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Chlorogenic acid with cytotoxic activity and other constituents from *Anacyclus valentinus* from Algeria

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Abstract: Anacyclus valentinus is a Saharan plant belonging to the Asteraceae family. This study identified and determined the structure of isolated compounds obtained from crude extract of *A. valentinus*. Their cytotoxic effects on two human cancer cell lines were examined. The extract of the aerial part of *A. valentinus* was fractioned by Solid Phase Extraction (SPE). The fractions obtained were analyzed by (NMR) spectroscopy and HPLC-DAD-MS. This is the first cytotoxic investigation of *A. valentinus*. The extracts of the plant were prepared, and their cytotoxic effects on two human cancer cell lines (A549, human lung adenocarcinoma; HepG2, hepatocellular carcinoma) were examined using the MTT assay. Several compounds have been identified. The results illustrated two newly identified compounds: chlorogenic acid and the β-glucosides derivative. In addition, C-glycosides (2 isomers of apigenin) were detected in the genus *Anacyclus*. The IC_{50} values of crude extracts of the aerial parts of *A. valentinus* against the A549 cell line were determined as 19.79 µg/mL. The values for the HepG2 cell line were 32.63 µg/mL. Chlorogenic acid was showed the highest cytotoxic activities on the A549 and HepG2 cell lines with IC50 values of 13.59 and 12.84 µg/mL, respectively.

Keywords: Anacyclus valentinus; chlorogenic acid; cytotoxic activity; flavonoids; β-glucosides; HPLC-DAD-MS.

INTRODUCTION

The healing potential of plant has been known for thousands of years. Plants produce a whole series of different compounds which are not of particular

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significance for primary metabolism, but can have a remarkable effect to other plants, microorganisms and humans. Currently, due to their effectiveness with low side effects, medicinal plants have a growing demand in the prevention and treatment of diseases as natural remedies. These organic compounds are defined as biologically active substances[1] and include polyphenols and essential oils.[2]

Anacyclus valentinus L. Aiton (*Asteraceae*), called "Guertoufa" in Arabic, is an annual plant restricted to the Mediterranean region, well represented in the western Mediterranean Basin, including Algeria[3] and Spain,[4] its distribution is related to annual mean temperature (BIO 1) and the precipitation of the coldest quarter (BIO19). Nevertheless, *A. valentinus* presents a high degree of intergeneric hybridization that makes its collection difficult. This plant is known for its anti-diabetic[5] and antifungal[6] effects. It is also used in some parts of the country as a food condiment.

The phytochemical constituents of *A. valentinus* revealed the presence of essential oil[7,8] which has a potential for use as a safe biocontrol agent to prevent food crops from fungal diseases and improve product quality and safety.[9]

Currently, have been identified by Gas Chromatography Mass Spectrometry (GC/MS) analysis, 29 components of which δ-3-carene (31 %), spathulenol (14.2 %), were the major compounds of this oil. The methanol extract of *A. valentinus* has been reported to contain quercetin, myricetin, kaempferol, luteolin and apigenin.[10,11]

The aim of this study was to identify and determine the structures of compounds isolated from different fractions of *A. valentinus* using spectroscopic methods, to determine their potential cytotoxic activities.

In the present study, phytochemical analysis of *A. valentinus*, revealed the presence of other phenolic acid and flavonoid compounds.

Thus, flavonoids have various bioactive effects, including anti-inflammatory, cardioprotective, antidiabetic, antiviral, and anticancer effects.

Drugs isolated from plants and microorganisms or synthesized after isolation constitute a significant proportion of anticancer agents used in cancer treatment.[12] Plants are a good source of antitumor compounds; indeed, some compounds initially derived from plants[13–16] have good potential as primary sources of chemotherapeutic drugs. Some chemotherapeutic agents, such as vinca alkaloids for leukemia, paclitaxel for breast cancer, and flavopiridol for colorectal cancer, have been derived from medicinal plants.[17]

Several studies have shown that herbal medicine may prevent tumor growth and exhibit cytotoxic activities on tumor cells without negative effects on normal cells.[18–20]

EXPERIMENTAL

Vegetal material

Anacyclus valentinus was collected during the period (April-May) in Tiaret region, the plant material was identified and validated by Prof. Dr. Hadjadj-Aoul.S (Botany Department, Oran1, Algeria). The plant was cleaned, washed with tap water and dried in the shade. Then it was weighed, coarsely ground, and collected in clean bags. Voucher specimens were identified and deposited in the herbarium of the Agricultural Institute in Algeries, Algeria.

Chemicals

Formic acid and HPLC grade methanol were purchased from Sigma-Aldrich. All chemicals: Acetone, methanol, and ammonium formiate were purchased from Sigma-Aldrich.

Sample preparation of the extracts

The following extracts were prepared: 25, 50,100 and 200 mg of the aerial parts of the plant were macerated with 4 mL of two solvents: (A: formic acid and methanol (99/1 v/v), and B: formic acid, acetone and water (60/39/1 v/v/v). The solutions were subjected to agitation by an extractor (Precelly® 24, Bertin Technologies S.A.S, France) according to the parameters 5100-3 x 40-040, during 10 minutes and 3 times, the liquid phase was evaporated.[21] Reagents were purchased from Sigma Aldrich.

HPLC-DAD -MS analysis

The chromatographic and mass analysis of the samples were carried out on a chain UPLC Waters coupled with a mass spectrometer (Brucker) by liquid chromatography with ultra-high performance with ionization by atmospheric pressure and an ionic trap door to obtain three levels of fragmentation.[22] The HPLC analysis was carried out on a Waters 2690 HPLC system equipped with a Waters 996 DAD (Waters Corp., Milford, MA) and Empower software (Waters).[23]

The Column used is C18, 1.7µm. The solvents were 1 % aqueous formic acid (solvent A) and water/formic acid (98:1) (Solvent B). The states of gradient conditions were as follows: from 0 to 6 min 98 % A, in 7min 82 % A, in 12 min at the 14 min 70 % A and 30 % B and at 27 min 25 % A min and 75 % B and in 32min 95 % B with a flow of 0.08mL/min. The volume of injection was the 0.5 µL and detection was carried out between 210 and 650 nm. Experiments were performed in negative and positive ion mode. The desolvation temperature was 300 °C. High spray voltage was set at 5000 V. Nitrogen was used as the dry gas at a flow rate of 75 mL min^{-1}. MS was carried out using helium as target gas. Identifications were achieved on the basis of the molecular ion mass, fragmentation, UV-visible spectra, and relative retention times or co-injection with standards.[24]

Breaking-up by cartouche SPE

The methanolic extract was dissolved in water (9 ml), filtered and fractionated by SPE using a Waters-SPE-PakRvac 20cc TC 18-5g cartridge. After cleaning with 5 mL of a solution of water/formic acid (99/1 v/v) and 5 mL of methanol/formic acid (99/1 v/v) and with a preconditioning with 5 ml of water/formic acid (99/1 v/v), the cartridge was loaded with the sample (2 ml). The analysts were eluted three times with 5 ml of methanol/water/formic acid (15/84/1 v/v/v), three times with 5 ml of methanol/water/formic acid (25/74/1 v/v/v), three times with 5 ml of methanol/water/formic acid (30/69/1 v/v/v), three times with 5 ml of methanol/water/formic acid (60/39/1 v/v/v). The protocol was repeated with 2 ml of sample until exhaustion. The fractions were combined and evaporated with a rotary evaporator.

The SPE makes it possible to extract and preconcentrer the sample in a liquid matrix, because is an adequate method for the preparation of samples.[25] The SPE is then followed of a chromatographic method of analysis.

Cytotoxic assay

Cell lines and culture medium

The following cancer cell lines were used in this study: A549 (human lung adenocarcinoma) and HepG2 (hepatocellular carcinoma). Cells were obtained from the National Cell Bank of Algiers (Pasteur Institute, Algiers).

MTT assay

The cytotoxic activity of crude extracts of the aerial parts of *A. valentinus* and chlorogenic acid compound was determined using an MTT colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay)

Cell viability was quantified using an MTT colorimetric assay (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay).[26] Cells were seeded into 96-well plates were incubated for 72 h at 37°C in a 5 % CO2 incubator. After that, chlorogenic acid compound and plant extracts at different concentrations 0.005, 0.01, 0.02,0,03 and 0.04 mg/mL were dissolved in 10 % (DMSO) and added to the cell culture to be tested against cell lines. The cells were incubated for 24, 48, and 72 hr to evaluate dose and time responsiveness.

Subsequently, 10 µL phosphate-buffered saline containing 5 mg/mL MTT was added to each well. After 4 h of incubation, the medium was discarded, and formazan blue crystals formed in the cells were dissolved in 100 mM DMSO. Reduced MTT concentrations were quantified using a microplate reader (Thermo Scientific Multiscan Spectrum) at 540 nm absorbance. The cytotoxic effects of the tested extracts were determined by comparing the optical density of the treated cells with that of the untreated cells. Cytotoxicity relative to controls was calculated using the following formula:

$$\% \text{ Cytotoxicity} = [(A_c - A_t)/A_c)] \text{ X}100 \quad (1)$$

Where A_c and A_t are the mean absorbances of the control and test wells, respectively.

Statistical analysis

The data were analyzed using SPSS software (Microsoft Corp., Chicago, IL, USA) (2007). We performed multiple treatment comparisons using one-way analysis of variance and Student's t-test. Differences were considered statistically significant at *p <0.05, *p <0.01, and *p <0.005. Data are presented as the mean ± standard deviation of three replicates.

RESULTS AND DISCUSSION

Identification of chromatographic peaks HPLC-DAD-MS

Analysis of each of these fractions showed several compounds eluting throughout the chromatographic profiles.

The profiles of the fractions appeared to be quite different, and most of the compounds were recovered in only one of them (Fig. 1). Moreover, some compounds eluting at the same retention time in the fractions showed different UV–visible properties.

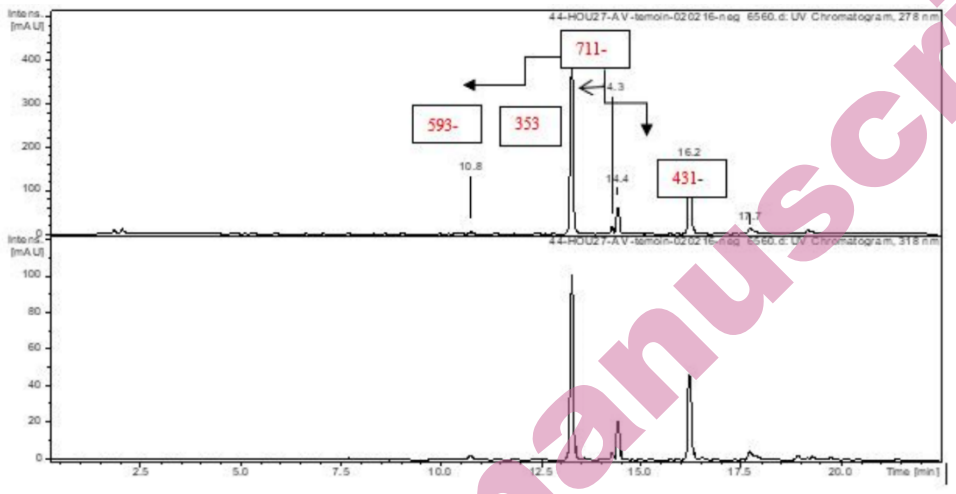


Fig. 1. Chromatogram of HPLC-DAD-MS of the methanolic extracted of the plant *A. valentinus*

The phenolic acid and flavonoid compounds are given in (Table I).

Table I. Phenolic components detected in *A. valentinus* aerial part in methanolic extract by HPLC-DAD –MS

Rt (min)	UV (λ max)	Mol. ion [M-H]–(m/z)	Fragmentation	Identified Compound
10.8	289	711	355 (100), 193 (100), 149 (23)	derivative β-glucosides
13.3	300	353	191 (100), 178 (9)	chlorogenic acid
14.3	293-305	711	355 (100), 549 (37), 369 (32), 271 (18)	dimer of derivative β-glucosides
14.4	271-336	593	472 (100), 353 (42), 503(36), 575 (19), 383 (27)	6,8-di-C-glucopyranosyl apigenin
16.2	295-319	711	355 (100), 193 (100), 149 (23)	Dimer of Derivative β-glucosides
17.7	269-340	431	311 (100), 431 (40), 413 (9)	Vitexin derivatives

The methanolic fraction presents the highest content of polyphenols and flavonoids in this study.

The molecular structure of the compounds found is the following: Figure 2 shows the structures of derivative β-glucosides and his dimer (A) and (B).

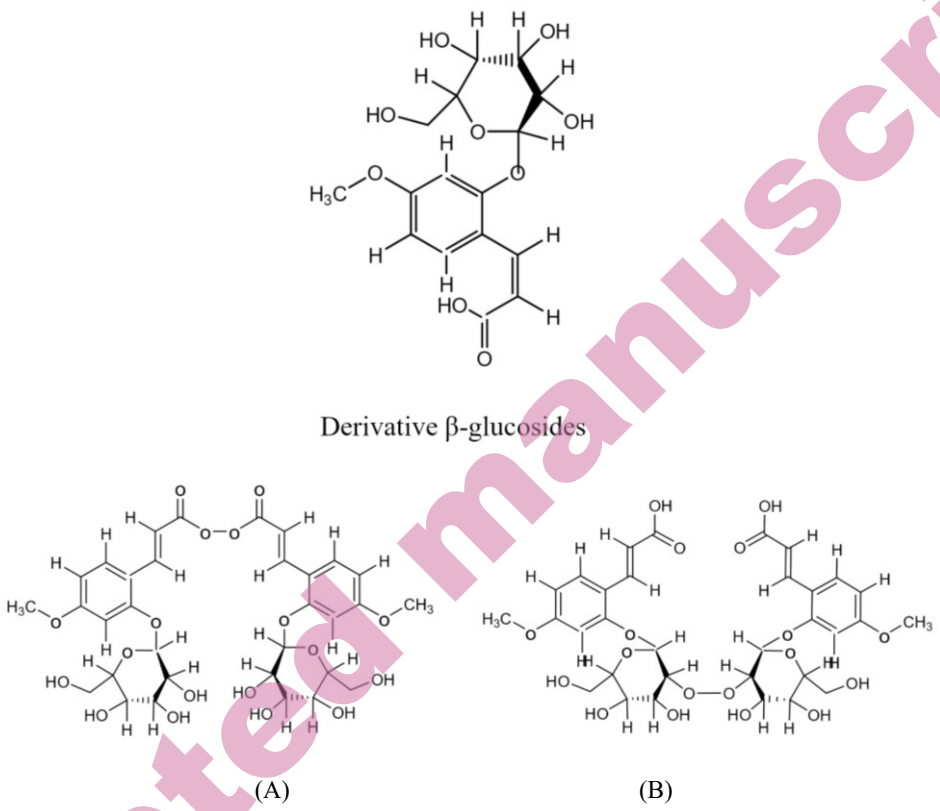


Fig. 2. Structures of derivative β-glucosides and its dimer (A) and (B)

Figure 3 shows major compounds chlorogenic acid, identified with a retention time of 13.3 min with absorption wavelength peak at 300nm, while (mass spectrum 593, had a retention time of 14.4 min with two absorption wavelength peaks (271nm, 336nm) and corresponds to flavone, 6,8-Di-C-glucopyranosyl apigenin (Fig. 4).

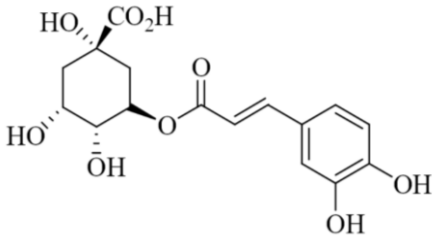


Fig. 3. Structure of chlorogenic acid

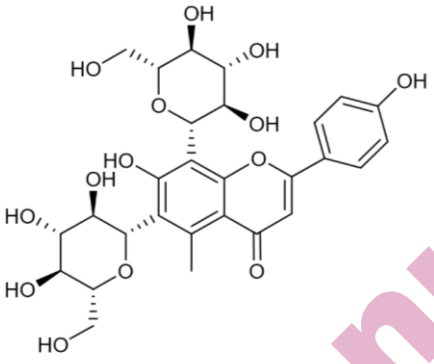
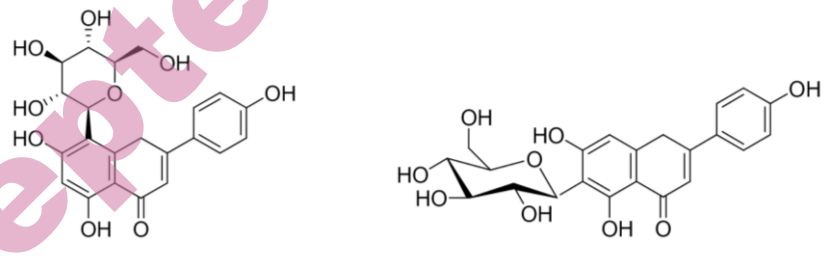


Fig. 4. Structure of 6,8-di-C-glucopyranosyl apigenin

Peaks with a retention time of 10.08 and 16.2 min correspond to the same mass 711 with the same fragmentation (355, 193, 149). The molecule is symmetric it would give only set of NMR signals as the monomer (Results no showed). This explains that they are isomers.

The last pic at 17,7 min, spectrum UV with 2 absorption wavelengths peaks (269 nm, 340 nm), and her mass spectrum 431 corresponds to flavone, derived from the vetexin (Fig. 5).



(A) (B)

Fig. 5. Structures of 8-C-glucosylapigenin (vitexin) (A) and 6-C-glucosylapigenin (isovitexin) (B)

Cytotoxic assay

As shown in graphs 1 and 2 (Fig. 6 and 7): The IC50 values of crude extracts of the aerial parts of *A. valentinus* against the A549 cell line were determined as 19.79 µg/mL. The values for the HepG2 cell line were 32.63 µg/mL.

The different extracts showed dose-dependent cytotoxic effects on the cancer cell lines. Chlorogenic acid exhibited the best cytotoxic effects on the A549 and HepG2 cell lines with IC50 values of 13.59 and 12.84 µg/mL, respectively.

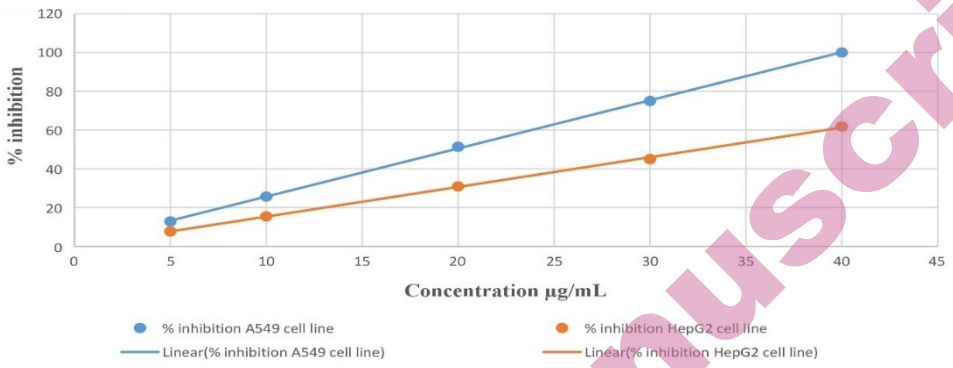


Fig. 6. Inhibition percentage of cell viability of crude extracts of the aerial parts of *A. valentinus* against the A549 cell line and the HepG2 cell line

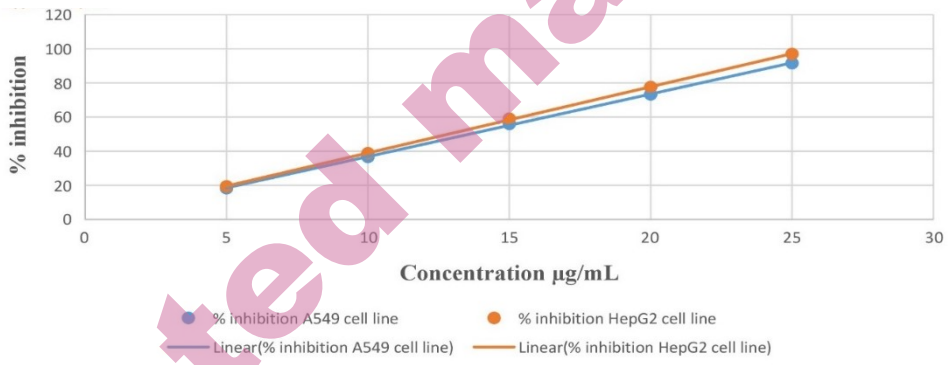


Fig. 7. Inhibition percentage of cell viability of chlorogenic compound against the A549 cell line and the HepG2 cell line

The use of *Anacyclus* species in folk medicine in North Africa is widely attributed to their recognized therapeutic properties, which are attributed to the presence of different compounds,[28–30] including terpenoids, flavonoids and alkaloids.[31]

The results obtained in this study are agreed with the composition of aerial parts of other species[32,33] of gender *Anacyclus* confirmed that the methanolic extract from the aerial parts of *A. maroccanus* and *A. radiatus* contained chlorogenic acid, that joint to rutin were the major identified compounds in these species.

Nevertheless, this is the first time that have been identified the following compounds: vitexin, isovitexin, and derivatives β-glucosides in *A. Valentinus* and suggest that the identified compounds may play a role in the bioactivities of the extracts, which needs further research.

Some flavones like vitexin and isovitexin are active components of many traditional Chinese medicines and were found in various medicinal plants. Vitexin

(8-C-Glucosylapigenin) has recently received increased attention due to its wide range of pharmacological effects, including but not limited to anti-oxidant, cytotoxic, anti-inflammatory, antispasmodic, and neuroprotective effects. Isovitexin (apigenin-6-C-glucoside), an isomer of vitexin, generally purified together with vitexin,[34] also exhibits diverse biological activities,[35,36] Flavonoids have various bioactive effects, including anti-inflammatory, cardioprotective, anti-diabetic, anti-viral and anti-cancer. For that, the knowledge of these compounds will help in formulating pharmaceutical products.

Our results showed that the plant extract of *A. valentinus* could significantly inhibit cancer cells. It could therefore be a preventive agent against the development of cancer cells.

The differents extracts and chlorogenic compound were exhibited antiproliferative activity against the two of the cell lines

Chlorogenic acid was isolated from *Anacyclus* species for the first time and showed the highest cytotoxic activities on the A549 and HepG2 breast cancer cell lines.

In fact, several studies[37–39] showed that chlorogenic acid, an important biologically active dietary polyphenol, is produced by certain plant species. Reduction in the risk of a variety of diseases.

CONCLUSION

This is the first phytochemical and cytotoxic investigation of an endemic species of A. *valentinus*. The identification of phenolic compounds in the air extract of *A. valentinus* was evaluated.

In the present study, phytochemical analysis of *A. valentinus*, revealed the presence of other phenolic acid and flavonoid compounds.

The compounds found in this study of the plant *A. valentinus* have many biological activities. Chlorogenic acid and methanolic extracts of *A. valentinus* showed significant anticancer activity against cancer cell lines. Herbal-based products have spanned hundreds of years and have been identified as a potent approach for the treatment of various human diseases, such as cancer.[40]

In future studies, the level of other chemical components of *A. valentinus* and the major extracts responsible for anticancer activity will be determined, and the molecular mechanisms of cell death will be elucidated in more detail.

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ИЗВОД

ХЛОРОГЕНА КИСЕЛИНА СА ЦИТОТОКСИЧНОМ АКТИВНОШЋУ И ДРУГИ САСТОЈЦИ *ANACYCLUS VALENTINUS* ИЗ АЛЖИРА

BAKHTA RAMLI[1], MOHAMED RABIE MOKRED[1], ABDELKADER HAMIANI[1], SALIMA BELLAHOUEL BENZINE[1], CHOUKRY KAMEL BENDEDDOUCHE[1], MARIA TERESA LAO[2], MARIE-LAURE FAUCONNIER[3] и NADIA KAMBOUCHE BOUZIDI[1]*

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Anacyclus valentinus је биљка Сахаре која припада породици Asteraceae. У овој студији су идентификована једињења изолована из сировог екстракта *A. valentinus* и одређена је њихова структура. Испитан је њихов цититоксични ефекат на две хумане канцерске ћелијске линије. Екстракт надземних делова *A. valentinus* је фракционисан методом екстракције на чврстој фази (SPE). Добијене фракције су анализиране NMR спектроскопијом и методом HPLC-DAD-MS. Ово је прва студија која анализира цитотоксичност *A. valentinus*. Цитотоксичност екстраката је тестирана на хуманим канцерским ћелијским линијама A549 (аденокарцином плућа) и HepG2 (хепатоцелуларни карцином) применом MTT теста. Идентификовано је више једињења, укључујући и два нова: хлорогена киселина и β-гликозидни дериват. Такође, детектовано је присуство Ц-гликозида (2 изомера апигенина). Вредност IC_{50} сировог екстракта надземних делова *A. valentinus* спрам A549 ћелијске линије је била 19,79 μg/mL, а спрам HepG2 32,63 μg/mL. Хлорогена киселина је испољила највећу цитотоксичност спрам A549 и HepG2 ћелијских линија, са IC50 вредностима 13,59, односно 12,84 μg/mL.

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