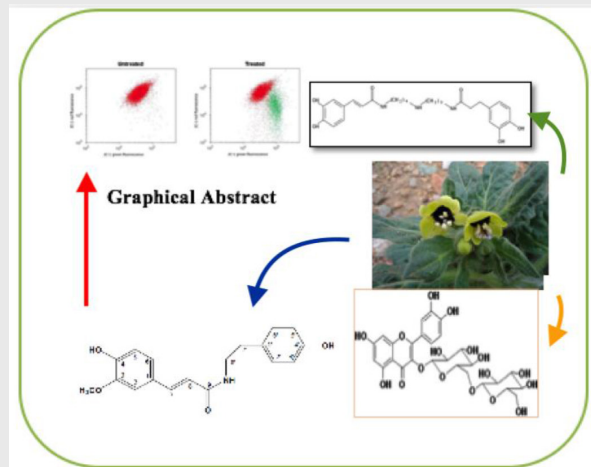
FC: Fraction C; **FF:** Fraction F; **P:** compound.

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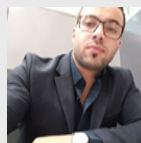
PICTORIAL ABSTRACT



SUMMARY

- Plant was collected from Aures Region Batna, Algeria
- Phytochemical screening lead to isolation of new compounds
- Isolated compounds have anticancer activity
- Isolated compounds induce apoptosis marked by Annexin V

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