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The Safety Evaluation of Verbascoside from the Viewpoint of Genotoxicity

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Abstract

Objective: This study aimed to evaluate the genotoxic potential of verbascoside using a standard battery of assays across bacterial and mammalian cell systems.

Materials and Methods: The genotoxic potential of verbascoside was evaluated using a standard battery of assays, including the bacterial reverse mutation (Ames) test, cytokinesis-block micronucleus (CBMN) assay, and alkaline Comet assay. The Ames test was performed on *Salmonella typhimurium* TA98 and TA100 strains, with and without S9 metabolic activation, at concentrations ranging from 1 to 1000 µg/plate. Chinese hamster ovary (CHO-K1) cells were used for the CBMN and Comet assays at concentrations between 25 and 200 µg/mL.

Results: The mutagenic index remained below 2.0 across all tested concentrations, showing no significant variation with increasing dose in the Ames test. No significant differences were observed in micronucleus frequency between the negative control and any concentration of verbascoside. The Comet assay results revealed no significant difference in DNA tail percentage between the negative control and verbascoside-treated groups.

Conclusion: Under the tested conditions, verbascoside showed no mutagenic or genotoxic effect in bacterial or mammalian cell models, supporting a favorable genotoxicity safety profile and warranting further pharmacological development.

Keywords: Micronucleus assay, Comet assay, Ames test, verbascoside, genotoxicity

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INTRODUCTION

Verbascoside (acteoside) is a phenylethanoid glycoside composed of a hydroxyphenethyl (hydroxytyrosol) moiety linked to a disaccharide (α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl) *via* a glycosidic bond, which is also esterified with a caffeic acid (Figure 1). First isolated in 1968 from *Verbascum* species, it has since been reported in more than 200 plant species across 23 plant families, including Scrophulariaceae, Lamiaceae, Plantaginaceae, Verbenaceae, Oleaceae, and Buddlejaceae (1-3).

Pharmacologically, verbascoside exhibits a broad range of biological activities, including anti-inflammatory (4-6), anti-ulcerogenic (7), antioxidant (8-10), antimicrobial (5), and analgesic effects (11). Mechanistic studies indicate attenuation of TAK1/JNK/AP-1 signaling, accompanied by increased SHP-1 phosphorylation, down-regulation of cyclooxygenase and nitric oxide synthase expression, and calcium-dependent inhibition of arachidonic acid and histamine release through phospholipase modulation (12-15). In line with its antioxidant potential, verbascoside strongly suppresses reactive oxygen species (ROS)-driven oxidation and has been shown to outperform vitamin C in comparative assays (16). Preclinical data further demonstrated its antidepressant and neuroprotective actions *via* enhanced dopamine biosynthesis and modulation of neuronal stress-response pathways (17), as well as vascular protection through NO pathway dependent effects and modest angiotensin-converting enzyme (ACE) inhibition (18,19).

Beyond its central and cardiovascular effects, verbascoside contributes to glycemic regulation by inhibiting α-amylase and sodium–glucose cotransporter 1 (SGLT1)-mediated glucose absorption (20,21), alleviates β-cell endoplasmic reticulum stress through PERK/eIF2α suppression (22), and reduces inflammatory signaling *via* MAPK/NF-κB pathway modulation in chondrocytes and hepatic cells (23). Verbascoside has also shown cytotoxic and antiproliferative effects in various cancer cell lines, including human myeloma, leukaemia, gastric carcinoma, colorectal carcinoma, oral squamous cell carcinoma, and glioblastoma (24).

Medicinal plants have long been important sources of pharmacologically active compounds, and many modern drugs originate from natural products. Nevertheless, the safety and efficacy of numerous herbal preparations remain insufficiently validated (25). As global use of herbal medicines increases, objective toxicological evaluation is essential. The assumption that "natural" equates to "safe" is misleading, since some plants produce toxic secondary metabolites capable of serious adverse effects. Accordingly, systematic safety testing, including *in vitro* cytotoxicity, toxicokinetic, and genotoxicity assessments, is critical to define safety margins for plant-derived compounds. Although verbascoside has been widely investigated for pharmacological potential, data on its genotoxic and mutagenic safety are limited and inconsistent, with some studies reporting no genotoxicity (26,27) and others indicating genotoxic effects (28). These discrepancies underscore the need for comprehensive evaluation. In this study, we therefore assessed the genotoxic potential of verbascoside using a standard assay battery comprising the Ames test, the micronucleus assay, and the Comet assay.

MATERIALS AND METHODS

Chemicals

Verbascoside was purified from *Globularia sintenisii* and its chemical structure was elucidated by NMR and MS analysis (29).

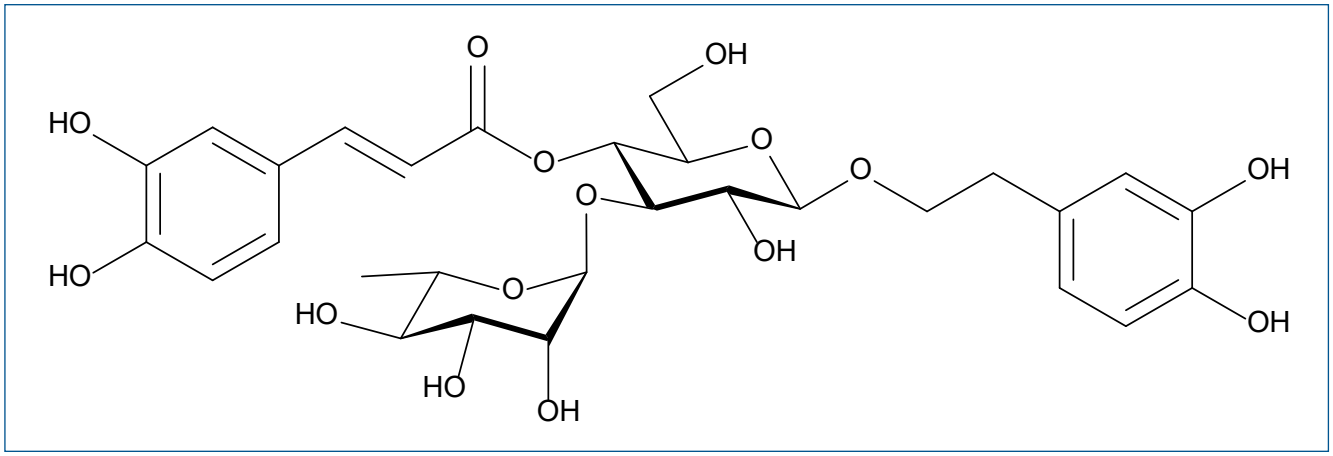

FIGURE 1. Chemical structure of verbascoside.

Genotoxicity Assessment

Mutagenicity Assays

The bacterial reverse mutation (Ames) plate-incorporation assay was carried out in a Turkish Accreditation Agency (*Türk Akreditasyon Kurumu*, TÜRKAK)-accredited facility (TS EN ISO/IEC 17025; AB-1764-T) in accordance with the method described by Maron and Ames (1983) (30). Histidine-auxotrophic *Salmonella typhimurium* strains TA98 and TA100 were maintained and used as recommended by the supplier. Testing was performed in triplicate at four concentrations (1–1000 µg/plate) across two independent experiments, both with and without metabolic activation (S9 from Aroclor™ 1254-induced rat liver). Dimethyl sulfoxide (DMSO; 50 µL/plate) was used as the vehicle control.

Positive controls were included as follows: −S9: 4-nitro-o-phenylenediamine (20 µg/plate) for TA98; sodium azide (1 µg/plate) for TA100. +S9: 2-aminofluorene (5 µg/plate) for TA98 and TA100. A result was judged positive when the revertant count showed at least a two-fold increase over the concurrent negative control. The mutagenic index (MI) was calculated as follows:

$$MI = A/B$$

where *A* represents the mean number of revertant colonies in the presence of the test compound and *B* represents the mean number of revertant colonies in the negative control. An MI value of ≥2 was considered indicative of a mutagenic effect.

Genotoxicity and Antigenotoxicity Assessment

Cell Line and Culture Conditions

Chinese hamster ovary (CHO-K1) cells (ATCC® CCL-61™) were grown in Ham's F-12 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA) and 1% penicillin–streptomycin (Gibco, NY, USA). Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere and passaged approximately every 3 days to maintain exponential growth.

Cytokinesis-Block Micronucleus Assay

CHO-K1 cells were seeded in 6-well plates (2×10^5 cells/well, 24 h), then exposed to test samples at 25–200 µg/mL. After 24 hours, cytochalasin B (3 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added for 24 hours to block cytokinesis. Cells underwent hypotonic treatment (0.075 M KCl), fixation (methanol:acetic acid 3:1, twice), slide preparation, and Giemsa staining (5% in Sorensen buffer, 5 min) (Sigma-Aldrich, St. Louis, MO, USA).

For each culture/treatment, 1000 binucleated cells were scored for micronuclei and the nuclear division index (NDI) were calculated using formulas described previously (31).

Alkaline Comet Assay

CHO cells were seeded in 6-well plates (3×10^5 cells/well) 24 hours before treatment. Cells were then exposed for 4 hours to the samples. Following treatment, cells were collected, centrifuged (5 minutes, 1000 rpm), and resuspended.

20 µL of the cell suspension was mixed with 180 µL prewarmed low-melting-point agarose (Sigma-Aldrich, St. Louis, MO, USA); 45 µL was layered onto high-melting-point agarose-precoated slides (Genaxxon Bioscience, Ulm, Germany), covered, set 3 minutes, and coverslips removed (two replicates/treatment).

Slides were lysed at 4°C in the dark, equilibrated 20 minutes in cold electrophoresis buffer, then electrophoresed at 25 V with current adjusted to 0.3 A for 20 minutes. After electrophoresis, slides were rinsed (distilled water → Tris, 5 min), fixed in ice-cold methanol (-20°C, 5 min), air-dried, stained with ethidium bromide (Bio Basic Inc., Markham, ON, Canada), and cover slipped.

DNA damage was scored under fluorescence microscope (BS 200 ProP; BAB Imaging System, Ankara, Türkiye) (31).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons against the control group. Analyses were conducted using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). A p value <0.05 was considered statistically significant.

RESULTS

Mutagenicity and Antimutagenicity Assays

As shown in Table 1, the positive controls yielded MI values >2 in both S9+ and S9− conditions for TA98 and TA100, confirming assay performance. Across all four verbascoside doses, MI values for TA98 and TA100 remained <2 regardless of metabolic activation, with no dose-related increase (Table 1). In summary, verbascoside showed no mutagenicity in either strain under S9+ or S9− conditions within the tested concentration range.

Results of Cytokinesis-Block Micronucleus Assay

As summarized in Table 2, verbascoside did not increase micronucleus (MN) frequency relative to the negative control at any concentration tested ($p>0.05$) and NDI values remained within the acceptable range of 1.3-2.2, indicating no relevant cytotoxicity under assay conditions.

Table 1. Ames mutagenicity results in *Salmonella typhimurium* TA98 and TA100, with and without metabolic activation (S9).

	TA98 (revertants/plate)	TA100 (revertants/plate)	MI (TA98)	MI (TA100)
Without S9				
Negative control	28.5 ± 4.5	192.5 ± 3.5	-	-
Positive control	998.0 ± 76.4 *	976.5 ± 17.7*	35.5	5.1
1000	23.0 ± 8.5	192.5 ± 19.1	0.8	1.0
100	27.5 ± 4.9	178.0 ± 19.8	1.0	0.9
10	34.0 ± 2.8	188.0 ± 14.1	1.2	1.0
1	27.5 ± 3.5	198.0 ± 5.7	1.0	1.0
With S9				
Negative control	39.5 ± 0.7	140.0 ± 22.6	-	-
Positive control	1451.5 ± 67.2 *	888.0 ± 5.7*	50.9	5.8
1000	32.5 ± 3.5	144.0 ± 12.7	1.1	1.0
100	29.0 ± 2.8	141.0 ± 5.7	1.0	1.0
10	38.0 ± 5.7	140.5 ± 21.9	1.3	1.0
1	39.0 ± 4.2	133.0 ± 7.1	1.4	1.0

Positive controls: in the experiment without metabolic activation, TA98; 4-nitro-o-phenylenediamine (20 µg / plate), TA100; sodium azide (1 µg /plate), in the experiment with metabolic activation, 2-aminofluorene (5 µg / plate) for both strains. Dunnett's multiple comparison test was carried out for statistical analysis. *p<0.01 versus negative control group. For all other treatment groups, p-values were found to be above 0.05.

Table 2. Percentage of micronuclei observed in CHO cell cultures treated with various concentrations of verbascoside and with doxorubicin as a positive control.

Sample	MN%	NDI
Negative control	1.04 ± 0.05	2.00 ± 0.01
Positive control (doxorubicin, 1 µM)	33.3 ± 2.05*	1.33 ± 0.04
Verbascoside 25 µg/mL	0.98 ± 0.01	2.01 ± 0.01
Verbascoside 50 µg/mL	0.86 ± 0.25	1.97 ± 0.09
Verbascoside 100 µg/mL	1.17 ± 0.31	2.00 ± 0.02
Verbascoside 200 µg/mL	1.28 ± 0.19	2.07 ± 0.10

MN%: Percentage of cells with micronuclei, **NDI:** Nuclear division index.

*p<0.01 versus the negative control (Dunnett's test). All verbascoside-treated groups did not differ significantly from the negative control (p>0.05).

In contrast, doxorubicin produced the expected significant elevation in MN frequency (p<0.01), confirming assay sensitivity. Overall, verbascoside showed no MN induction and no cytotoxicity up to 200 µg/mL in CHO cells.

Results of Alkaline Comet Assay

DNA strand-breaks were evaluated in CHO cells by the alkaline Comet assay and expressed as %DNA in tail (100 cells/condition; analyzed with the BAB microscope software). Typical Comet images observed with ethidium bromide staining, are shown in Figure 2.

As shown in Figure 3, verbascoside produced no significant change in %DNA in tail versus the negative control across the tested concentrations ($p>0.05$). In contrast, doxorubicin (positive control) caused a significant increase in %DNA in tail ($p<0.01$). Although modest rises were noted at 100 and 200 µM verbascoside, these did not reach statistical significance ($p>0.05$).

DISCUSSION

Verbascoside, one of the most common phenylethanoid glycosides, possesses numerous biological activities, including analgesic, anti-inflammatory, anticancer, neuroprotective, antiulcer and antispasmodic. Given its increasing interest as a potential therapeutic and nutraceutical compound, the evaluation of its genotoxic safety is essential, as untested herbal products may pose toxicological risks despite their natural origin.

Previous studies evaluating the genotoxic potential of verbascoside have yielded inconsistent results. Santoro et al. (28) showed that verbascoside isolated from *Kigelia africana* induced structural chromosome aberrations and sister chromatid exchanges in human lymphocytes, accompanied by a reduction in mitotic index, suggesting a potential clastogenic effect. However, contribution of co-isolated constituents or extraction related artefacts cannot be completely excluded in this study. In contrast, Henn et al. (26) reported that verbascoside (1–50 µg/mL) was non-genotoxic in human fibroblasts and V79 cells using the alkaline Comet assay and that extracts containing verbascoside from *Aloysia* species were non-mutagenic in the Ames test. These findings were further supported by *in vivo* and alternative model studies. For instance, a long-term dietary rabbit study revealed no induction of chromosome aberrations or sister chromatid exchanges in peripheral lymphocytes and even showed a tendency toward reduced cytogenetic damage over time in treated groups (32).

Similarly, negative results were obtained in the *Drosophila melanogaster* SMART assay (27). Acute and subacute toxicity studies in mice further indicated a high safety margin, with an intraperitoneal LD_{50} exceeding 5 g/kg and no treatment related systemic toxicity following 21-day administration (33).

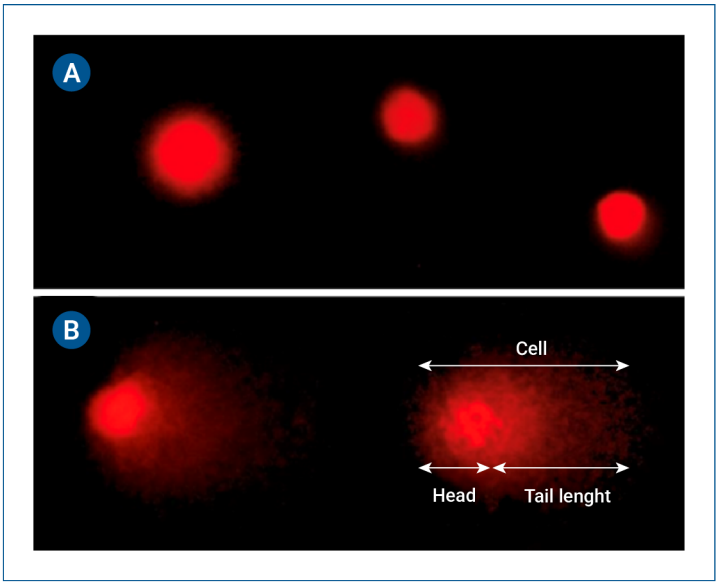

FIGURE 2. Example images of the Comet assay on CHO-K1 cells. A) Control group, B) Cells exposed to 1.5 µM doxorubicin, displaying increased DNA damage, as indicated by the presence of Comet tails.

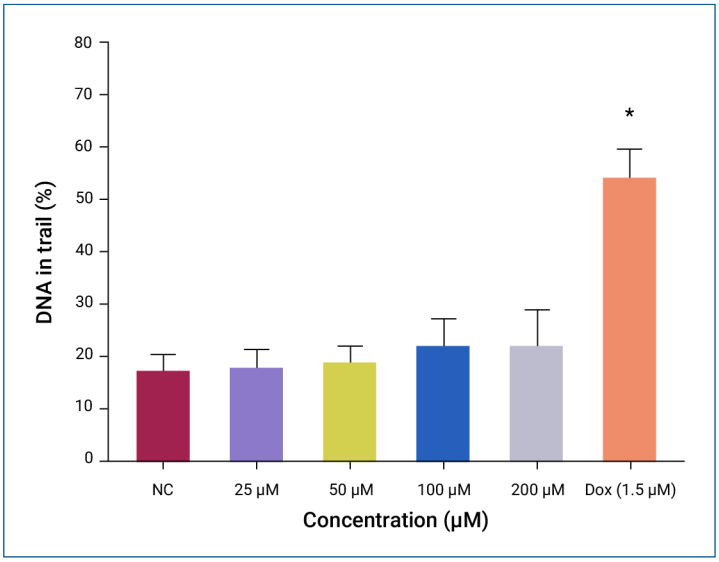

FIGURE 3. Percentage of DNA in tail observed in the Comet assay for CHO cell cultures treated with different concentrations of verbascoside and with doxorubicin (Dox) as a positive control.

*$p<0.01$ versus negative control group. For all other treatment groups, p-values were found to be above 0.05.

In the present study, verbascoside was evaluated using a battery of complementary genotoxicity assays, including the Ames test, the cytokinesis-block micronucleus assay, and the Comet assay. In the Ames test, the mutagenic index remained below the threshold value of 2.0 at all tested concentrations, indicating a lack of mutagenic effect in bacterial reverse mutation systems. In the micronucleus assay, verbascoside did not induce a statistically significant increase in micronucleus frequency compared with the negative control, suggesting the absence of clastogenic or aneugenic effect in mammalian cells. Furthermore, Comet assay analysis did not reveal a significant increase in DNA strand breaks, as reflected by unchanged DNA tail percentages across treatment groups. Although slight numerical increases were observed at higher concentrations (100 and 200 µM), these

changes were not statistically significant and did not exhibit a clear dose response relationship, limiting their biological relevance.

A major strength of the present study is the integrated use of three distinct genotoxicity endpoints, enabling the detection of gene mutations, structural/numerical chromosomal aberration and DNA strand breaks within a single experimental framework. This approach strengthens the reliability of the negative findings and reduces the likelihood of false negative interpretation that may arise from reliance on a single assay. Nevertheless, several limitations should be acknowledged. First, the present study was restricted to *in vitro* test systems and therefore does not account for complex *in vivo* factors such as absorption, metabolism, tissue distribution and long-term exposure. Second, mechanistic endpoints such as oxidative DNA base damage or DNA repair modulation were not specifically investigated and may warrant further targeted exploration.

Several mechanistic considerations may explain why verbascoside consistently appears non-genotoxic. Verbascoside is a strong antioxidant and radical scavenger capable of reducing intracellular ROS, chelating transition metals and stabilizing free radicals through its phenolic structure. Since oxidative stress is a major driver of DNA strand breaks and chromosomal damage, its ROS modulating activity may inherently limit DNA lesion formation (34). Additionally, verbascoside has been shown to enhance endogenous antioxidant defenses (e.g., SOD, CAT, GSH systems) and suppress inflammatory signaling, further reducing oxidative stress related genotoxicity (35). Thus, the biochemical properties of verbascoside are consistent with the absence of mutagenic or clastogenic findings observed in the present study's assays.

Taken together, the current findings in conjunction with published *in vitro* and *in vivo* studies, indicate that verbascoside does not exhibit mutagenic or genotoxic effect under the tested conditions. The single report suggesting clastogenicity appears to be an exception rather than the prevailing trend and may reflect experimental or matrix specific factors. Importantly, to the best of our knowledge, this is the first study to evaluate the genotoxic safety of verbascoside using the combined application of the Ames test, micronucleus assay and Comet assay within a single experimental design. Therefore, the present work provides a substantial and methodologically rigorous contribution to the toxicological characterization of verbascoside and supports its continued investigation as a bioactive phytochemical with a favorable genotoxic safety profile.

Ethical Approval: Ethical approval was not required for this study, as all experiments were conducted exclusively *in vitro* using established bacterial strains and commercially available cell lines, without the involvement of human participants or experimental animals.

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

Author Contributions: Concept – M.H., H.K., A.A.; Design – E.N.K., E.Ö., G.E., M.H., H.K.; Supervision – M.H., H.K., A.A.; Fundings – M.H., H.K.; Materials – M.H., H.K.; Data Collection and/or Processing – E.N.K., E.Ö., G.E., A.G.K.; Analysis and/or Interpretation – E.N.K., E.Ö., G.E., A.G.K.; Literature Review – E.N.K., E.Ö., G.E., A.G.K.; Writer – M.H.; Critical Reviews – H.K., A.A.

Conflict of Interest: The author declares no conflict of interest.

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