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ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF THE HEPATOTOXIC EFFECTS OF SOME HERBAL PRODUCTS BY *IN VITRO* METHOD

BAZI BİTKİSEL ÜRÜNLERİN HEPATOTOKSİK ETKİLERİNİN İN VİTRO YÖNTEMLE ARAŞTIRILMASI

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ABSTRACT

Objective: *This study investigated the hepatotoxic effects of the Ayurvedic plants Atractylis lancea, Stevia rebaudiana, and Senecio vulgaris exclusively through in vitro methods. Although many individuals use herbal products to support general health or relieve various symptoms, these products are often perceived as inherently safe due to their botanical origin. Such misconceptions may encourage their uncontrolled short- and long-term use, thereby posing potential health risks. In this context, the hepatotoxic potential of diterpene glycosides and pyrrolizidine alkaloids derived from Asteraceae species was examined in vitro using human hepatocellular carcinoma (HepG2) cell lines to provide insights into their safety profiles.*
Material and Method: *Two complementary cytotoxicity assays—MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and LDH (lactate dehydrogenase)—were applied to HepG2 cells cultured in vitro. Both assays are well-established, widely used, and reliable for assessing hepatocellular injury.*
Result and Discussion: *The results emphasize the necessity of systematic toxicological evaluation of herbal products widely used in traditional medicine. Clarifying the mechanisms of xenobiotic-induced hepatotoxicity through in vitro studies is essential for risk assessment and for the predicting of potential health hazards. This study provides further insight into the hepatotoxic potential of Ayurvedic herbal products and highlights the need for cautious use within public health contexts.*
Keywords: *Ayurvedic plants, hepatotoxicity, HepG2 cells, in vitro*

ÖZ

Amaç: *Bu çalışmada, Ayurvedik bitkiler olan Atractylis lancea, Stevia rebaudiana ve Senecio vulgaris'in hepatotoksik etkileri yalnızca in vitro yöntemlerle araştırılmıştır. Günümüzde birçok birey, genel sağlığını desteklemek veya belirli semptomları hafifletmek amacıyla bitkisel ürünleri tercih etmektedir. Ancak bu ürünler, bitkisel kökenli olmaları nedeniyle çoğunlukla tamamen*

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güvenli oldukları yönünde yanlış bir algıyla değerlendirilmektedir. Bu yanlış inanış, kısa ve uzun vadeli kontrolsüz kullanımı teşvik ederek potansiyel sağlık risklerini artırmaktadır. Bu bağlamda, Asteraceae familyasından elde edilen diterpen glikozitler ve pirolizidin alkaloidlerin hepatotoksik potansiyeli, insan hepatoselüler karsinom (HepG2) hücre hattı kullanılarak in vitro koşullarda incelenmiş ve güvenlik profilleri hakkında bilgi sunulmuştur.

Gereç ve Yöntem: *İki tamamlayıcı sitotoksisite testi-MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür) ve LDH (laktat dehidrogenaz)-in vitro koşullarda kültüre edilmiş HepG2 hücrelerine uygulanmıştır. Her iki test de hepatoselüler hasarı değerlendirmede iyi tanımlanmış ve yaygın olarak kullanılan yöntemlerdir.*

Sonuç ve Tartışma: *Bulgular, geleneksel tıpta yaygın olarak kullanılan bitkisel ürünlerin sistematik toksikolojik açıdan değerlendirilmesinin önemini vurgulamaktadır. Kseno-biyotiklerin neden olduğu hepatotoksisitenin mekanizmalarının in vitro olarak aydınlatılması, risk değerlendirmesi ve potansiyel sağlık tehlikelerinin öngörülmesi açısından kritik öneme sahiptir. Bu çalışma, Ayurvedik bitkisel ürünlerin hepatotoksik potansiyeline dair mevcut bilgi birikimine katkı sağlamakta ve halk sağlığı bağlamında dikkatli kullanım gerekliliğini ortaya koymaktadır.*

Anahtar Kelimeler: *Ayurvedik bitkiler, hepatotoksisite, HepG2 hücreleri, in vitro*

INTRODUCTION

In recent years, the hepatotoxic potential of some herbal remedies commonly used in traditional medical systems such as Ayurveda has emerged as a significant topic of interest in modern toxicology. Some compounds, particularly glycoside and alkaloid compounds, have been shown to cause serious liver dysfunction. Plants such as Atractylis lancea, Stevia rebaudiana, and Senecio vulgaris belonging to the Asteraceae family pose a potential risk of toxicity due to their bioactive components such as Atractyloside A, Stevioside, Senecionine, and Senecionine N-Oxide, despite their traditional uses [1].

Ayurveda, an ancient system of traditional medicine, originated in the Indian subcontinent. According to Ayurvedic principles, the universe is composed of five elements: Vayu (Air), Jala (Water), Aakash (Space), Prithvi (Earth), and Teja (Fire), which constitute the three fundamental humors—Vata dosha, Pitta dosha, and Kapha dosha—that govern the basic physiological functions of the human body. This triad is called the "Tridosha," and each is consists of five subcategories. While Ayurvedic practitioners maintain that this system is a complete medical model, Ayurveda has been described as lacking the scientific and rigor and methodological approaches necessary for diagnosing and treating disease [2].

Identifying the ingredients involved in cases of hepatotoxicity associated with Ayurvedic medicines presents significant challenges due to factors such as product mislabeling, absence of labeling, presence of toxic additives or contaminants, and the complex polyherbal composition of herbal preparations. Therefore, it is important for clinicians to have precise knowledge of potentially hepatotoxic herbs in patients with liver damage associated with Ayurvedic herbal medicines [3].

Liver injury occurs through cellular stress, drugs, specific immune reactions, disruption of intracellular ion balance, disruption of mitochondrial membrane potential, apoptosis, or bile acid-dependent mechanisms [4]. This condition, defined as hepatotoxicity, arises through hepatotoxins of medicinal, chemical, dietary, or plant origin. Hepatotoxicity generally happens through mechanisms such as cellular stress formation, alteration of mitochondrial membrane permeability, and activation of pro- apoptotic proteins [5].

Many pharmaceutical agents, including antibiotics, analgesics, antiemetics, and anticancer drugs, can also cause hepatotoxicity [6,7]. Today, various test kits are being developed using human hepatoma cells (HepG2) to assess cytotoxicity, one of the most widely used parameters in measuring hepatotoxicity. In these tests, using two different cytotoxicity methods, such as MTT and LDH, is recommended to understand the causes of cell loss [8].

People often use herbal products without scientific evaluation, leading to various health risks [9]. The global burden of hepatotoxicity affects more than fifty million people worldwide [10]. In this study, the widely used plants *Atractylis lancea*, *Stevia rebaudiana*, and *Senecio vulgaris*, whose toxicity mechanisms have not yet been clarified in the literature, were evaluated; the hepatotoxic effects of the glycoside and alkaloid chemicals contained in these plants were investigated *in vitro* on the HepG2 cell

line by two different cytotoxicity methods.

It has been reported that Atractyloside A, one of the active ingredients of the *Atractylis lancea* plant, exhibits antiviral activity against the influenza B virus and can alleviate the damage caused by this virus by regulating M2 macrophage polarization [11]. However, its toxicological mechanism has not been fully elucidated, and therefore it was investigated within the scope of the study.

Stevia rebaudiana has been traditionally used as a natural sweetener, and glycosides such as Stevioside and Rebaudioside A found in its leaves are 250–300 times sweeter than sucrose [12-14]. Although the toxicological effects of Stevia are reported as safe in the literature [15], it is important to evaluate the potential hepatotoxic effects of Stevia by *in vitro* and in silico methods.

Senecio vulgaris is rich in pyrrolizidine alkaloids (PA), and Senecionine and Senecionine N-Oxide are the major constituents responsible for the toxic effects of this plant. It is reported that these alkaloids cause liver damage by being converted into reactive metabolites via the CYP450 system, especially leading to hepatic sinusoidal obstruction syndrome [16,17].

The increasing use of herbal medicines worldwide, lack of regulation, and a false sense of security create the need for serious toxicological evaluation. Cases of acute and chronic liver injury associated with herbal products such as Jin Bu Huan and Ma-Huang illustrate this situation [18,19]. The fact that women are more frequently affected by such hepatotoxicity suggests a gender-based susceptibility [20].

Determination of the toxicological profile of plants such as Senecio, Atractylis, and Stevia has become necessary considering the lack of standardization, contamination risk, and interaction potential [21]. This study aimed to elucidate the toxic effects of these plants by applying *in vitro* hepatotoxicity assessment methods.

The HepG2 human liver cell line was chosen for this study due to its several advantages, such as ethical compliance, low cost, and reproducibility. It is widely used in toxicology studies due to its metabolic activity, ease of culture, and extensive literature support [22,23].

MATERIAL AND METHOD

Materials and Instruments Used

The chemicals, cell lines, reagents, and laboratory equipment used in this study were obtained from international suppliers to ensure experimental accuracy and reproducibility. Key compounds tested included Atractyloside A, Stevioside, Senecionine, and Senecionine N-Oxide. All materials were prepared and stored under sterile conditions in accordance with the manufacturers' instructions. The HepG2 cell line was cultured at 37 °C, 5% CO_2, and under humidified incubation conditions throughout the study.

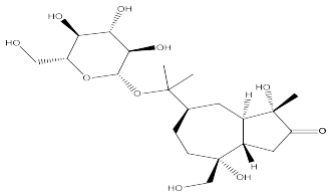
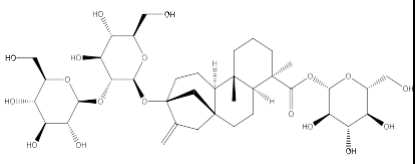
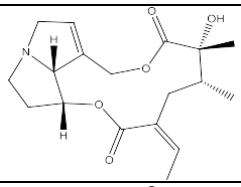
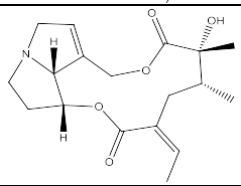
Solution Preparations

The medium was composed of DMEM/F-12 supplemented with 10% (v/v) fetal bovine serum, 1% antibiotic mixture (penicillin/streptomycin), and 2 mM L-glutamine. MTT and LDH solutions were freshly prepared according to the manufacturers' instructions. Stock solutions for Stevioside, Atractyloside A, Senecionine, and Senecionine N-Oxide were prepared based on their solubility and stability. The solutions were stored under appropriate conditions at +4 °C or −20 °C until use.

Cell Culture and Cytotoxicity Assay Procedures

HepG2 cells were incubated in 75 cm² culture vials in DMEM medium containing 10% FBS, 1% antibiotics, and L-glutamine. When the cells reached 80% confluence, they were detached using 0.25% trypsin/EDTA, collected by centrifugation, and subjected to viability analysis using trypan blue staining to determine cell count. Counting was performed using a Neubauer slide and a Juli Br cell imaging system. The cell suspension was adjusted to 1×10^4 cells/well for transfer to a 96-well plate. The subculturing and proliferation process of HepG2 cells is illustrated in Figure 1, which presents original micrographs generated by the authors from original experimental data**.**

Table 1. Names, IUPAC names, and chemical structures of plant-derived standards used in the study

Compaunds	
Atractyloside A IUPAC:(3S,3aR,5R,8R,8aS)-3,8-dihydroxy-8-(hydroxymethyl)-3-methyl-5-[2-[(2S,3R,4S,5S,6R)- 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2- yl]oxypropan-2-yl]-3a,4,5,6,7,8a-hexahydro-1H-azulen- 2-one.	
Stevioside IUPAC:[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (1R,4S,5R,9S,10R,13S)-13-[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl]oxy-5,9- dimethyl-14-methylidenetetracyclo[11.2.1.01,10.04,9]hexadecane-5- carboxylate	
Senecionine IUPAC:(1R,4Z,6R,7R,17R)-4-ethylidene-7-hydroxy- 6,7-dimethyl-2,9-dioxa-14- azatricyclo[9.5.1.014,17]heptadec-11-ene-3,8-dione	
Senecionine N-Oxide IUPAC:(1R,4Z,6R,17R)-4-ethylidene-7-hydroxy-6,7- dimethyl-14-oxido-2,9-dioxa-14- azoniatricyclo[9.5.1.014,17]heptadec-11-ene-3,8-dione	

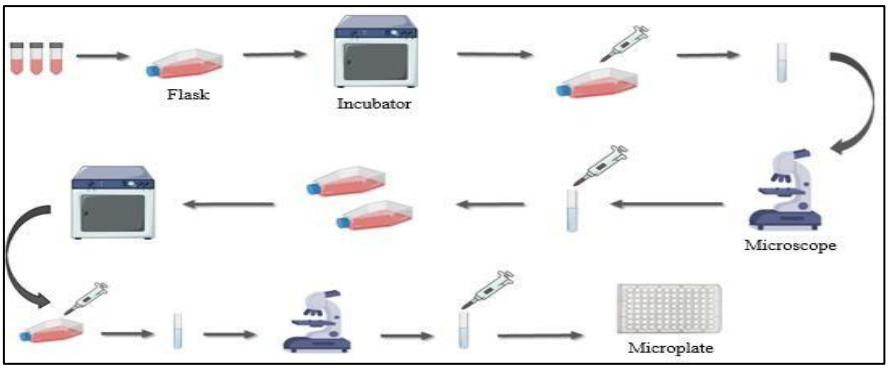


Figure 1. Subculturing and proliferation of HepG2 cells

Preparation of Test Substances and Administration Concentration

The study tested the hepatotoxic effects of four different phytochemicals: Stevioside (12.5–200 µM) [24], Atractyloside A (2.5–40 µM) [25], Senecionine (50–800 µM) [26], and Senecionine N-Oxide (100–1600 µM) [27]. All test substances were dissolved in DMEM, passed through sterile filters, and stored as stock solutions. These concentration ranges were selected based on prior in vitro hepatotoxicity assessments demonstrating reproducible cytotoxic responses within physiologically relevant, non-lethal exposure intervals [24–27]. The structures of the plant compounds used in the study were modeled using ChemDraw software and are presented in Table 1.

Cytotoxicity Test Protocols MTT Test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to assess cell viability following compound exposure according to the original method described by Mosmann (1983) [28]. During the test, 100 µl of the prepared HepG2 cell suspension was seeded into each well and incubated for 24 hours before treatment. After 24 hours of compound exposure, 20 µL of MTT solution was added to each well and the plates were incubated for an additional 3 hours. At the end of incubation, the resulting formazan crystals were dissolved using DMSO, and absorbance readings were measured at 570 nm (reference 650 nm).

$$\text{Viability} = 100\times\frac{(\text{OD570e-OD650e})}{(\text{OD570b-OD650b})}$$

OD570e; OD650e: Absorbance value measured in the exposure group
OD570b; OD650b: Absorbance value measured nm in the negative control group

The % viability values were used to construct concentration–response curves using the Hill function, and IC_{50} values were calculated accordingly [29]. Formazan production was considered an indicator of mitochondrial metabolic activity. Experiments were performed in three technical replicates and repeated across three independent passages. The step-by-step workflow of the MTT assay is illustrated in Figure 2, which was created by the authors from original experimental data.

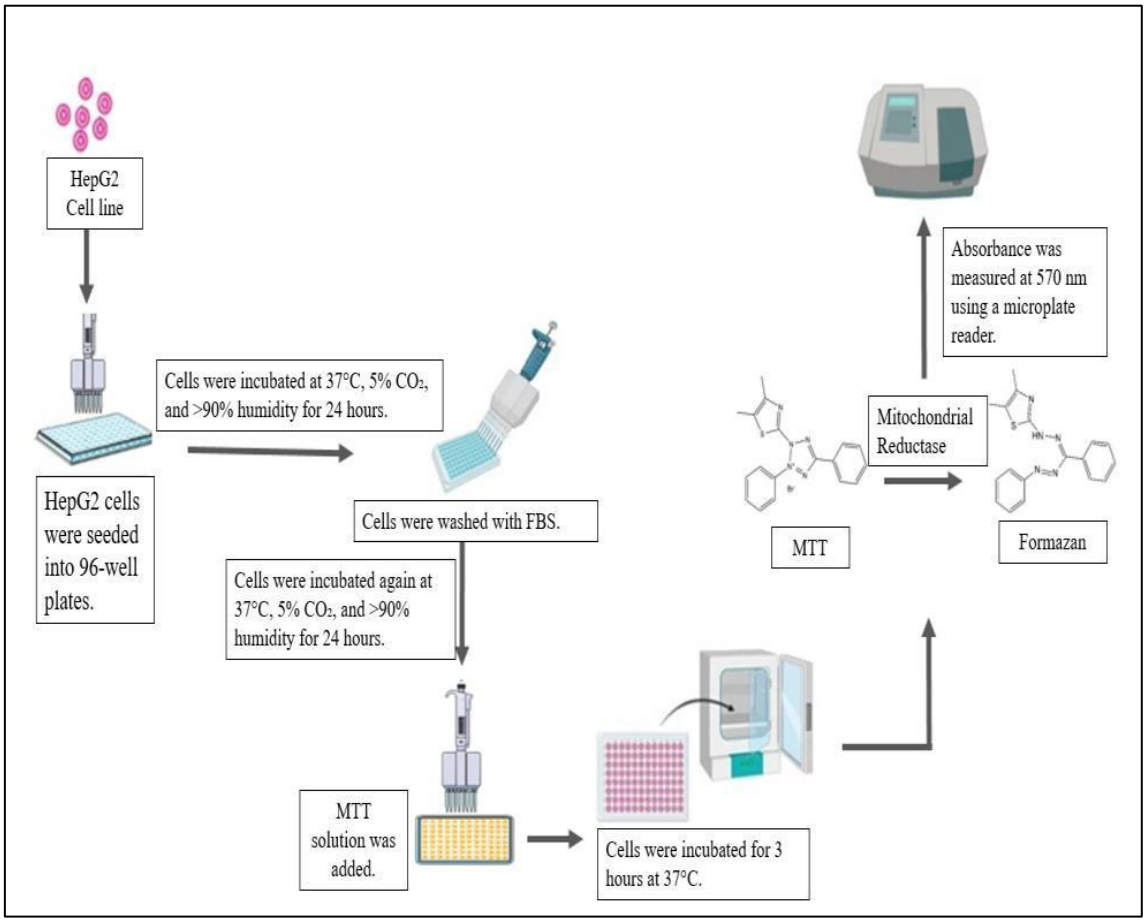


Figure 2. Experimental Procedure of the MTT Cytotoxicity Assay in HepG2 Cells

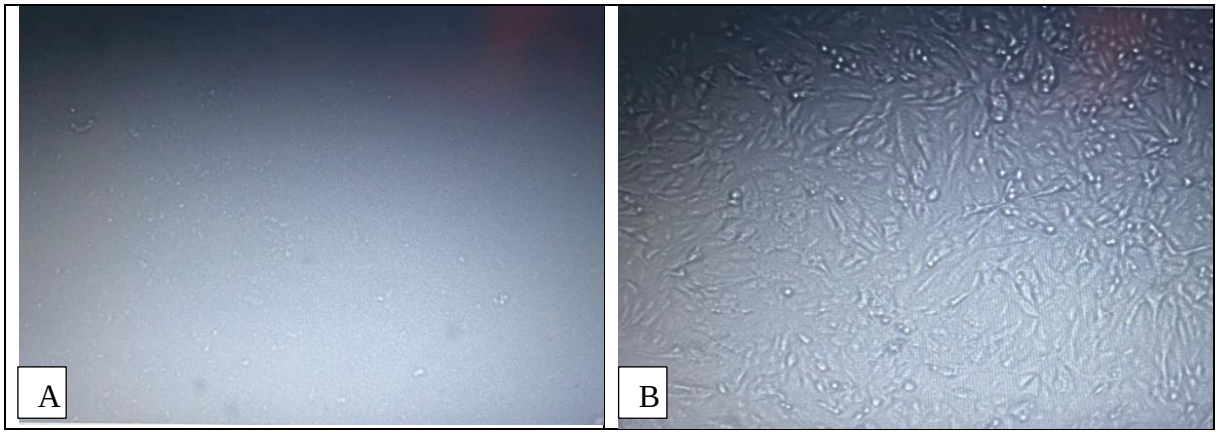


Figure 3. A. HepG2 cells treated with Triton X-100 (positive control). B. Untreated HepG2 cells (negative control)

LDH Test

The lactate dehydrogenase (LDH) assay was performed to evaluate membrane integrity by detecting extracellular LDH release using the standard method described by Decker and Lohmann-Matthes (1988) [30] and the manufacturer's ELK Biotechnology protocol [31]. After compound treatment, culture supernatants were collected, and LDH activity was quantified using an ELISA-based sandwich immunoassay, with absorbance measured at 450 nm. Triton X-100 was used as the positive control, and untreated cells served as the negative control. Figure 3 displays representative microscopic images of HepG2 cells, where panel A depicts Triton X-100–treated cells (positive control) and panel B shows untreated cells (negative control), prepared by the authors from original micrographs.

Data Evaluation

Concentration–response curves were generated using absorbance values obtained from the MTT assay, and IC_{50} values were calculated using the Hill function [29]. LDH data were qualitatively compared with MTT findings to ensure consistency between mitochondrial activity and membrane integrity. Statistical evaluation was based on descriptive analysis, whereby mean values were calculated from three technical replicates performed across three independent passages.

RESULTS AND DISCUSSION

The structurally and toxicologically diverse nature of stevioside, atractyloside A, senecionine, and senecionine N-oxide suggested differential effects on hepatocellular viability and membrane integrity. Therefore, cellular responses were assessed using complementary assays reflecting metabolic activity and cytotoxic membrane damage. The cytotoxic effects of these four different plant compounds used in the study, on the HepG2 cell line were evaluated using *in vitro* MTT and LDH assays.

According to the MTT assay results, a concentration-dependent decrease in cell viability was observed for all compounds. Atractyloside A was determined as the most potent cytotoxic compound with an IC_{50} value of 29 ± 0.871 µM. The IC_{50} value of Stevioside was calculated as 120 ± 1.012 µM, indicating moderate toxicity. The IC_{50} values of Senecionine and Senecionine N-Oxide were 555 ± 22.046 µM and 946 ± 12.685 µM, respectively, indicating lower cytotoxic effects compared to Atractyloside A and Stevioside. The concentration-dependent cytotoxic effects of the tested compounds on HepG2 cell viability, as determined by the MTT assay, are displayed in Figure 4.

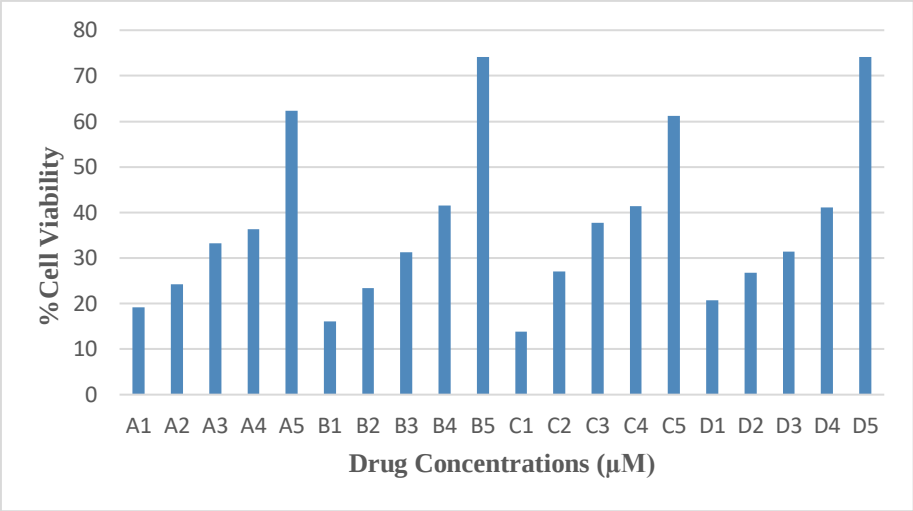


Figure 4. MTT cytotoxicity findings at different concentrations for the four tested compounds
A: Atractyloside A (A1: 2.5 µM, A2: 5 µM, A3: 10 µM, A4: 20 µM, A5: 40 µM)
B:Stevioside (B1: 12.5 µM, B2: 25 µM, B3: 50 µM, B4: 100 µM, B5: 200 µM)
C: Senecionine (C1: 50 µM, C2: 100 µM, C3: 200 µM, C4: 400 µM, C5: 800 µM)
C: Senecionine N-oxide (D1: 100 µM, D2: 200 µM, D3: 400 µM, D4: 800 µM, D5: 1600 µM)

LDH release assay results were also consistent with the MTT assay, and disruptions in cell membrane integrity were quantitatively measured. The highest LDH release was observed at 5 µM and 2.5 µM concentrations of Atractyloside A, and these values were similar to the positive control Triton X. Maximum LDH release was obtained at 100 µM for Stevioside (0.6036 ng/ml), at 200 µM for Senecionine (0.5667 ng/ml), and at 400 µM for Senecionine N-Oxide (0.6452 ng/ml). The LDH standard curve used for quantification is shown in Figure 5, while the LDH (ng/ml) values of the plant-derived chemical standards at different concentrations are presented in Table 2 and their graphical representation is provided in Figure 6.

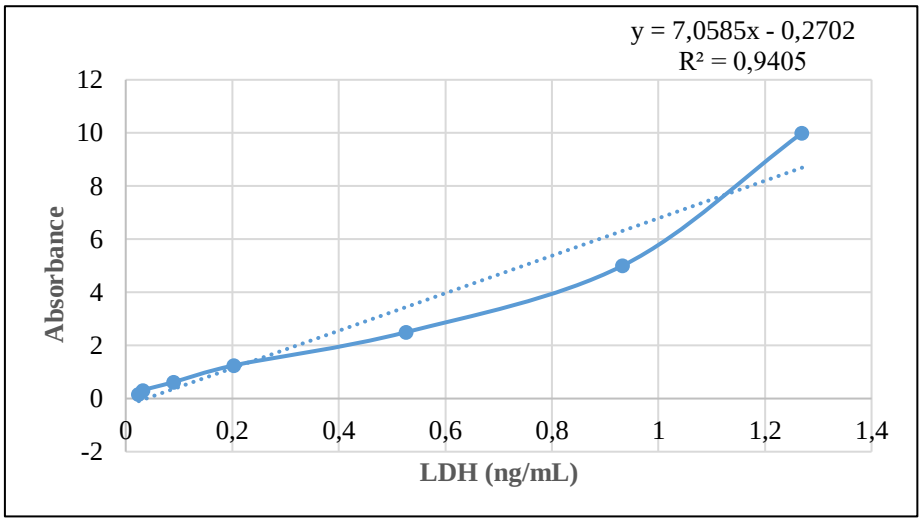


Figure 5. The graph illustrates the linear relationship between LDH concentration (ng/ml) and absorbance values (a.u.). The regression equation and R^2 value displayed on the curve were used to quantify LDH release in the samples

Table 2. LDH (ng/ml) values obtained from the LDH release assay for each compound at different concentrations

Standards	Concentration (µM)	LDH (ng/ml)
Atractyloside A	20	0.7156
	10	0.5528
	5	1.5094
	2.5	1.2476
	1.25	1.5457
Stevioside	200	0.4868
	100	0.6036
	50	0.4427
	25	0.3787
	12.5	0.5161
Senecionine	400	0.5667
	200	0.4149
	100	0.3387
	50	0.3834
	25	0.6452
Senecionine N-Oxide	400	0.6452
	200	0.6346
	100	0.5362
	50	0.3887
	25	0.4610

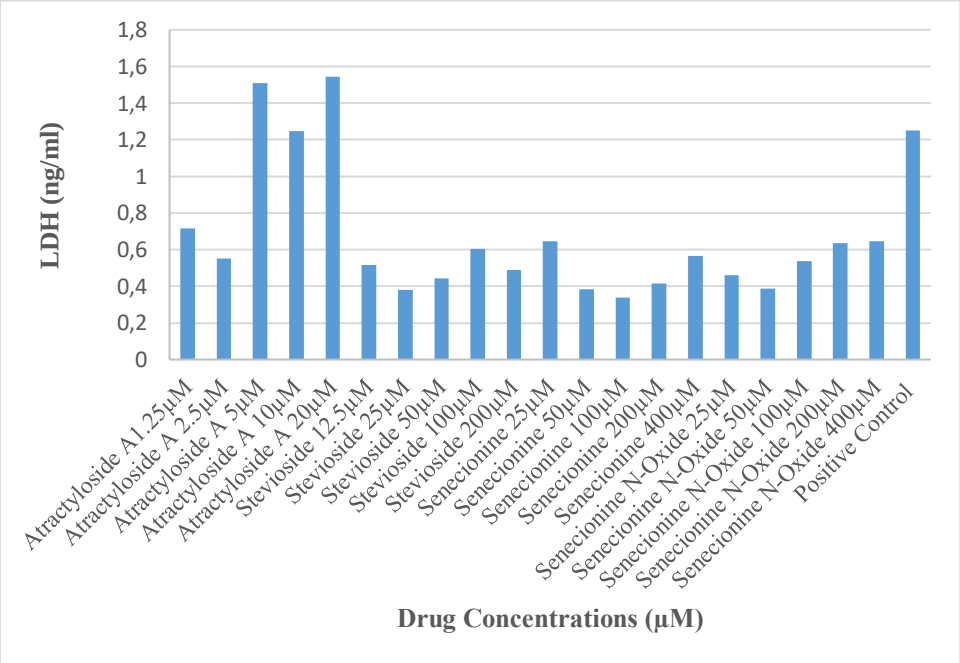


Figure 6. LDH (ng/ml) values of compounds at different concentrations.
Each bar represents the LDH release (ng/ml) measured for a specific concentration of each test compound. The numerical values next to the bars correspond to the measured LDH amount at that concentration

Although no apparent concentration-dependent increase in LDH was detected, Atractyloside A was found to have a significant effect on both mitochondrial activity and cell membrane damage. These results indicate that Atractyloside A has a high toxic potential toward HepG2 cells, while Stevioside has moderate toxicity, and Senecionine and Senecionine N-Oxide are less toxic [32,33].

These findings demonstrate that the combined use of the MTT and LDH assays in this study provides reliable and complementary information regarding cell viability and membrane damage.

In recent years, interest in traditional medicine systems, particularly Ayurvedic herbal products, has been growing worldwide. According to the World Health Organization, more than 80% of the world's population relies on herbal medicine for primary healthcare [34]. However, this tendency, together with the uncontrolled use of many herbal products that have not been adequately evaluated scientifically, can lead to serious toxicological consequences [35,36].

In this study, we compared four plant standards commonly used in Ayurvedic medicine, namely Atractyloside A, Stevioside, Senecionine, and Senecionine N Oxide, regarding their glycoside or alkaloid nature and evaluated their in vitro cytotoxic effects on the HepG2 cell line. Cell viability, mitochondrial activity, and membrane integrity were measured using MTT and LDH assays.

Atractyloside A was identified as the most potent cytotoxic compound, with a low IC_{50} value (29 ± 0.871 µM) and a high LDH release level in both MTT and LDH assays. These findings indicate that Atractyloside A impairs energy metabolism by inhibiting mitochondrial ADP/ATP transporters and damages cell membrane integrity, leading to rapid cell death [37,38].

Although Stevioside is widely considered safe [39], the present study revealed a measurable cytotoxic response in HepG2 cells at higher concentrations, with 41.5% cell death at 100 µM and an IC_{50} of 120 ± 1.012 µM. Similar observations have been reported previously, suggesting that dose-dependent hepatocellular effects may occur under in vitro conditions [40,41].

The IC_{50} values of Senecionine and Senecionine N-Oxide, which are pyrrolizidine alkaloids, were found to be 555 ± 22.046 µM and 946 ± 12.685 µM, respectively. The lower IC_{50} value of Senecionine suggests that it has higher cytotoxic toxicity than the N-Oxide derivative. This can be explained by the difficulty of the N-Oxide form in passing through the cell membrane and its lower bioavailability due to the metabolic activation process [42,43].

LDH assay findings also supported these data; Atractyloside A exhibited the highest LDH release at concentrations of 5 µM and 2.5 µM. Maximum LDH release was observed at concentrations of 100 µM for Stevioside, 200 µM for Senecionine, and 400 µM for Senecionine N-Oxide. However, the concentration dependence of LDH release for Stevioside and pyrrolizidine alkaloids was limited, and IC_{50} calculation was not possible.

Our study aimed to scientifically analyze the often overlooked but significant toxic potential of herbal products and evaluated the effects of Ayurvedic compounds on liver cells at the molecular level. Our findings demonstrate that just because herbal products are "natural" does not necessarily mean they are safe; in fact, some ingredients can produce serious hepatotoxic effects.

These findings indicate that Atractyloside A, in particular, should be carefully evaluated for medicinal uses. Similarly, given the structural diversity and distinct toxicity profiles of pyrrolizidine alkaloids, the uncontrolled use of these compounds should be limited to protect public health. While the toxic effects of compounds such as Senecionine have been previously documented in the literature [44,45], our study confirms these findings in a human cell line.

The order obtained from our *in vitro* data—Atractyloside A > Stevioside > Senecionine > Senecionine N-Oxide—showed that glycoside compounds have higher cytotoxic potential than pyrrolizidine alkaloids. This can be explained by the mitochondrial destructive effect of Atractyloside A and the apoptotic pathway activation of Stevioside in certain cell lines [41,46].

All these data highlight the importance of systematically evaluating the toxicological profiles of herbal products. It is important to recognize that herbal ingredients, primarily when used in formulations containing multiple combinations, can exhibit toxic interactions and cause severe organ damage. Therefore, supporting traditional medicine with modern toxicological methods and subjecting these products to preclinical studies is imperative for public health.

Legal regulations in Turkey regarding the licensing of herbal products are important steps in this regard. However, with the proliferation of Ayurvedic herbal products in the Turkish market, the

toxicological profiles of the active ingredients in these products must be carefully examined. The Ministry of Agriculture and Forestry's regulations based on positive plant lists play a critical role in this regard [47].

In conclusion, this study has demonstrated that *in vitro* toxicity analyses based on scientific methods are effective in assessing the safety of herbal products and should become an essential tool for public health. The analyses clearly demonstrate that compounds with low IC_{50} values, such as Atractyloside A, despite their widespread use in traditional medicine, should be handled with caution due to their potential toxicity. Furthermore, although Stevioside is considered safe, its potential for cytotoxic effects at specific concentrations necessitates re-evaluation of these products from a pharmaceutical perspective.

In this context, it is recommended that other diterpene glycosides and alkaloids from different classes be evaluated *in vitro* in prospective studies. Furthermore, the composition of herbal products should be standardized, pharmaceutical quality control processes should be implemented, and healthcare professionals should be informed about this issue.

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AUTHOR CONTRIBUTIONS

Concept: N.C., Ö.C.Ü.; Design: N.C., Ö.C.Ü.; Control: N.C., Ö.C.Ü.; Sources: N.C., Ö.C.Ü.; Materials: N.C., Ö.C.Ü., B.Y.D., G.E.D.; Data Collection and/or Processing: N.C., Ö.C.Ü., G.E.D.; Analysis and/or Interpretation: N.C., Ö.C.Ü., B.Y.D., G.E.D.; Literature Review: N.C., Ö.C.Ü.; Manuscript Writing: N.C., Ö.C.Ü.; Critical Review: N.C., Ö.C.Ü., B.Y.D.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest related to this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval was not required for this study.

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