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Protective Effects of Aqueous Propolis Extract from the Artvin-Hatila Region of Türkiye Against Zinc Oxide-Induced Genotoxicity: Antimutagenic, Antioxidant, and Phytochemical Evaluation

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ABSTRACT

This study investigated the protective effects of aqueous propolis extract from Türkiye's Artvin-Hatila region against zinc oxide (ZnO)-induced genotoxicity in *Allium cepa* root meristem cells. At 100 mg/L, the extract significantly reduced ZnO-related cytogenetic damage, increasing the mitotic index by 143% and lowering micronucleus frequency by 66.6% compared to the positive control. Chromosomal aberration rates also approached those of the negative control at this dose. Antioxidant assays demonstrated strong activity, with a DPPH IC_{50} of 0.004 mg/mL, FRAP value of 7.44 mg TE/g, and CUPRAC value of 0.30 mmol TEAC/g. HPLC analysis revealed 16 bioactive compounds, notably caffeic acid, epicatechin, and ferulic acid. These results highlight the extract's potential as a natural antigenotoxic and antioxidant agent, effective within an optimal concentration range against environmental genotoxins such as ZnO.

Keywords: Propolis, Antimutagenicity, Antioxidant Activity, Phenolic Composition, *Allium cepa*, HPLC Analysis

Türkiye'nin Artvin-Hatila Bölgesinden Elde Edilen Propolisin Sulu Ekstraktının Çinko Oksit Kaynaklı Genotoksisiteye Karşı Koruyucu Etkileri: Antimutajenik, Antioksidan ve Fitokimyasal Değerlendirme

ÖZ

Bu çalışma, Türkiye'nin Artvin-Hatila bölgesinden elde edilen sulu propolis ekstraktının çinko oksit (ZnO) kaynaklı genotoksisiteye karşı *Allium cepa* kök meristem hücrelerinde koruyucu etkilerini araştırmıştır. 100 mg/L dozunda ekstrakt, pozitif kontrolle karşılaştırıldığında mitotik indeksi %143 oranında artırmış ve mikronükleus oluşumunu %66,6 oranında azaltarak ZnO kaynaklı sitogenetik hasarı önemli ölçüde azaltmıştır. Bu dozda kromozomal anomali oranları da negatif kontrol değerlerine yaklaşmıştır. Antioksidan analizlerde ekstrakt güçlü etki göstermiş; DPPH IC_{50} değeri 0,004 mg/mL, FRAP değeri 7,44 mg TE/g, CUPRAC değeri ise 0,30 mmol TEAC/g olarak belirlenmiştir. HPLC analizinde başta kafeik asit, epikateşin ve ferulik asit olmak üzere 16 biyolojik etkili bileşik tespit edilmiştir. Bulgular, bu ekstraktın belirli bir doz aralığında çevresel genotoksinlere karşı doğal bir antigenotoksik ve antioksidan ajan olarak potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: Propolis, Antimutajenik Etki, Antioksidan Aktivite, Fenolik Bileşik Profili, *Allium cepa*, HPLC Analizi

INTRODUCTION

Zinc oxide (ZnO) is widely utilized across various industries, including electronics, paints, rubber, textiles, cosmetics, and food packaging, due to its semiconducting nature, strong ultraviolet absorption, and antimicrobial properties [1, 2]. The use of ZnO in nanoparticulate form has become increasingly common, as its enhanced surface area contributes to improved product performance. However, this also increases the likelihood of environmental dissemination. Waste products generated during the manufacture and application of ZnO may enter soil and surface water systems, where the potential for biological interaction and adverse effects in living organisms becomes a growing concern [3]. Plants, in particular, are vulnerable to ZnO accumulation due to root uptake mechanisms, and several studies have reported inhibited root development, mitotic disruption, and chromosomal abnormalities following exposure [2, 4]. Although ZnO is not classified as a heavy metal, it shares similar cytotoxic characteristics by promoting oxidative stress at the cellular level, which may in turn damage genetic material [5]. Consequently, the long-term biological impacts of ZnO exposure in living systems remain a subject of active investigation.

In light of these concerns, the protective effects of natural antioxidant compounds against potential genotoxic and mutagenic agents have attracted increasing scientific

attention. Among these bioactive substances, propolis—a resinous material collected by *Apis mellifera* bees from botanical sources—has emerged as a candidate of interest due to its complex composition, which includes high levels of phenolic compounds, flavonoids, aromatic acids, and essential oils [6, 7]. While the chemical makeup of propolis varies depending on its botanical and geographical origin as well as the extraction method employed, numerous studies have documented its antioxidant, anti-inflammatory, antimicrobial, and antimutagenic activities [4, 8]. Extracts rich in polyphenols and flavonoids have been shown to support redox homeostasis, mitigate DNA damage, and suppress cytogenetic anomalies [5, 9]. These bioactivities are particularly relevant in experimental models involving oxidative damage. Furthermore, aqueous propolis extracts are generally considered more physiologically compatible and less toxic than ethanolic forms, making them more favorable for therapeutic use in humans [4, 11]. However, the extent to which propolis confers protection against widely used environmental agents such as ZnO remains inadequately characterized.

Due to ethical and logistical considerations, plant-based systems are frequently preferred for evaluating the genetic toxicity of environmental pollutants. The *Allium cepa* assay, in particular, is widely recognized for its effectiveness in assessing alterations in cell division dynamics and chromosomal structure following exposure to genotoxic substances [3, 11]. The rapidly dividing meristematic cells of *A. cepa* root tips exhibit pronounced cellular responses to toxic agents, enabling precise quantification of indicators such as reduced mitotic index, abnormal metaphase formations, micronucleus occurrence, and chromosome fragmentation [12]. Because of its simplicity, cost-effectiveness, and high sensitivity, the *Allium* test has gained broad acceptance by environmental health organizations as a valid cytogenetic model [1]. Furthermore, the strong correlation between its outcomes and those observed in mammalian systems makes it a robust platform not only for toxicity screening but also for evaluating the efficacy of natural protective compounds [2, 5].

To date, most studies evaluating the protective effects of propolis against metal oxide-induced genotoxicity have primarily utilized ethanolic extracts, and the majority of these have focused on agents other than ZnO [13, 14, 15, 16]. In particular, studies specifically investigating the protective effects of aqueous propolis extract against ZnO-induced genotoxicity are either extremely limited or absent, especially in plant-based assay systems. This gap has not yet been systematically addressed in the literature. This study, therefore, addresses a notable gap by systematically examining the genoprotective and antioxidant potential of a water-based propolis formulation using the *A. cepa* model.

In this context, the present study aimed to evaluate the cytogenetic recovery potential of an aqueous propolis extract—sourced from the Artvin-Hatila region of Türkiye—against ZnO-induced toxicity in *A. cepa* root meristem cells. The extract was administered at five different concentrations (25, 50, 100, 200, and 400 mg/L) for 12 h, and its effectiveness in mitigating ZnO-associated damage was assessed genotoxicity markers (mitotic index, chromosomal aberrations). Additionally, the biochemical activity of the extract was analyzed via antioxidant capacity assays (DPPH, FRAP, CUPRAC), total phenolic and flavonoid content determination, and phenolic compound profiling using HPLC. This multifaceted evaluation highlights not only the possible modulatory effects of propolis on ZnO-induced cytogenotoxicity in a plant-based model but also presents preliminary evidence supporting its potential as a natural bioprotective agent with a favorable safety profile. By employing a concentration gradient approach, the study also generated data on dose–response relationships, thereby contributing to the broader scientific understanding of propolis efficacy in the context of environmental toxicant exposure. Accordingly, this work represents one of the first systematic efforts to examine the protective potential of propolis against ZnO toxicity within a well-established plant-based assay model, in alignment with current literature.

MATERIAL and METHODS

Material Supply and Preparation of Propolis Extract

The propolis used in this study was collected in 2023 from beehives located in the natural environment of Hatila Valley National Park in Artvin Province, Türkiye. The raw propolis samples were stored at room temperature in a cool, dry, and dark place until use to preserve their chemical integrity.

For extract preparation, the propolis was first ground into small pieces and then dissolved in distilled water to obtain five different concentrations: 25, 50, 100, 200, and 400 mg/L. Each solution was stirred continuously using a magnetic stirrer at 25 °C for 24 h. Following the incubation period, the suspensions were filtered through Whatman No.1 filter paper to remove any particulate matter. The resulting aqueous extracts were used for the experimental applications. All extraction procedures were carried out under controlled conditions and protected from direct sunlight to minimize potential oxidative degradation of the bioactive compounds.

Determination of Total Phenolic and Flavonoid Contents

Measurement of Total Phenolic Content

The total phenolic content of the aqueous propolis extract was determined using the Folin–Ciocalteu colorimetric method, based on the procedure described by Singleton et al. (1999) [17]. This method is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds, resulting in the formation of a blue-colored complex that absorbs maximally at 760 nm. The intensity of the color produced is proportional to the concentration of phenolic compounds in the sample. A calibration curve was prepared using gallic acid as the standard, and the results

were expressed as mg gallic acid equivalents (GAE) per gram of dry sample.

Measurement of Total Flavonoid Content

Total flavonoid content was measured according to a modified version of the method developed by Zhishen et al. (1999) [18]. The technique is based on the formation of stable complexes between flavonoids and aluminum chloride ($AlCl_3$). The aluminum ions interact with the C-4 keto group and C-3 or C-5 hydroxyl groups of flavones and flavonols, as well as with ortho-dihydroxy groups in the A and B rings, producing a yellow complex measurable at 510 nm. Quercetin was used as the reference standard in the concentration range of 0.03125 to 1.0 mg/mL, and a calibration curve was constructed accordingly. Results were expressed as mg quercetin equivalents (QE) per gram of dry sample.

Evaluation of DPPH Radical Scavenging Capacity

The free radical scavenging activity of the propolis extract was assessed based on the discoloration of DPPH (2,2-diphenyl-1-picrylhydrazyl), following a modified procedure adapted from Brand-Williams et al. (1995) [19]. A stock DPPH solution was prepared by dissolving the stable radical in methanol (≥99.8% purity) at a concentration of 4 mg per 100 mL. Serial dilutions of the extract were prepared in order to assess concentration-dependent antioxidant behavior. For each assay, 0.750 mL of DPPH solution was added to an equal volume of sample solution.
The resulting mixtures were incubated at ambient temperature in the absence of light to prevent photodegradation of the DPPH radical. After the incubation period, the absorbance of each sample was measured at 517 nm using a UV-Vis spectrophotometer. The percentage of radical inhibition was calculated for each concentration, and a dose–response curve was constructed. From this curve, the IC_{50} value—defined as the concentration of extract required to neutralize 50% of the DPPH radicals—was calculated and expressed in mg/mL.

Assessment of Ferric Ion (Fe^{3+}) Reducing Antioxidant Capacity (FRAP Method)

The ferric reducing antioxidant power (FRAP) assay was applied to evaluate the overall antioxidant capacity of the propolis extract by measuring its ability to convert ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) form in an acidic medium, which generates a blue-colored chromophore [16, 17]. In the assay, 3 mL of freshly prepared FRAP reagent was combined with 100 µL of either the test sample or blank (extraction solvent) in a glass tube. The mixture was incubated at a constant temperature of 25 °C, and the absorbance was recorded at 593 nm over a 4 min period. A standard curve was constructed using serial dilutions of $FeSO_4 \cdot 7H_2O$ in the range of 100–1000 µmol/L to determine the antioxidant reducing capacity of the extract. The final results were expressed as micromoles of Fe^{2+} equivalents per gram of dry extract.

Quantification of Antioxidant Capacity via Cupric Ion Reduction (CUPRAC Assay)

The antioxidant activity of the propolis extract was further analyzed using the CUPRAC (Cupric Reducing Antioxidant Capacity) assay, which is based on the reduction of Cu^{2+} to Cu^{+} in the presence of neocuproine under near-neutral conditions. This redox reaction generates a colored Cu^{+}-neocuproine chelate that shows peak absorbance at 450 nm. The assay was standardized using Trolox, a water-soluble analog of vitamin E, across a calibration range of 0.03125 to 1.0 mM. The antioxidant potential of the samples was expressed as Trolox Equivalent Antioxidant Capacity (TEAC), which offers a comparative metric for antioxidant efficiency relative to the Trolox standard [18].

High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds

Phenolic constituents of the aqueous propolis extract were separated and identified using high-performance liquid chromatography (HPLC) equipped with a diode array detector (DAD). Chromatographic separation was achieved on an ACE 5 C18 column (250 mm × 4.6 mm i.d.) maintained at 35 °C. Two different gradient elution methods were employed to optimize the separation of a wide range of phenolic compounds.
In the first method, the mobile phase consisted of solvent A (acetonitrile) and solvent B (1.5% acetic acid in water). The gradient started with 15% A and 85% B, gradually shifting to 40% A and 60% B over a 29-min run. Detection wavelengths were set at 250, 270, and 320 nm. The system utilized a quaternary pump (flow rate: 0.7 mL/min), a vialsampler for 10 µL injections, and a thermostated column compartment.
In the second protocol, the mobile phase was modified using methanol (≥99.8% purity) (A) and 1.5% aqueous acetic acid (B). The gradient was initiated at 10% A and 90% B, adjusted to 40% A and 60% B by 29 min, further ramped to 60% A and 40% B until 40 min, and finally transitioned to 90% A and 10% B by the 53rd min. Chromatographic signals were monitored at 280, 290, 320, 370, and 535 nm. Phenolic compounds were identified by comparing their retention times and UV absorption spectra with those of authentic standards.

Experimental Groups and Application Design

The *Allium cepa* bulbs (2n = 16) used in this study were selected based on uniform size and overall health. The outer scales were carefully removed without damaging the primary meristematic regions at the root tips. Prior to treatment, the bulbs were immersed in distilled water for 72 h to initiate root development. Once the roots reached an average length of approximately 1.5–2 cm, the bulbs were randomly assigned to experimental groups.
The experimental groups were organized as follows:

- Negative control group: bulbs treated with distilled water only
- Positive control group: bulbs exposed to 680 mg/L zinc oxide (ZnO) solution
- Treatment groups: bulbs pretreated with 680 mg/L ZnO solution followed by exposure to aqueous propolis extract at concentrations of 25, 50, 100, 200, and 400 mg/L for 12 h

In the experimental procedure, bulbs were first treated with 680 mg/L ZnO solution for 12 h. Subsequently, they were transferred into solutions containing different concentrations of aqueous propolis extract and subjected to a second 12h treatment. At the end of the exposure period, five bulbs exhibiting the most pronounced root growth were selected from each concentration group. Root tips were excised approximately 0.5–1 cm from the apex and fixed in ethanol:glacial acetic acid solution (3:1, v/v) for 24 h. Fixed samples were then hydrolyzed in 1 N HCl at 60 °C for 5 min. Following hydrolysis, the root tips were stained with Schiff's reagent in preparation for cytogenetic analysis.

The ZnO concentration used in the treatment solution (680 mg/L) was selected based on findings from previous studies evaluating the genotoxic and cytotoxic effects of ZnO nanoparticles in the *A. cepa* model [2].

Statistical Analysis

All experimental procedures were conducted in three independent replicates (n = 3). The data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software version 22.0. One-way analysis of variance (ANOVA) was used to assess differences among groups, followed by Dunnett's post-hoc test for multiple comparisons when appropriate. Prior to conducting ANOVA, the assumptions of normality and homogeneity of variances were tested using the Kolmogorov–Smirnov and Levene's tests, respectively. These preliminary tests confirmed that the assumptions for ANOVA were satisfied. A p-value of less than 0.05 was considered statistically significant in all analyses.

RESULTS and DISCUSSION

Total Phenolic Content

In this study, the total phenolic content (TPC) of the aqueous propolis extract obtained from the Artvin-Hatila region was determined as 5.99 ± 1.03 mg GAE/g dry sample (Table 1). This value is consistent with those reported for aqueous propolis extracts in previous studies and aligns with the range of 4.3–6.5 mg GAE/g observed in samples collected from different regions of Türkiye [4, 10]. Although organic solvents such as ethanol often yield higher extraction efficiency, water-based solvents are preferred in biological assays due to their lower toxicity, especially in experiments involving living systems [9].

In addition to the quantitative phenolic content, HPLC analysis revealed the presence of several biologically active phenolic acids in the aqueous extract, such as caffeic acid, chlorogenic acid, and 3,4,5-tri-O-caffeoylquinic acid, which are known for their potent antioxidant and free radical scavenging properties [10].These phenolic constituents have been previously described in the literature for their antioxidant, anti-inflammatory, cytotoxic and DNA-protective effects [5, 6, 20, 21]. The presence of such compounds corresponds well with the strong antioxidant performance observed in DPPH, FRAP, and CUPRAC assays conducted in this study.

The relatively high phenolic concentration observed here also corresponds with the increased mitotic index and decreased chromosomal aberrations following ZnO exposure, suggesting that phenolic compounds may contribute to cellular defense mechanisms under genotoxic stress. This interpretation is supported by previous findings indicating that structural variations among phenolics influence not only antioxidant potential but also antimutagenic activity [7, 8]. Therefore, the ability of aqueous propolis extracts to exert biologically relevant effects despite their lower solvent strength highlights their potential as a safe and functional plant-based intervention, particularly in the context of environmental toxicology.

Table 1. Bioactive and ntioxidant properties of aqueous propolis extract

Sample analyzed	Total phenolic content (mg GAE/g dry sample)	Total flavonoid content (mg QE/g dry sample)
Propolis extract	5.99±1.03	0.53±0.04

Total Flavonoid Content

In this study, the total flavonoid content (TFC) of the aqueous propolis extract was determined as 0.53 ± 0.04 mg QE/g dry sample (Table 1). Although this value is relatively low compared to those reported for aqueous propolis extracts in the literature, it remains within expected limits when considering the limited solubility capacity of water-based solvents [10]. Nonetheless, it has been stated in various studies that even such concentrations can exert measurable biological effects in living systems when extracted with aqueous media [7].

The chemical composition of propolis is known to vary depending on factors such as geographical origin and extraction technique. Studies utilizing HPLC and LC-MS/MS analyses have identified several flavonoid compounds—such as pinocembrin, chrysin, kaempferol, galangin, and apigenin—as major contributors to the antioxidant and antimutagenic effects of propolis [6, 7, 26]. These compounds have been shown in numerous studies to exhibit free radical scavenging properties within antioxidant mechanisms, particularly in

maintaining membrane integrity and mitigating DNA damage [7].

In the present study, the measured flavonoid content was observed to align with the partial recovery of mitotic activity and reduction in chromosomal abnormalities induced by ZnO exposure, suggesting a possible modulatory role of flavonoids against cytogenotoxic stress. Furthermore, the inherently low solvent toxicity of aqueous extracts supports their applicability as a safer alternative in experimental biological models. These findings indicate that propolis can exert biologically relevant effects not only through ethanol-based extracts with high flavonoid content but also through more biocompatible formulations.

DPPH Free Radical Scavenging Capacity

In this study, the DPPH radical scavenging activity of the aqueous propolis extract was determined as 0.004 ± 0.00 mg/mL IC_{50} (Table 2). This value indicates a strong antioxidant capacity, demonstrating that the extract can effectively neutralize DPPH radicals even at low concentrations. Achieving such a low IC_{50} value with a water-based extract is particularly notable and supports the presence of biologically active compounds within the propolis sample.

In a study by Volpi and Bergonzini (2006) [7], the IC_{50} values of aqueous propolis extracts were reported to range between 0.005–0.012 mg/mL, placing the current result at the lower boundary of this range. This suggests that propolis collected from the Hatila region possesses a rich antioxidant profile and that regional origin may influence biological activity. Castro et al. (2014) [5] emphasized that the antioxidant effect of propolis is not solely associated with total phenolic or flavonoid content but also closely linked to the structural diversity of individual compounds.

When interpreted alongside the observed reduction in genotoxic stress under ZnO exposure, the high radical scavenging activity revealed by the DPPH assay suggests that propolis may alleviate cellular oxidative damage. This effect can be explained by antioxidant constituents such as flavonoids and phenolic acids, which are known to support membrane stability and contribute to the maintenance of mitotic activity [6, 8].

Additionally, in order to contextualize the antioxidant capacity of the aqueous propolis extract, the IC_{50} value was compared to that of a standard antioxidant (Trolox), which was determined to be 0.0038 mg/mL under the same experimental conditions. The extract's IC_{50} value of 0.004 mg/mL is remarkably close to that of Trolox, indicating a comparable free radical scavenging potential. This comparison further highlights the strong antioxidant nature of the propolis sample, even in aqueous form, and supports its potential use as a natural alternative to synthetic antioxidants.

In conclusion, the potent antioxidant effect demonstrated by this low IC_{50} value suggests that aqueous propolis extracts may serve not only as supportive agents but also as potential protectants in biological systems.

Table 2. Antioxidant activity profile of the aqueous propolis extract based on IC_{50} and TEAC values

Sample analyzed	IC_{50} DPPH Inhibition (mg/ml)	FRAP (µmol $FeSO_4.7H_2O$/g sample)	CUPRAC (mmol TEAC/g sample)
Propolis extract	0.004±0.00	7.44±0.12	0.30±0.00
Standard	0.0038±0.0	-	-

The IC_{50} value of the standard antioxidant (Trolox) was derived from the corresponding calibration curve.

Ferric Reducing Antioxidant Power (FRAP)

In this study, the ferric reducing antioxidant power (FRAP) of the aqueous propolis extract was determined to be 7.44 ± 0.12 mg TE/g dry sample (Table 2). This value is considerably higher than many of the previously reported FRAP values for water-based propolis extracts, suggesting a potentially strong reducing capacity. The FRAP assay is a widely accepted spectrophotometric method that evaluates the ability of antioxidants to reduce Fe^{3+} ions to their Fe^{2+} form, thereby reflecting the cumulative reducing effect of the compounds present in the extract [22, 23].

In various studies, FRAP values for aqueous propolis extracts have generally been reported within the range of 1.5–5.0 mg TE/g [24, 25]. Therefore, the result obtained in this study may indicate that propolis sourced from the Hatila region contains a rich composition of phenolic and flavonoid compounds extractable with water. However, it should be noted that the chemical composition of propolis can vary greatly depending on regional flora and climatic conditions [26].

Several phenolic acids and flavonoids—such as caffeic acid, ferulic acid, apigenin, and chrysin—have been reported to show high activity in FRAP assays [27, 28], and this aligns with the phenolic profile observed in our HPLC analysis. When considered alongside the observed reduction in ZnO-induced genotoxic effects, the high FRAP value measured in this study supports the idea that propolis may contribute to the maintenance of cellular redox balance. Nonetheless, this outcome should be interpreted cautiously, as it may be specific to the experimental system and extraction conditions applied.

Total Reducing Capacity via CUPRAC Assay

In this study, the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) value of the aqueous propolis extract was determined as 0.30 ± 0.00 mmol TEAC/g dry sample (Table 2). This value indicates that the extract exhibits a notable capacity to reduce cupric ions and reflects a substantial level of total antioxidant activity in water-based formulations. Compared to other spectrophotometric assays, the CUPRAC method offers advantages such as operation under near-physiological pH and the ability to evaluate both hydrophilic and lipophilic antioxidants [29, 30].

CUPRAC values reported in propolis extracts from various geographical regions generally range between 0.15 and 0.37 mmol TEAC/g. For instance, Kalogeropoulos et al. (2009) [24] reported this range for Greek propolis, while Aliyazicioglu et al. (2013) [31] found similar values in Iranian samples. When compared with the present findings, the Hatila propolis extract demonstrates a competitive antioxidant potential, suggesting the presence of water-extractable bioactive constituents.

Compounds such as apigenin, pinocembrin, chrysin, and caffeic acid, which are frequently found in propolis, have been reported to exhibit high reducing activity in CUPRAC assays [26, 32]. These molecules contribute significantly to redox regulation due to their ability to chelate transition metals and to act as electron donors. This observation is consistent with the phenolic profile obtained via HPLC in this study.

The CUPRAC result, when considered alongside the reduction in ZnO-induced genotoxicity, suggests that propolis may play a regulatory role in maintaining intracellular redox balance. The ability of flavonoids to form complexes with cupric ions may contribute to this effect, which further supports their potential function in mitigating metal ion-induced oxidative stress [30].

In conclusion, the strong CUPRAC performance of the aqueous propolis extract highlights its potential as a functional food component, natural antioxidant, or therapeutic adjuvant. These findings suggest that water-based propolis formulations may offer meaningful redox activity without relying on organic solvents.

HPLC Phenolic and Flavonoid Compound Profile

In this study, a total of 16 bioactive compounds consisting of ascorbic acid, phenolic and flavonoids were quantitatively identified in the aqueous propolis extract obtained from the Artvin-Hatila region through HPLC analysis (Table 3). This diversity exceeds the commonly perceived limitations regarding the compositional richness of water-based extracts and reveals that the water-extractable fraction of propolis may also possess a complex chemical profile worth investigating.

According to the profile obtained, phenolic acids such as progallol (45.25 mg/L), caffeic acid (40.94 mg/L), and coumaric acid (39.8 mg/L), along with flavonoids such as catechin (24.5 mg/L), epicatechin (23.06 mg/L), and quercetin (6.3 mg/L), were found to be major contributors to the antioxidant potential of the extract (Table 3). These compounds have been previously reported as key components in the biological activity of propolis [6, 26].

In addition, the ascorbic acid content of the extract was measured as 3.38 mg/mL (Table 3). Although this value may vary across different propolis samples, ascorbic acid is a well-known potent antioxidant and has been shown to act synergistically with phenolic compounds [33]. Indeed, some studies have demonstrated that ascorbic acid at concentrations ranging from 0.1 to 1 mM has a superoxide and hydroxyl radical scavenging capacity comparable to that of high-concentration propolis extracts [34]. Thus, the natural presence of ascorbic acid in the extract may enhance the overall biological effect of the phenolic profile.

In another study on Turkish propolis, caffeic acid was reported as the dominant compound in water-based extracts, and its concentration was found to be higher than in ethanol-based extracts [4]. The caffeic acid level detected in the Hatila propolis is consistent with these findings. Moreover, the presence of other phenolic acids such as ferulic acid (25.2 mg/L) and chlorogenic acid (11.31 mg/L) indicates that the total redox capacity of the extract relies not only on one or two dominant compounds but rather on a wide spectrum of phenolic structures [10].

The flavonoids identified in this study (such as quercetin, catechin, epicatechin) are known to be closely associated with the high antioxidant capacities observed in the CUPRAC, DPPH, and FRAP tests [27, 30]. Flavonoids have been reported to exert protective effects against genotoxic stress through mechanisms such as redox regulation, metal ion chelation, and enhancement of membrane stability.

However, it should be noted that the phenolic and flavonoid profile obtained may vary depending on geographical origin, plant flora, climatic conditions, and seasonal variability. Therefore, conducting comparative analyses with samples collected from other regions will be valuable in enhancing the generalizability of these findings.

In conclusion, the HPLC-derived phenolic and flavonoid profile strongly supports the potential biological activity of the aqueous propolis extract used in this study—not only in terms of structural diversity but also regarding the interaction of these compounds with antioxidant systems. This comprehensive biochemical background aligns with the extract's observed mitigation of ZnO-induced genotoxic stress and supports its potential use as a functional food ingredient or natural bioactive agent.

Table 3. Bioactive constituents identified in the aqueous propolis extract via quantitative HPLC profiling

No	Compounds	Propolis extract (mg/L)
	Vitamin	
1	Ascorbic acid	3.38
	Phenolics	
2	Gallic acid	4.74
3	3,4 hydroxy benzoic acid	1.10
4	Vanillic acid	3.61
5	Syringic acid	4.4
6	Coumaric Acid	39.8
7	Caffeic acid	40.94
8	Ferulic acid	25.2
9	Rosmarinic acid	1.14
10	Progallol	45.25
11	Chloragenic acid	11.31
12	Resvaratrol	N/D
13	Oleuropein	N/D

	Flavanoids	
14	Catechin	24.5
15	Epicatechin	23.06
16	Rutin	0.9
17	Myricetin	N/D
18	Qercetin	6.3
19	Apigenin	N/D
20	Cyanidin cloride	N/D
21	Hesperitin	N/D
22	Kaempferol	N/D
23	Baicalin	2.16
24	Chrysin	N/D

N/D: Non determined

Mitigating Effects of Propolis on Zinc Oxide-Induced Genotoxic and Cytogenetic Alterations in Allium cepa Root Cells

In this study, the effects of aqueous propolis extract applied following zinc oxide (ZnO)-induced genotoxic stress were comprehensively evaluated in terms of mitotic activity and chromosomal abnormalities in *A. cepa* root meristematic cells. According to the data obtained, the mitotic index (MI) in the positive control group (ZnO treatment) was significantly reduced compared to the negative control ($p < 0.05$), confirming the suppressive impact of ZnO on cell division dynamics (Table 4).

Among the propolis-treated groups, only the 100 mg/L concentration significantly ameliorated the ZnO-induced decrease in the mitotic index (MI), maintaining a value (6.59 ± 1.35) close to that of the negative control. In contrast, mitotic activity was suppressed at both lower and higher doses—specifically 25, 50, 200, and 400 mg/L—with MI values in the 25 mg/L (1.91 ± 0.53) and 400 mg/L (2.28 ± 0.62) groups falling even below that of the ZnO-treated group (Table 4). These findings suggest that the aqueous extract of propolis contains compounds capable of modulating the cell cycle and that this effect is likely dose-dependent.

A significant decrease in the mitotic index indicates an increased number of cells failing to reach the M phase of the cell cycle, which may reflect cell death, delays in mitotic progression, or suppression of DNA synthesis [35, 36]. According to Lemme and Marin-Morales (2009) [37], such a reduction in MI can serve as an indicator of the biochemical impact of the exposed substance on the growth and development of the organism. Likewise, Mercykutty and Stephen (1980) [38] reported that a decline in the number of dividing cells may reflect the mitodepressive effects of natural compounds such as propolis.

In our study, the increase in MI observed at the 100 mg/L concentration suggests that propolis at this dose may possess protective potential against genotoxic effects (Table 4). Similarly, Çavuşoğlu (2020) [60] reported that moderate concentrations of propolis enhanced mitotic activity in *A. cepa*, indicating a supportive role at specific doses. These findings imply that the antioxidant compounds in propolis may contribute to the regulation of DNA repair, cell division, and chromosomal stability when administered within an optimal concentration range.

Comparable findings in the literature are consistent with the results of this study. For instance, Celik and Aslantürk (2010) [39], Oyeyemi and Bakare (2013) [40], Chukwujekwu and Van Staden (2014) [41], and Roberto et al. (2016) [42] have demonstrated that different concentrations or regional variations of propolis exert varying effects on the mitotic index. While certain doses were reported to have inhibitory effects, others showed a predominantly protective role.

Table 4. Effects of aqueous propolis extract on the mitotic activity and cell division phases in *A. cepa* under zinc oxide stress

TRM	ECN	MI± SD	TN CP	TN CM	TN CA	TN CT
NC	3000	6.73± 1.39	64	80	51	38
PC	3000	2.71± 0.39*	47	44	16	7
25 mg Pr	3000	1.91± 0.53*	71	20	4	8
50 mg Pr	3000	2.85± 1.53*	67	15	24	48
100 mg Pr	3000	6.59± 1.35	188	67	41	60
200 mg Pr	3000	3.64± 0.93*	45	36	30	20
400 mg Pr	3000	2.28± 0.62*	18	43	21	0

*Significant at $p<0.05$. TRM: Treatments, NC: Negative Control, PC: Positive Control, Pr: Propolis, ECN: Examined cell number, MI±SD: Mitotic index±Standart deviation, TNCP: Total number of cells at prophase, TNCM: Total number of cells at metaphase, TNCA: Total number of cells at anaphase, TNCT: Total number of cells at telophase

When evaluated alongside MI data, the type and frequency of chromosomal abnormalities further elucidate the extent of genotoxic effects. In this study, chromosomal aberrations observed in *A. cepa* root cells were analyzed to assess the impact of aqueous propolis extract on ZnO-induced genotoxicity. The findings revealed that the degree of chromosomal damage varied depending on the concentration of propolis applied.

In the ZnO-exposed positive control group, the most frequently observed cellular abnormalities included disturbed prophase (2.57 ± 0.41), chromosome stickiness (1.86 ± 0.59), C-metaphase (0.71 ± 0.76), bridge formation (0.43 ± 0.79), and multipolar anaphase (0.43 ± 0.79) (Table 5, Figue 1). These findings indicate that ZnO induces substantial disruptions in the mechanisms of cell division [4, 5]. In particular, C-metaphase events are characterized by the disorganization of metaphase chromosomes in the cell center due to inhibition of spindle fiber formation. This anomaly was first described by Levan (1938) [43] and is associated with agents that act similarly to colchicine by delaying centromere separation. This mechanism also

plays a direct role in the observed decrease in mitotic index [44].

Similarly, chromosome stickiness, commonly observed in the high-dose group, reflects toxic effects associated with disruptions in the protein matrix of chromatin and is generally attributed to depolymerization of DNA [45, 46, 47]. Additionally, the observed bridge formations are thought to result from replication errors or faulty reunification of broken chromosomes, while multipolar anaphase arises due to the irregular formation of spindle fibers, leading to abnormal chromosome segregation and contributing to micronucleus formation [48, 49].

In the groups treated with propolis, a clear dose-dependent effect was observed. In the 25 mg/L group, the total number of aberrant cells (6.67) was close to that of the ZnO group, with a particularly high rate of disturbed prophase (5.33 ± 2.55), suggesting that this low dose was insufficient to suppress genotoxic stress. In the 400 mg/L group, the total abnormality rate reached a high level of 10.44, with notably elevated frequencies of chromosome stickiness (1.11 ± 0.27), C- metaphase (4.33 ± 0.02), and bridge formation (2.33 ± 0.61) (Table 5; Figure 1). These findings imply that rather than stabilizing cell division, high-dose propolis may impose additional chromosomal stress through a potential cytotoxic effect [37, 40].

In contrast, the abnormality rate in the group treated with 100 mg/L propolis (1.33) was nearly identical to that of the negative control (1.42), and all types of abnormalities were found to be minimal (Table 5). This indicates that the 100 mg/L dose provided optimal protective effects and successfully mitigated ZnO-induced genotoxicity. Similar findings have previously been reported, highlighting the DNA-protective role of propolis at this concentration [39, 50, 51].

In conclusion, this study demonstrates that propolis has the potential to preserve cellular structural integrity under genotoxic stress; however, this effect is clearly dose-dependent. Specifically, the 100 mg/L dose exhibited a markedly protective profile, both in terms of total chromosomal abnormalities and specific types of damage.

Table 5. Dose-dependent effects of aqueous propolis extract on chromosomal aberrations in *Allium cepa* root tip cells under zinc oxide stress

Treatments	Disturbed prophase	Ch. stickness	C-metaphase	Ch. disrupted	Laggard ch.	Vagrant ch.	Bridge formation	Multipolar Anaphase	Multipolar anaphase with forward ch.	Multipolar anaphase with ch. bridge	Diagonal telophase with vagrant ch.	Total abberant cells
Negative control	-	0.33±0.77	0.33±0.82	0.25±0.45	-	-	0.50±0.02	-	-	-	-	1.42
Positive control	2.57±0.41	1.86±0.59	0.71±0.76	-	0.14±0.36	0.29±0.76	0.43±0.79	0.43±0.79	0.29±0.49	0.29±0.49	0.14±0.38	7.14*
25 mg Propolis	5.33±2.55	0.56±0.22	0.44±0.53	-	-	0.11±0.33	-	0.11±0.33	-	-	0.11±0.33	6.67*
50 mg Propolis	0.56±0.88	0.37±0.93	1.01±0.22	-	0.17±0.51	0.3±1.00	1.00±1.22	-	-	-	1.88±2.75	3.43
100 mg Propolis	0.58±0.28	0.11±0.71	0.08±0.73	-	-	-	0.56±0.13	-	-	-	-	1.33
200 mg Propolis	-	0.28±0.75	0.83±0.72	-	-	2.14±0.81	-	0.33±0.02	-	-	0.33±0.02	4.92
400 mg Propolis	2.67±0.02	1.11±0.27	4.33±0.02	-	-	-	2.33±0.61	-	-	-	-	10.44*

*Significant at $p<0.05$, Ch: Chromosome.

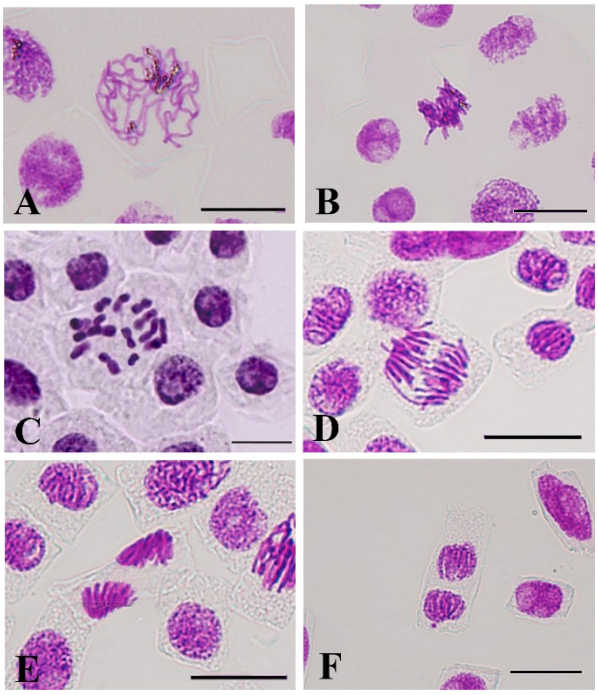

Figure 1. Mitotic Disruptions in *A. cepa* Root Tips Following ZnO Exposure and Propolis Intervention. (A) Disturbed prophase; (B) Chromosome stickness; (C) C- metaphase; (D) Chromosome bridge; (E) Diagonal telophase; (F) Micronucleus in telophase. Scale bars 20µm.

The MN assay is widely used in genotoxicity studies as a sensitive biomarker for detecting DNA damage and chromosomal disruptions. MNs are formed from chromosome fragments or entire chromosomes that fail to be incorporated into the daughter nuclei during mitosis. Therefore, the presence of MNs is considered a reliable indicator of the genotoxic potential of a substance [52, 53].

In this study, the effect of propolis at various doses on micronucleus (MN) formation was evaluated under genotoxic stress induced by ZnO exposure (Figure 1F). In the ZnO-treated positive control group, the MN frequency was recorded as $0.12 \pm 0.02\%$, indicating that ZnO exerts harmful effects on DNA and chromosomal integrity (Table 6). A dose-dependent response was observed among the propolis treatment groups. Applications of 50 mg/L ($0.02 \pm 0.01\%$) and 100 mg/L ($0.04 \pm 0.07\%$) significantly reduced MN frequency, yielding values close to those of the negative control (Table 6). This suggests that the phenolic and flavonoid constituents of propolis may help mitigate oxidative stress caused by ZnO, thereby reducing DNA damage [7, 54]. Similarly, previous studies have reported that natural compounds with antioxidant properties, when applied at low concentrations, are effective in suppressing micronuclear damage induced by genotoxic agents [29].

However, in the group treated with 400 mg/L of propolis, the MN frequency increased to $0.36 \pm 0.13\%$, representing the highest value among all groups (Table 6). This finding suggests that at high concentrations, propolis may disrupt the intracellular redox balance and exert pro-oxidant effects, thereby triggering DNA damage [27]. Notably, this increase in MN frequency parallels the elevated incidence of chromosomal abnormalities such as C-metaphase and anaphase bridges observed at the same dose.

In conclusion, the genotoxicity-reducing effect of propolis clearly exhibits a dose-dependent profile. While 50 and 100 mg/L concentrations demonstrated protective effects in terms of MN frequency, the 400 mg/L dose appeared to exert detrimental effects on cellular systems. Accordingly, propolis may be considered a potential natural agent for mitigating ZnO-induced genotoxicity when used within an appropriate dosage range.

Table 6. Frequency of micronucleus in *A. cepa* cells at different concentrations of propolis extracts

Treatments	Examined Cell Number	Micronucleus (%)
Negative Control	3000	-
Positive Control	3000	0.12±0.02
25 mg Propolis	3000	0.06±0.00
50 mg Propolis	3000	0.02±0.01
100 mg Propolis	3000	0.04±0.07
200 mg Propolis	3000	0.11±0.27
400 mg Propolis	3000	0.36±0.13*

*Significant at $p<0.05$

CONCLUSION

In this study, the potential protective effects of the aqueous extract of propolis, obtained from the Artvin-Hatila region of Türkiye, were comprehensively evaluated against genotoxic stress induced by ZnO exposure in *A. cepa* root cells. ZnO is a metal oxide widely used in various industries, including cosmetics, paint, plastics, and electronics, and is known to exert cellular and genetic-level toxicity when released into the environment. In this context, the present study investigated whether the cytogenetic damage caused by ZnO in a model system could be alleviated by a natural agent such as propolis.

The analyses revealed that, particularly at a concentration of 100 mg/L, the aqueous propolis extract exhibited mitosis-supporting properties and suppressive effects on ZnO-induced chromosomal abnormalities and MN formation. The cytogenetic parameters obtained at this dose were comparable to those of the negative control group. On the other hand, protective efficacy was insufficient at both lower (25 mg/L) and higher (400 mg/L) concentrations, and in some parameters, genotoxicity was even exacerbated. These findings suggest that the effects of propolis on cell division and genomic stability are clearly dose-dependent.

These findings not only demonstrate the potential modulatory effect of aqueous propolis extract on ZnO-induced cytogenotoxic stress but also emphasize the critical importance of selecting an appropriate dose to ensure biological safety. The *A. cepa* model used in this study is a widely accepted bioindicator system for detecting environmental mutagens, and the data obtained

here may form a basis for future studies involving higher-order systems such as animal or human cell lines.

Considering the extensive industrial use of ZnO, identifying natural and low-toxicity protective compounds against such potential genotoxic agents holds particular relevance for environmental and public health. Although further validation at the molecular level and testing in more advanced biological systems are required, the present study suggests that propolis could be a promising candidate compound for mitigating genotoxic damage to genetic material.

In this regard, the findings underscore not only the biological efficacy of propolis as a natural product but also the necessity of developing low-toxicity and biologically safer solutions to counteract environmental stressors caused by metal oxides. The aqueous formulation obtained through water-based extraction presents a safe application range in experimental biological systems, rendering it a viable candidate not only in laboratory conditions but also in agricultural production systems and environmentally friendly product formulations. Therefore, this study has the potential to provide interdisciplinary contributions to the fields of environmental toxicology, plant biotechnology, and natural protective agent research.

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